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RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES INCLUDING BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY, INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY, PSYCHIARTY, SURGERY, AND VETERINARY MEDICINE, VOLUME I

Walter Reed Army Institute of Research
Washington, D. C.

30 June 1974

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RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

INCLUDING

BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY, INTERNAL MEDICINE, NUCLEAR MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

(Projects, tasks, and work units are listed in Table of Contents)

Annual Progress Report
1 July 1973 - 30 June 1974

Volume I

Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington, D. C. 20012

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences - National Research Council.

SUMMARY

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498 introducing each work unit report, and names of investigators are given at the beginning of each report.

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PROJECT 3A161101A91C
IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00
In-House Laboratory Independent Research

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6513	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. SMSN INSTR'N	8A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71C	00	176
b. CONTRIBUTING	61101A	3A161101A91C	00	CH FR 105
c. [redacted]	CARDS 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Mechanisms of Transmission of Hepatitis Viruses

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 010100 Microbiology 03500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 74	2.5	93
CURRENT FISCAL YEAR 75	2.5	129

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Hepatitis; (U) Virus; (U) Antigen; (U) Immunology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To define the epidemiology of hepatitis in military populations in order to establish methods for reducing disability from hepatitis. Emphasis is on developing and applying sensitive and specific methods for detection of hepatitis viruses/antigens and antibodies to determine host factors important in resistance to disease and infection in military personnel.

24 (U) New methods for identification and antigenic analysis of hepatitis viruses are under development. The immune response of patients infected with hepatitis viruses is studied to define sensitive parameters of infection and to define factors critical in immunity. The epidemiology of hepatitis B in military populations is defined.

25 (U) 73 07 - 74 06 The standard commercial radioimmunoassay (Ausria) was found highly sensitive but not specific for HBsAg detection. False positive reactions were frequent in humans with antibody to guineapig proteins or with CPM less than 10 standard deviations greater than controls. HBsAg was found in saliva and urine of Hepatitis B patients during the period of antigenemia. An increased prevalence of Hepatitis B infections in family members of HBsAg carriers was shown. HBsAg from Hepatitis B patients at Fort Hood and USAREUR were nearly uniformly AYW, a subtype commonly associated with drug-related Hepatitis B epidemics. Preliminary data on the prevalence of Hepatitis B core antibody (anti-HBc) in hepatitis cases and HBsAg carriers at Fort Hood suggest that this antibody reflects current or recent Hepatitis B replication in humans. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1973 - 30 June 1974.

* Available to contractors upon originator's approval.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 105 Mechanisms of transmission of hepatitis viruses

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Description

To define the epidemiology of hepatitis in military populations in order to establish methods for reducing disability from hepatitis. Emphasis is on developing and applying sensitive and specific methods for detection of hepatitis viruses/antigens and antibody to determine host factors important in resistance to disease and infection.

Progress

I. Epidemiology of Hepatitis B infection in military populations

A. Field studies at Fort Hood

1. Description of Hepatitis B outbreak

Beginning in 1970 a sustained outbreak of Hepatitis B has occurred at Fort Hood, Texas. The incidence of hepatitis reached a peak of 19 cases/1,000 average strength/year in August 1972 (Fig. 1). This rate is 10 times the average incidence at Fort Hood prior to the onset of the outbreak and 5 times the CONUS rate. The outbreak was thought to be entirely related to illicit drug abuse, but as described in another section (II-B) non-parenteral transmission may have accounted for possibly 25% of the clinical cases. Initial cases included recent Vietnam veterans, but with time, the attack rate peaked long after the return of most Vietnam veterans.

A relationship between food or water supply and hepatitis cases could not be established. No evidence of fecal contamination of the water supply could be documented. Clusters of cases were distributed sporadically among the 4 military units (2nd Armor Division, 1st Cavalry, 13th Support Brigade, and III Corps). The attack rate was highest among lower ranking enlisted personnel (Fig. 2). Many hepatitis cases had received disciplinary demotion and were overtly hostile to

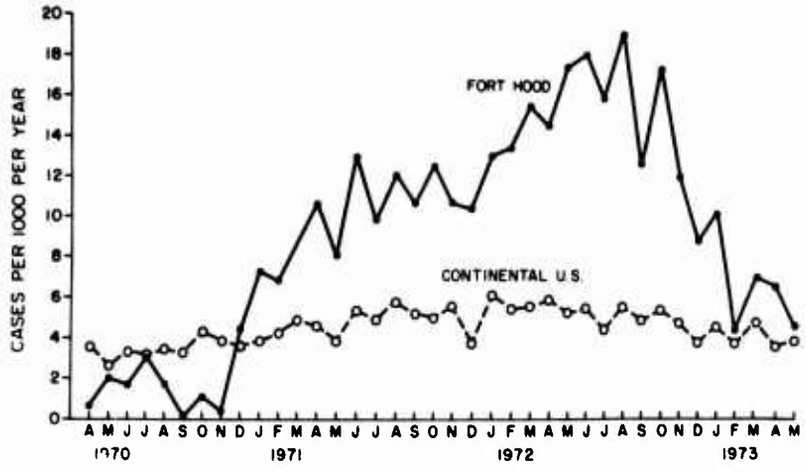


Figure 1. Incidence of viral hepatitis in Army personnel at Fort Hood, Texas, and in Army personnel in the entire continental United States, April 1970 - May 1973

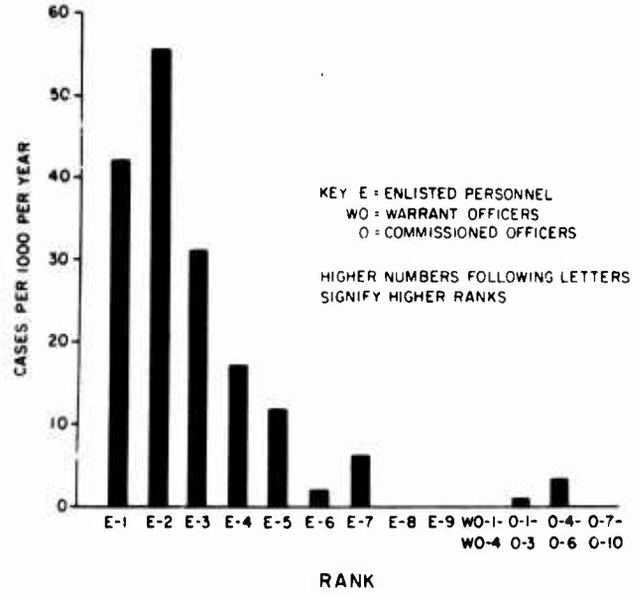


Figure 2. Incidence of viral hepatitis by military rank, Fort Hood, Texas, 1 January - 30 June 1972

military life in general. At least one-third of the cases admitted to using intravenous drugs within the recognized incubation period of Hepatitis B infection. Sharing of wine and smoking "pot" were common habits among cases.

2. Serologic survey of Fort Hood personnel

A serologic prevalence survey of post personnel was undertaken to:

a) Determine the proportion of acute hepatitis cases admitted to Darnall Army Hospital which were Hepatitis B

b) Define the prevalence of HB_S Ag and anti-HB_S in randomly selected troop units and from units with clinical cases of hepatitis

c) Determine the prevalence of HB_S Ag and anti-HB_S in populations at risk of acquiring Hepatitis B infection

From Feb to Apr 1973, 38 consecutive hepatitis admissions to Darnall Army Hospital were studied for etiology. By both CEP and RIA, 17 (44.6%) were positive for HB_S Ag. The 16 patients with antigen concentration sufficient for subtyping were all HB_S Ag/AYW except for 1 of ADW subtype. Of the 21 hepatitis patients without HB_S Ag in acute specimens, 3-month convalescent sera were obtained on 9 and 6 (66%) had developed anti-HB_S by PHA. Given the limitations of this study, it appears that current antigen and antibody assays can determine Hepatitis B etiology in over 80% of patients with acute HBV.

Although the prevalence of HB_S Ag in index case units is twice that of randomly selected units, this difference is not statistically significant. There is little difference in the prevalence of anti-HB_S in these groups (Table 1).

Table 1 - Correlation of serologic data (HB_S Ag and anti-HB_S Ag) with the presence of a hospitalized index case of acute Hepatitis B infection

Combat troops	No. tested	HB_S Ag^+	Anti-HB_S
Index case unit	853	1.4%	15.6%
Random control unit	1,042	0.7%	16.2%

AMEDD personnel had the same prevalence of HB_S Ag and anti-HB_S as randomly selected combat troops (Table 2). A high prevalence of HB_S Ag was found in the dental company which experienced a minor outbreak of Hepatitis B infection with 6 cases of acute hepatitis being observed over a 10-week period. It is tempting to speculate that this subpopulation outbreak is related to work performed. Dental personnel routinely work without gloves in a field contaminated with blood; acquisition of HB_S Ag might therefore be possible through a small break in the skin. Also, transmission could occur by aerosols of saliva containing HB_S Ag created by high speed drills.

Table 2 - Prevalence of HB_S Ag and anti-HB_S in AMEDD personnel

	No. tested	HB_S Ag+	Anti-HB_S
All AMEDD personnel	1,863	.9%	15.6%
Dental Co.	164	3.65%	15.2%

Correlation of serologic data with questionnaire replies revealed that soldiers having OCONUS duty had a much greater prevalence of anti-HB_S than those of the same age without such experience ($x^2 = 29$ $P < .001$), Table 3. No difference was found for HB_S Ag. No difference was found in the prevalence of HB_S Ag or anti-HB_S for factors such as quarters (on or off post), marital status, or history of blood transfusion (Tables 4 & 5).

Table 3 - Prevalence of HB_S Ag and anti-HB_S based on history of overseas active duty tour

History of OCONUS tour	HB_S Ag+	Anti-HB_S*
Yes	1.0%	19.4%
No	1.1%	11.1%

* Adjusted for age (prevalence of anti-HB_S is statistically significant; $x^2 = 29.0$, $P < .001$)

Table 4 - Percentage of personnel anti-HB_S^+ and HB_S Ag^+ according to location of housing. Adjusted for age and history of OCONUS

Location of housing	% with anti-HB_S	% with HB_S Ag	Number
On post	17.9	1.0	1639
Off post	14.1	1.1	1319
TOTAL	16.0	1.0	2958

Table 5 - Correlation of serological survey for HB_S Ag and anti-HB_S with marital status. Rates adjusted for age and history of OCONUS tour

Marital status	% anti-HB_S Ag+	% HB_S Ag^+	Number
Single	15.8	1.1	1,377
Married	15.6	1.0	1,581
TOTAL	16.0	1.0	2,958

Soldiers admitting to having clinical hepatitis at some time in the past were more likely to have anti-HB_S than those not having hepatitis (Table 6). Of interest is the comparison of anti-HB_S among health personnel and combat troops at Fort Hood, Texas. Young men (approximate age, 18-20 years) with no overseas experience have a prevalence of anti-HB_S comparable to officers entering the U. S. Army Medical Corps who are 8-12 years older. This serological observation may possibly be related to the difference in socio-economic background of the respective groups. A similar notation has been reported by Cherubin et al. concerning the civilian populations in New York City (ghetto vs middle class vs wealthy).

Table 6 - Correlation of serum anti-HB_S with history of clinical hepatitis; $x^2_c = 14.7$, $P < .001$

Clinical hepatitis	% with anti-HB_S	Number tested
Yes	35.2	54
No	14.8	1,104
TOTAL	15.7	1,158

B. Prevalence of Hepatitis B antigen and antibody in military health personnel attending service schools at Fort Sam Houston (FSH), Texas

The serologic survey of health personnel at Fort Sam Houston (see WRAIR Annual Report 1973, pages 260-269) was expanded to encompass a broader base to study prevalence of HB_S Ag and anti-HB_S. The survey includes officers in the following Corps: MC, DC, VC, MSC, ANC and AMSC. Sera were obtained on various officers in these Corps who were attending advanced courses or entering military service. Sera were also collected on enlisted personnel entering the Army Medical Training Center, as well as those attending the various advanced technical schools at Fort Sam Houston. A summary of the survey appears in Table 7. Differences between the prevalence of HB_S Ag and anti-HB_S in officers (0.56% and 3.3%) were not significantly different from enlisted personnel (0.32% and 5.8%).

As previously noted, physicians entering the U. S. Army appear to have anti-HB_S (8.8%) more frequently than officers of other health profession Corps (4%). This increase could be secondary to physician's increased age and patient contact. More complete demographic data on officers entering the Army are presently being analyzed by computer. Current projects in this survey include an attempt to rebleed all those who entered the survey in 1972 in order to estimate the acquisition rates for HB_S Ag and/or anti-HB_S of health personnel on active duty for 2 years.

At present there are no further plans to continue bleeding FSH personnel. This project defined the prevalence of HB_S Ag and anti-HB_S in health personnel and may establish the risk to physicians and

health personnel of acquiring HBV infections over a 2-year observation period.

Table 7 - Prevalence of HB_S Ag and anti-HB_S in officer and enlisted health personnel attending various schools at FSH, Texas, 1973-74

Personnel:	Summary of FSH survey for HB_S Ag and anti-HB_S, 1973-74				
	Number tested	HB_S Ag		Anti-HB_S	
		RIA+	CEP+	Ad cells	Ay cells
Officers	2,688	15	9	90	87
Enlisted	1,541	5	4	89	83

C. Preliminary investigation of Hepatitis B infection in Germany

Since Sept 1974 small numbers of sera from acute clinical cases of hepatitis hospitalized at USAH, Nuremberg, Germany, were forwarded by CPT Willard Cates, MC. In the past 24 months, USAH, Nuremberg, has admitted large numbers of hepatitis cases, reflecting a hepatitis epidemic in USAREUR. From the limited epidemiologic data available, this epidemic seems comparable to the outbreak at Fort Hood, Texas, USA; in Germany, however, attack rates appeared to be higher (27/1000/year, as compared to peak attack rate at Fort Hood of 19/1000/year), with greater than 100/1000/year in some combat units. Acute cases were predominately young, low-ranking white soldiers and were strongly associated with the illicit use of drugs. A summary of sera on acute hepatitis cases from Germany is recorded in Table 8.

Initial CEP testing revealed marked differences in positive reactions between USAREUR and WRAIR labs. Subsequent coded panels of sera sent to 10th medical lab demonstrated close correlation of CEP results in USAREUR with WRAIR results. Based on crude data, it appears that the hepatitis B epidemic is comparable to the experience at Fort

Hood, Texas and other previously recognized Hepatitis B outbreaks in the military, including Fort Bragg, NC in 1970, Camp LeJeune, NC in 1970, and in Vietnam. The USAREUR Hepatitis B rates, based on clinical disease, are the highest observed in the military. Since Hepatitis B infection appears to be a persistent and important military medical problem, there is a strong military requirement for research into its epidemiology, control and prevention. Until a Hepatitis B vaccine is available, the only possible methods of prevention are elimination of drug abuse or prophylaxis with hyperimmune B globulin. Initial reports by Krugman(1971), Conrad (1972), and Prince (1974) suggest that there may be a protective effect against infection by gamma globulin containing anti-HB_S. Whether hyperimmune gamma globulin would significantly protect against drug-induced Hepatitis B in USAREUR can only be established in field studies.

Table 8 - Results of testing 104 sera from USAREUR cases of hepatitis by RIA & CEP

Date	No. tested	No. CEP^+ USAREUR Lab	No. CEP^+ WRAIR	No. RIA^+ WRAIR
Sep 73	30	9	20*	21
Dec 73**	40	28	28	28
Apr 74**	34	NR	17	23
TOTAL	104		65	72

*All but 3 specimen tested (of total 65 CEP^+ sera) are HB_S Ag/ayw; other: 2 adw, 1 adr

**Specimen in transit from USAREUR 1 month at ambient temperature

NR - No record of USAREUR test results sent

II. Clinical research of HB_S Ag

A. Specificity and sensitivity of radioimmunoassay for Hepatitis B antigen

Solid phase radioimmunoassay (RIA) is one of the most sensitive and convenient techniques for detecting Hepatitis B antigen (HB_S Ag) in serum (Cossart, 1973). An unresolved problem in the use of this technique is the level of radioactivity in counts per minute (CPM) that is considered a positive test. A further difficulty has been reported recently by Sgouris (1973), Prince (1973), and Alter (1973), who have demonstrated that many sera which are positive by RIA but negative by counterelectrophoresis (CEP) are false positive reactions due to non-specific human antibody against guinea pig proteins used in the test system. Resolution of these problems is required in order to avoid misclassification of sera tested by RIA.

The sera used in this study were obtained during a serologic survey for HB_S Ag which was conducted at Fort Hood, Texas during February and March 1973. The survey was part of an investigation of an outbreak of viral hepatitis in soldiers (Section I A & B). Sera were collected from 6026 military and civilian personnel with no signs or symptoms of hepatitis. In addition, sera were obtained from 85 patients with acute hepatitis.

The solid phase radioimmune assay for HB_S Ag (Ausria, Abbott) was performed as described by Ling and Overby. Briefly, 0.1 ml of serum to be tested was placed in plastic tubes coated with GP HB Ab and incubated for 16 hours at room temperature. Each tube was washed with five 2-ml aliquots of Tris (hydroxymethyl) aminomethane buffer. After thorough aspiration of the remaining buffer from the bottom of the tube, 0.1 ml of I^{125} labeled GP HB Ab, was added for 90 minutes at room temperature and the tubes were washed as described above and counted in a Model 1185 Searle (Nuclear Chicago) gamma counter. The manufacturer's negative control serum was dispensed 10 times into separate tubes and processed as above for all runs.

All sera positive by RIA (i.e., CPM >BSD above the mean of 10 replicates of the negative control) were tested in counterelectrophoresis against rabbit anti-HB_S. All CEP^+ sera were checked for HB_S Ag specificity by immunodiffusion against rabbit HB_S Ab and standard HB_S Ag. CEP^+ sera were considered HB_S Ag^+ if lines of identity with standard HB_S Ag were obtained. Sera positive by RIA but negative by CEP were analyzed for specificity to HB_S Ag by means of the following absorption test: 0.1 ml aliquots of the serum to be tested were incubated at room temperature for 2 hours with 0.025 ml of each of 4 absorption sera, normal guinea pig serum, guinea pig anti-HB_S, normal rabbit serum, and rabbit anti-HB_S. In addition, duplicate aliquots were run without absorption. Following incubation, 0.1 ml of the absorbed serum was pipetted into Ausria tubes and processed as above.

Since rabbit and guinea pig anti-HB_S in preliminary tests routinely decreased radioactive counts of CEP positive sera by 80% or more, RIA positive, CEP negative sera showing a decrease in CPM in excess of 80% with rabbit or guinea pig anti-HB_S, but not by normal rabbit or guinea pig sera, were considered specific for HB_S Ag.

The sera obtained from 6026 asymptomatic subjects at Fort Hood were tested for HB_S Ag by RIA and CEP (see Table 9). According to one's choice of three recommended criteria for RIA-test positivity (Ginsberg 1972, Ling 1972), RIA was 2.2, 2.5, or 3.2 times more "sensitive" than CEP (positive RIA test: > 3 S.D., 5 S.D. above mean, or > 2.1 times mean, respectively).

Table 9 - Hepatitis B antigen positivity in sera from 6026 asymptomatic people using RIA and CEP

Test results		Subjects	
CEP	RIA	No.	%
Positive	CPM > 3 SD	48	0.79
Negative	CPM > 2.1 X M	56	0.93
Negative	2.1 X M > CPM > 5 SD	15	0.25
Negative	5 SD > CPM > 3 SD	33	0.55
	TOTAL	152	2.52

Key: SD = standard deviations above the mean of the negative control

XM = times the mean of the negative control; CPM = radio activity in counts perminute

The 152 sera which were positive by RIA were tested for specific reactivity for HB_S Ag. Each of the 48 sera which were positive by CEP as well as by RIA gave precipitin lines of identity with HB_S Ag standards used for subtyping with rabbit antisera.

Absorption tests were performed to determine the specificity of RIA positive, CEP negative sera for HB_S Ag. The sera were classified as true or false positives according to the following criteria: True positive, sera showing an 80 percent or more decrease in CPM after absorption with guinea pig or rabbit anti-HB_S but not with normal animal sera; false positive-antiguinea pig protein, - sera blocked by normal guinea pig serum; and indeterminate false positive, - sera inhibited by neither specific anti-HB_S nor by normal animal sera. The latter form of false positive was labeled "indeterminate" because its mechanism is unknown, whereas the former was referred to as "antiguinea pig protein" to indicate non-specific reactivity to guinea pig protein used in the test. Examples of the results obtained after absorption testing, together with their interpretation, are shown in Table 10.

Using the criteria outlined above, 68 (44.8%) of the 152 RIA-positive sera were classified as true positives, 10 (6.6%) were false positives - anti-guinea pig protein, and 74 (48.6%) were indeterminate false positives. Fifty-five (74%) of the sera classified as indeterminate false positives had radioactivity counts between 3 SD units above the mean and 2.1 times the mean of the negative control. The counts in this range were too low to yield conclusive results with absorption tests. Fourfold concentration of 6 of these sera failed to resolve the problem, primarily because the counts did not show a proportionate increase with concentration.

Four RIA-positive, CEP negative sera with radioactivity counts greater than 20 SD units above the negative control mean were not inhibited by animal sera containing anti-HB_S against subtype D revealed significant inhibition (Table 11).

There was a correlation between the degree of elevation of radioactivity counts found on RIA testing and the proportion of tests classified as true or false positives after absorption tests (Table 12). The higher the counts in SD units above the mean of the negative control, the more likely were the sera to be truly positive. All of the 49 sera with counts above 50 SD units were classified as true positives, while only 19 (18.4%) of the 103 sera with counts between 3 and 50 SD units were so classified; 72% of the true positive sera had CPM in excess of 50 SD units.

The sensitivity and specificity of the RIA test for HB_S Ag in sera obtained from asymptomatic people was compared to that in sera obtained from patients with acute hepatitis (Table 13). As compared to the results of CEP testing, RIA appeared to be relatively more sensitive in asymptomatic individuals than in patients; however, the difference was not statistically significant ($x^2 = 0.5$ $P > .25$), and the increase in sensitivity over CEP for patients as well as asymptomatics was 27%. No non-specific reactions for HB_S Ag occurred in patients.

Table 10 - Absorption of RIA-positive sera to determine specificity for Hepatitis B antigen: representative test results in counts per minute, using Ausira Kits

Serum no.	Unabsorbed sera		Absorbed sera				Interpretation
	CEP	RIA	Normal guineapig serum	Guinea-pig Anti-HB_S	Normal rabbit serum	Rabbit anti-HB_S	
032	Positive	10,051	8,352	826	8,600	852	True positive
F 80	Negative	7,263	7,650	1,049	5,430	287	True positive
F 1226	Negative	1,372	442	452	1,329	1,388	False positive - antiguinea pig protein
F 1769	Negative	1,883	472	399	1,295	838	False positive - antiguinea pig protein
M 744	Negative	739	775	794	863	728	Indeterminate false positive
M 1895	Negative	518	383	428	646	594	Indeterminate false positive
Positive control: rabbit anti-guinea pig protein	-	16,175	511	492	13,461	16,256	False positive - antiguinea pig protein

Table 11 - Selective inhibition of an RIA positive, CEP negative serum by hepatitis B antibody specific for subtype D

Absorption serum	Counts per minute
None (unabsorbed)	5554
Normal guineapig serum	5864
Guineapig anti-HB_S Y specificity	3174
Guineapig anti-HB_S D specificity	630

Table 12 - Frequency distribution of absorption - tested sera according to the counts obtained in RIA for hepatitis B antigen

Counts (SD's above mean)	True positives	False positives	
		Antiguineapig protein	Indeterminate
3-	2 (2.9)	0 -	31 (41.9)
5-	6 (8.8)	2 (20.0)	24 (32.4)
10-	1 (1.5)	6 (60.0)	11 (14.9)
20-	10 (14.7)	2 (20.0)	8 (10.8)
50-	27 (39.7)	0 -	0 -
100-	12 (17.6)	0 -	0 -
200-	10 (14.7)	0 -	0 -
TOTAL	68 (100.0)	10 (100.0)	74 (100.0)

Note: Figures in brackets represent percentages of total. Positivity criterion of the manufacturer (i.e., 2.1 times mean) ranged between 4.4 and 13.6 SD units on 38 runs, and averaged 8.3 SD units

Table 13 - Sensitivity and specificity of RIA for hepatitis B antigen in sera from 85 hepatitis patients and from 6,026 asymptomatic people

	Number patients	Number Asymptomatics
True positives:		
RIA(+), CEP(+)	30	48
RIA(+), CEP(-)	8	20
TOTAL	(38)	(68)
False positives:		
Anti-guineapig protein	0	10
Indeterminate	0	74

The solid phase RIA is reported to be up to 500 times more sensitive than CEP in detecting HB_S Ag in serial dilutions of known positive sera (Cossart, 1973). Used as a screening test in blood donors, RIA has detected as many as 10 times the number of positives detected by CEP (Prince, 1973). Recently, most of this apparent difference in sensitivity has been found to be due to non-specific reactions to guinea pig and other proteins in the test system (Alter, 1972) and revised estimates of sensitivity now indicate that RIA will detect only 2 to 3 times the number of "true positives" as CEP. Our results suggest that the actual difference in sensitivity is much more modest, being only on the order of one-quarter greater than CEP. Whether the discrepancy between our results and those of others is due to differences in technique, or in populations sampled, remains to be determined.

Abbott Laboratories has recently modified the RIA kit by adding guineapig serum to the radioactive labeled anti-HB_S (Leers, 1973). By this step it is hoped that the problem with non-specific reactivity to guineapig protein will be overcome. Although we have had limited experience with the new test system, the data in this report suggest that its use would have resulted in only a 10% decrease in the number of

false positives detected by RIA.

The majority of false positives reactions appear to be what we have called "indeterminate false positives." The exact cause of this reaction is unknown, but it is presumably due to other serum components within the test system. In this survey none of 49 asymptomatic subjects whose sera were initially classified as indeterminate false positive had abnormal test results when rebled 3 months later; while, in contrast, each of 7 subjects who were classified as anti-guineapig false positive was RIA positive 3 months later, and 28 (90%) of 31 true positive asymptomatics were again RIA positive after 3 months. These findings indicate that the factors responsible for indeterminate false positive tests were transient in their occurrence, and may be due to temporally associated phenomena in the host or in the test system. A new, as yet unlicensed, RIA test from Abbott appears to significantly decrease this second type of false positive reaction by altering time and temperature of incubation (Personal communication, Abbott Labs).

Absorption tests may occasionally reveal that sera which are strongly positive by RIA but negative by CEP cannot be inhibited either by normal guineapig serum or by guineapig anti-HB_S. In this study and in at least one other (Cossart, 1973) the cause was found to lie in subtype specificity of the anti-HB_S used in the inhibition tests. Sera containing subtype Y may be missed unless subtype specific antisera are used; most commercial anti-sera are usually satisfactory for subtype D (Cossart, 1973). RIA positive, CEP negative sera with relatively low radioactivity counts (CPM $<$ 2.1 times mean) are difficult to subtype by absorption-inhibition testing because of weak reactivity. However, when inhibition of sera with elevated CPM can be obtained by anti-HB_S differing specificities (anti-ayw vs anti-adw), it is possible to confirm not only the presence of HB_S Ag but to suggest that the individual serum was of the subtype inhibited by the appropriate anti-HB_S. Theoretically, it may be possible to distinguish HB_S Ag determinants W and R by this method (Bancroft, 1972).

For research purposes, our laboratory uses 3 SD units above the mean on 2 different occasions as the criterion of a positive RIA test. Sera that are negative by CEP then undergo absorption tests for specificity. However, as demonstrated in Table 13, the results of specificity testing can largely be inferred from the RIA test itself: high radioactivity counts ($>$ 50 SD above the mean) provide reasonable assurance that the serum is a true positive, whereas low counts ($<$ 20 SD above the mean) indicate the strong probability that the serum is a false positive.

With serially diluted CEP^+ sera, HB_S Ag can be routinely detected out to the level of 10^{-4} of 10^{-5} using 3 SD above the mean as indicative of HB_S Ag. However, an individual serum obtained from a clinical source having CPM equal to points on a standard HB_S Ag titration curve cannot be assumed to contain HB_S Ag without specificity testing.

The need for further evaluation and refinement of the RIA test for HB_S Ag is obvious. However, it remains the most sensitive of the available tests, and the problems of specificity can be overcome by means of appropriate procedures.

B. Comparison of Hyland and Abbott radioimmunoassay test for HB_S Ag

The anticipated advantages of increased sensitivity in HB_S Ag detection has given rise to a variety of RIA tests. The only licensed test, Ausria RIA (Abbott) employs guineapig anti-HB_S. Hyland Labs have developed an RIA test which uses goat anti-HB_S. A comparison of these 2 RIA kits against a panel of HB_S Ag sera at WRAIR was undertaken. The procedure for the Ausria test has been described elsewhere (Section II-A). The method for the Hyland RIA test is described in pages 10-15 of the Hyland Manual. The advantages of the Hyland test are mainly in the conservation of time required for completion of the test (four hrs vs 20 hrs for Abbott test).

A summary of the serum panel tested is listed in Table 14:

Table 14 - Summary of Abbott vs Hyland RIA test for HB_S Ag

	Abbott	Hyland
A positive control (HB_S Ag^+)	2/3 positive	0/3 positive
B positive control (HB_S Ag^+)	3/3 positive	3/3 positive
WRAIR samples:		
HB_S Ag positive by CEP^+	17/17 positive	17/17 positive
RIA^+ (Ausria) (CEP-)	5/5 positive	4/5 positive
Abbott negative control	0/10 positive	3/10 positive**
Abbot false positive	1/1 positive*	0/1 positive

*Not inhibited by specific anti-HB_S

**Positive values of Abbott negative control serum fall into ratio range 2.0-2.6

In general there appears to be a difference in detecting HB_S Ag. The Hyland RIA test did not detect the A positive control (Hyland control) nor 1 RIA^+ (Ausria), CEP- serum.

The Hyland kit did not identify the one serum known to be false positive by Ausria. Hyland detected with greater sensitivity than Ausria its own coded sensitivity panel, a dilution curve of HB_S Ag.

Since this panel was done, Hyland has modified its anti-HB_S to contain 20% normal goat serum which will hopefully eliminate nonspecific reactions to goat protein. Further testing of the Hyland test is in order before routine use can be advocated.

C. Detection of anti-HB_S by passive hemagglutination (PHA) and radioimmunoassay inhibition (RIAI)

PHA (Vyas 1970) is a rapid and sensitive method for detecting anti-HB_S. The presence of anti-HB_S is determined by agglutination of human type O erythrocytes coated with HB_S Ag/adw or ayw subtype in the presence of antibody. Advantages in sensitivity are outweighed by the expense of the assay and the short half-life of coated erythrocytes (less than 3 days). With the development of sensitive methods for detecting HB_S Ag (i.e., RIA), it appeared that a comparably sensitive assay for anti-HB_S might be made by inhibiting standard quantities of HB_S Ag (RIAI) (Bancroft 1973).

A comparison between PHA and RIAI of the sensitivity for detecting anti-HB_S was made from a serum panel identified as having antibody by PHA. As previously noted, when screening large numbers of sera by PHA with red cells coated with either HB_S Ag/adw or HB_S Ag/ayw, 2 types of reactions were observed: 1) Most sera agglutinate red cells labeled with either adw and ayw HB_S Ag (Table 15); and 2) Some sera agglutinate only one type of cell. A sample of these sera were titered to an end point by PHA. The same sera were then diluted 2-fold serially and preincubated with standard HB_S Ag of ayw or adw subtype. A 50% or more recuation in counts per minute of HB_S Ag preincubated with serum containing antibody by PHA as compared to controls (a dilution correction factor was applied) was taken as evidence of anti-HB_S. Table 15 compares the sensitivity of PHA to RIAI for detecting anti-HB_S using 2 different concentrations of HB_S Ag in RIAI. The RIAI test proved less sensitive than PHA for detection of anti-HB_S.

Tested were 15 sera that had titers of anti-HB_S by PHA ranging from 1:32-1:4056 against ayw coated red cells and 1:16-1:4056 to adw coated red cells. Only 4 of the 7 sera with PHA HB_S Ag/ayw titers $\geq$ 512 showed anti-HB_S by RIAI and but one of 8 sera with a PHA titer $<$ 512 had antibody by RIAI. Comparable results were obtained when the same sera were run against HB_S Ag/adw.

Table 15 - Detection of anti-HB_S by PHA and RIAI

Serum number	RIAI titer* HB_S Ag/ayw		PHA titer ay red cells	RIAI titer* HB_S Ag/adw		PHA titer ad red cells
	1:50	1:100		1:50	1:100	
F-4	1:4	1:4	1:32	0	0	1:16
F-49	0	1:4	1:1024	1:8	1:16	1:512
F-52	0	0	1:128	0	1:8	1:256
F-80	0	0	1:32	0	1:4	1:1024
F-93	C	0	1:64	0	1:8	1:2048
F-96	0	0	1:512	0	0	1:128
F-123	1:8	1:16	1:1024	1:64	1:128	1:4056
F-143	0	1:4	1:128	0	1:8	1:2048
F-180	0	0	1:256	1:4	1:8	1:512
F-188	1:8	1:16	1:4056	1:16	1:64	1:1024
F-189	0	0	1:2048	0	0	1:128
F-200	1:8	1:16	1:4056	1:32	1:64	1:4056
F-204	0	0	1:256	0	0	1:256
F-212	1:8	1:16	1:4056	1:16	1:32	1:1024
F-220	0	0	1:128	0	1:4	1:4056

* Dilution of HB_S Ag used

Sera that agglutinated only one type of HB_S Ag coated red cells (adw or ayw) showed anti-HB_S only to the same subtype of HB_S Ag by RIAI.

D. Detection of antibody to Dane particle cores

Recently a second antigen-antibody system differing from HB_S Ag has been described for HBV; the antigen is the core antigen of Dane particles (Almeida 1971). A complement fixation test to detect anti-HB_C (anti-core antigen) has been developed by Hoofnagle (1973) at the National Institutes of Health. A small panel of sera from acute

hepatitis cases at Fort Hood, Texas, has been tested by CF for the presence of anti-HB_C. A summary of this work is listed in Tables 16 and 17. Paired acute and convalescent sera of 10 HB_S Ag^+ and 5 HB_S Ag^- hepatitis cases were tested. Eight of 10 soldiers with hepatitis and HB_S antigenemia had detectable anti-HB_C during their acute illness; three months following acute illness, when HB_S Ag was no longer detectable by RIA, all 10 had anti-HB_C. Five of the 8 with anti-HB_C in acute serum had a 4-fold or greater drop in their titer at three months.

Table 16 - Anti-HB_C detection in Fort Hood hepatitis cases with only acute serum specimens

HB_S Ag^+ serum no.	Anti-HB_C titer	3-mo. convalescent serum	Anti-HB_C titer
69	1:4	A-83	1:16
452	1:11	A-88	1:4
480	1:32	A-94	1:64
172	1:256	A-102	1:64
145	1:512	A-106	1:128
60	1:1024	A-107	1:512
44	1:256	A-113	1:32
155	1:4	A-115	1:32
491	1:2048	A-124	1:4
33	1:512	A-128	1:16
HB_S Ag^- serum no.			
46	AC*	A-103	1:4
499	AC	A-108 AC	AC
130	1:400	A-109 AC	1:8
66	AC	A-110	4:0
179	1:16	A-114	1:16

* Serum anticomplementary

Table 17 - Anti-HB_c detection in Fort Hood hepatitis cases with only acute serum specimens

HB_s Ag^+		HB_s Ag^-	
Serum number	Core Ab titer	Serum number	Core Ab titer
2204	1:512	2311	1:256
2205	1:256	2316	-
2206	1:4	2318	AC
2209	1:1024	2324	-
2214	1:256	2328	-
2223	1:2048	2330	1:16
2228	1:4	2333	-
2231	1:32	2334	1:32
2242	1:256	2335	-
2243	1:256	2336	1:128

In an additional 10 patients with Hepatitis B (and only acute serum available for testing), 8 had detectable anti-HB_c in acute serum. In this small sample, 16 of 20 Hepatitis B patients with antigenemia had anti-HB_c.

Of 15 HB_s Ag^- acute hepatitis cases (10 only single specimen), 5 had anti-HB_c. Four additional people had anti-complementary activity. Convalescent sera from five acute hepatitis cases without HB_s Ag or anti-HB_c in acute sera were tested for anti-HB_c and an additional 2 people had anti-HB_c 3 months after hepatitis onset.

These preliminary findings suggest that anti-HB_c tests in combination with assays for HB_s Ag and anti-HB_s may provide increased sensitivity in etiologic diagnosis of HBV.

A comparison of HB_s Ag^+ and $^-$ soldiers with acute hepatitis at Fort Hood revealed no difference in levels of SGOT or bilirubin of an average hospitalization duration of 28 vs 18 days, respectively.

E. Studies of HB_S Ag carriers

1. Hepatitis B_S antigen and antibody in families of asymptomatic HB_S Ag carriers

In order to define the risk of Hepatitis B infection in close contacts of an asymptomatic carrier of HB_S Ag, a serologic survey of family contacts of carriers identified at Fort Hood, Texas was undertaken. There was a high prevalence of hepatitis B infection in family contacts as compared to controls (Table 18). Presumably, the acquisition of HB_S Ag in families of carriers is by other than the parenteral route. In view of the high prevalence of HBV infections in family members of asymptomatic HB_S Ag carriers, it is recommended that they be tested for HB_S Ag and anti-HB_S. Those with HB_S Ag should be evaluated by liver function tests and liver biopsy is indicated if persistent abnormalities are noted.

Table 18 - Hepatitis B infection in families of HB_S Ag carriers and controls

	Carrier families	Control families
Number SPOUSES tested:	17	11
With HB_S Ag	0	0
With anti-HB_S	10	2
With past history of hepatitis	2	2
Number CHILDREN tested:	35	22
With HB_S Ag	2	0
With anti-HB_S	4	1
With past history of hepatitis	4	0

2. Viral antibody titers in asymptomatic HB_S Ag carriers, Hepatitis B cases, and normal controls

The immunologic mechanism responsible for persistent HB_S antigenemia is still an enigma. Patients with known defects in their immune system (those with lymphatic malignancies, leukemia, renal dialysis patients, etc.) have a high prevalence of antigenemia. A possible reason for the carrier state is related to immunosuppression due to the underlying disease and/or its treatment.

Some people, however, carry HB_S Ag in their blood chronically without evidence of any clinical disease and with entirely normal liver functions. Preliminary work with in vitro systems measuring T-cell (Thymus) function indicate that a defect in cellular immunity (CMI) may be important in the pathogenesis of the asymptomatic carrier (Yeung, 1971; Dudley, 1972; Smith, 1971). The CMI defect, however, is not total anergy because of normal in vivo response to classical skin test antigens (mumps, trichophyton, Monilia and streptokinase, streptodornase (Irwin, 1972, unpublished data).

In an effort to determine if HB_S Ag carriers differed from normals in antibody response (B-cell) to common viruses, a survey of asymptomatic HB_S Ag carriers, Hepatitis B patients and normal controls was undertaken at Fort Hood. A standard complement fixation test was used to measure antibody titers to cytomegalovirus, Herpes simplex, Varicella, mumps, Rubeola, respiratory snycytial virus, mycoplasma, influenza A and B, adenovirus and lymphogranuloma venereum. As shown in Table 19 there was no difference in the distribution of CF antibody titers among the groups.

Thus, it would appear that asymptomatic HB_S Ag carriers have a distribution of antibody titers to common viral antigens similar to control patients. Therefore, if asymptomatic carriers are immunologically tolerant to HB_S Ag, the tolerance is very narrow and possibly limited only to HB_S Ag since their immune response to other viruses as measured by CF antibody titers is normal.

F. Detection of Hepatitis B_S Ag in saliva, urine and stool

Hepatitis B antigen (HB_S Ag) has been detected in random samples of various body fluids, excretions, or secretions including saliva (Ward, 1972), urine (Tripatzis, 1971; Apostolov, 1971; Ogra, 1973), stool (Grob, 1971), seminal fluid, breast milk and menstrual blood. Since ingestion of serum containing HB_S Ag has been shown to transmit Hepatitis B infection orally (Krugman, 1967), the presence of HB_S Ag in these fluids implies the potential for transmission of Hepatitis B virus (HBV) by the non-parenteral route. The purpose of this survey is to determine the excretion of HB_S Ag in saliva, urine, and stool of acute hepatitis cases and asymptomatic HB_S Ag carriers for excretion of HB_S Ag.

Table 19 - Viral antibody titers in acute hepatitis B cases, asymptomatic carriers and controls

Virus	Cumulative %					
	4	4	8	16	32	64
CMV:						
Hepatitis B	47.6	32.3	61.8	90.3	100.00	-
Control	29.5	38.5	61.2	88.2	100.00	-
Carrier	31.2	37.4	62.4	90.5	100.00	-
HERPES:						
Hepatitis B	38.0	42.7	47.4	71.2	95.0	100.00
Control	34.0	36.2	40.7	58.8	97.4	100.00
Carrier	18.7	28.0	34.2	56.0	96.6	100.00
MUMPS:						
Hepatitis B	47.6	52.3	90.3	100.00	-	-
Control	38.6	56.7	79.4	95.3	-	100.00
Carrier	37.5	68.7	84.3	93.6	-	100.00
RUBEOLA:						
Hepatitis B	14.3	42.8	76.1	95.1	100.00	-
Control	29.5	47.6	74.6	92.7	97.2	100.00
Carrier	21.8	59.3	87.4	96.7	100.00	-
MYCOPLASMA:						
Hepatitis B	38.0	71.3	95.1	100.00	-	-
Control	56.8	77.2	95.3	100.00	-	-
Carrier	59.3	71.8	96.8	-	100.00	-

Cont'd on next page

Table 19 - Cont'd

LGV:						
Hepatitis B	33.3	71.3	100.00	-	-	-
Control	40.4	64.2	85.6	100.00	-	-
Carrier	46.6	73.2	89.8	96.4	-	100.00
VARICELLA:						
Hepatitis B	57.1	76.1	95.1	100.00	-	
Control	23.8	61.9	88.0	92.7	100.00	-
Carrier	23.3	59.9	89.9	93.2	100.00	-
RESPIRATORY SYN:						
Hepatitis B	33.3	80.9	95.2	100.00	-	-
Control	19.5	50.5	93.3	98.0	100.00	-
Carrier	26.6	53.2	89.8	96.4	100.00	-
INFLUENZA B:						
Hepatitis B	4.8	38.1	80.9	100.00	-	-
Control	9.0	54.4	93.0	97.5	100.00	-
Carrier	9.3	43.6	84.2	100.00	-	-
ADENOVIRUS:						
Hepatitis B	19.0	57.0	90.3	100.00	-	-
Control	20.4	52.2	84.0	100.00	-	-
Carrier	21.8	53.0	90.5	100.00	-	-
INFLUENZA A:						
Hepatitis B	-	-	23.8	66.6	85.6	100.00
Control	-	13.4	36.1	86.1	100.00	-
Carrier	3.1	15.6	37.4	81.1	96.7	100.00

Saliva, urine and stool samples were collected from the following populations: (1) Patients with hepatitis hospitalized at Darnall Army Hospital, Fort Hood, Texas, and at Walter Reed General Hospital,

Washington, D. C.; (2) Patients hospitalized on the orthopedic service and non-hospitalized military personnel without hepatitis served as controls; (3) Asymptomatic carriers of HB_S Ag, all of whom had normal liver function tests and persistent antigenemia for at least 3 months.

Approximately 5-10 mls of saliva, and 100 mls of first morning urine were collected twice a week on all hepatitis cases and controls during the course of hospitalization. A stool specimen was obtained once a week. Saliva and urine samples were collected in sterile 1 and 4 ounce jars respectively and stored at -20°C until the time of testing for HB_S Ag. In 6 hepatitis patients, 24-hour urine collections were obtained. Blood was collected from all patients, carriers and controls studied and the serum stored at -20°C. Convalescent samples of blood, urine, and saliva were obtained on most acute hepatitis cases approximately 3 months later. Reagent strips (Bili-Labstix-Ames Co.) were used to detect blood in saliva and urine. Hematest tablets (Ames Co.) were used for testing for blood in stool.

Because HB_S Ag was not found in any unconcentrated urine samples, morning urine samples (100 ml) were concentrated 100 times by filtration across PM 30 Amicon membranes. Following the concentration procedure the supernate as well as a 1-2 ml eluate of the PM-30 membrane were stored at -20°F until testing for HB_S Ag. Twenty-four hour urine samples were concentrated 250-500 times over a single membrane and processed as above.

Stool samples (20 Gms) were homogenized in 100 ml of Tris buffer and then concentrated 100 times as above. Aliquots (.2 cc) of supernatant and eluates were adjusted to a pH of 2.0 with HCl, since preliminary tests with acid treatment of stool with added serum containing HB_S Ag^+ tended to increase the counts per minute of HB_S Ag recovered. Both acid treated and untreated concentrated samples were tested for HB_S Ag.

Aliquots (0.1 ml) of concentrated urine and stool specimens and unconcentrated saliva and serum samples were processed as described for serum by radioimmune assay (RIA) using the Ausria test kit. Saliva was not concentrated because of limited quantities. HB_S Ag positive controls of saliva, urine, and stool were made by diluting counterelectrophoresis (CEP) positive HB_S Ag sera (10^{-1} - 10^{-6}) in the respective material obtained from normal individuals without antigenemia. For each experiment, in addition to these dilution curves, 10 replicates of an HB_S Ag negative control of saliva, concentrated urine, or stool were included. A specimen yielding counts per minute (CPM) $>$ 5 standard deviations above the mean of the 10 replicates of the appropriate control was considered to be a positive test. Because false positive RIA reactions have occurred with the RIA test (Sgouris, 1973; Prince, 1973; Alter, 1973), all samples $>$ 5SD were tested for specificity by an inhibition test using antibody to surface antigen (anti-HB_S) made in guineapigs and rabbits to purified HB_S Ag.

HB_S Ag was detected in the saliva of 25% of patients with acute HB_S Ag^+ hepatitis, 34% of asymptomatic HB_S Ag carriers, and in one case, 5%, of acute hepatitis without demonstrable antigenemia. This latter patient had HBV infection confirmed by anti-HB_S rise in convalescent serum (Table 20). Because of limited volumes of saliva, only unconcentrated samples of saliva (0.1 ml saliva) were tested. The lack of correlation between HB_S Ag and detectable blood in individual saliva specimens is shown in Table 21.

Table 20 - Detection of HB_S Ag in unconcentrated saliva by RIA

Group	Serum	No. HB_S Ag^+ / No. tested	Number samples tested	No. with HBs Ag
Acute hepatitis	HB_S Ag^+	5/20	84	12
	HB_S Ag^-	1/20	66	1
Asymptomatic carriers	HB_S Ag^+	14/41	41	14
Non-hepatitis patients	HB_S Ag^-	0/35	82	0
Healthy controls	HB_S Ag^-	0/77	77	0

Table 21 - Relationship between salivary HB_S Ag and blood in individual specimens from 61 antigenemic persons

Saliva	HB_S Ag detected	HB_S Ag not detected	Total
Blood detected	16	59	75
Blood not detected	10	40	50
TOTAL	26	99	125

* Blood was detected in 43/92 HBs Ag negative control specimens from 45 people.

Using 100 X concentrations of urine, positive RIA tests were obtained from 26 of 63 (41%) people with HB_S Ag with acute hepatitis and asymptomatic carriers (Table 22). Five of 105 control subjects (4%) also yielded a positive RIA test. Specificity for HB_S Ag generally was not commonly confirmed on 100 ml concentrated urine samples because of low counts per minute (CPM); only 7 of the 26 positive samples were indeed confirmed as HB_S Ag. However, when 24-hour urine collections from one of three antigenemic patients tested were concentrated 500 X, specificity for HB_S Ag was confirmed in one patient with positive urine. Blood or greater than a trace of protein were not detected in any urine samples. HB_S Ag detected had normal BUN levels.

Table 22 - Presence of HB_S Ag in concentrated samples of urine by RIA

Urine samples	Serum	No. with RIA / No tested	No. samples tested	HBs Ag specificity confirmed / positive RIA test
First morning urine concentrated 100 X:				
Acute hepatitis	HB_S Ag$^+$	4/20	73	0/6
	HB_S Ag$^-$	1/18	65	0/1
Asymptomatic carriers	HB_S Ag$^+$	7/43	45	7/19
Patients	HB_S Ag$^-$	5/35	70	0/5
Healthy controls	HB_S Ag$^-$	0/67	67	0
24-hr. urine samples concentrated 500 X:				
Acute hepatitis	HB_S Ag$^+$	1/3	12	5/5
	HB_S Ag$^-$	0/3	12	0

Table 23 lists the presence of HB_S Ag in stool. Although 5 of 19 stool samples from patients with hepatitis gave positive RIA tests, they could not be confirmed as HB_S Ag due to low CPM. A comparison of the CPM by RIA of serial 10-fold dilutions of known HB_S Ag^+ controls (made by diluting CEP^+ HB_S Ag in normal saliva, urine, or stool) to clinical samples of saliva, urine and stool from antigenemic people, revealed that CPM for concentrated urine and stool clinical samples were low and located in the range of 10^{-3} to 10^{-4} points on the HB_S Ag dilution curve. HB_S Ag was found in unconcentrated clinical saliva specimens.

Table 23 - HB_S Ag in stool by RIA

	Serum	Number people	Number samples	Number HB_S Ag^+
Acute hepatitis	HB_S Ag^+	7	11	2*
	HB_S Ag^-	4	5	3*
Asymptomatic carriers	HB_S Ag^+	3	3	0
Normal controls	HB_S Ag^-	5	5	0

*Specificity for HB_S Ag by RIA not confirmed due to low CPM. No blood found in stool.

There was no definite correlation of HB_S Ag in saliva, urine or stool with antigenemia during the course of hospitalization. This probably is due to the sporadic detection of antigen in sequential saliva, urine and stool specimens as shown in Table 24.

Samples of blood, urine and saliva were obtained on 11 of 20 antigenemic individuals approximately 3 months after onset of hepatitis B. HB_S Ag was detected in the blood of only one individual at that time and in the saliva of another person, but not in any urine specimen. From 9 of 20 acute hepatitis cases without HB_S Ag studied 3 months into convalescence, HB_S Ag was not detected in any samples of blood, urine or saliva though 6 had developed anti-HB_S by passive hemagglutination.

Table 24 - Patterns of HB_S Ag detection in saliva, urine and stool

	Days							
	1	5	10	20	30	45	60	90
Patient 1 (Acute hepatitis):								
Serum HB_S Ag	+	+	+	+	+	+	+	ND
Saliva HB_S Ag	+	+	0	+*	+	0	+*	ND
Urine HB_S Ag	0	0	0	0	0	0	0	ND
Stool HB_S Ag	ND	0	ND	0	ND	ND	ND	ND
Patient 2 (Acute hepatitis):								
Serum HB_S Ag	+	+	+	+				0
Saliva HB_S Ag	0	0	0	0		No samples obtained		0
Urine HB_S Ag	+	+	0	0				0
Stool HB_S Ag	ND	+**	0	ND				ND
Patient 3 (Asymptomatic carrier):								
Serum HB_S Ag	+	+	+	+				+
Saliva HB_S Ag	0	ND	+	+		No samples obtained		0
Urine HB_S Ag	+	0	ND	+				+
Stool HB_S Ag	ND	ND	ND	ND				ND

*Presence of blood in saliva.

ND - Not done.

**Specificity for HB_S Ag not confirmed.

In this survey, aside from serum, HB_S Ag was most frequently found in unconcentrated saliva. The detection of HB_S Ag in the saliva as well as other substances was sporadic and its presence was not dependent on duration of antigenemiz or the presence or absence of HB_S Ag in previous saliva samples in acute hepatitis cases.

As suggested by Ward and confirmed in this survey, blood in saliva appears to occur with high frequency. However, in this study HB_S Ag may be found in saliva without detectable blood. Indeed, HB_S Ag was detected in 20% of all saliva specimens tested whether or not they contained detectable blood.

In urine obtained from individuals with acute hepatitis, and asymptomatic carriers, HB_S Ag was detected only after 100-fold or greater concentration. In all cases the CPM obtained by RIA on these concentrated clinical specimens were low. (As compared with dilution curves of HB_S Ag, the concentration of HB_S Ag in clinical specimens of urine or stool is estimated to be 10^{-5} - 10^{-7}, that of serum HB_S Ag used for comparison).

Some stool specimens from hepatitis cases yielded low CPM which could not be confirmed as HB_S Ag. Intestinal inhibitors of HB_S Ag as well as biodegradation of HB_S Ag are known to occur in stool

Except for the saliva of one individual, HB_S Ag was not found in saliva or urine of patients 3 months after onset of Hepatitis B. However, persistent HB_S Ag by CF in the urine has been reported following clearance of antigenemia; however the specificity of the antigen detected in this paper was not confirmed.

Our findings suggest that if HB_S Ag is present in the serum, it is potentially in the saliva, urine, and/or stool. While a correlation between HB_S Ag with infectivity in excretions remains to be determined, until infectivity tests are possible, these excretions should be assumed to be infectious during antigenemia.

III. Characterization of HB Ag

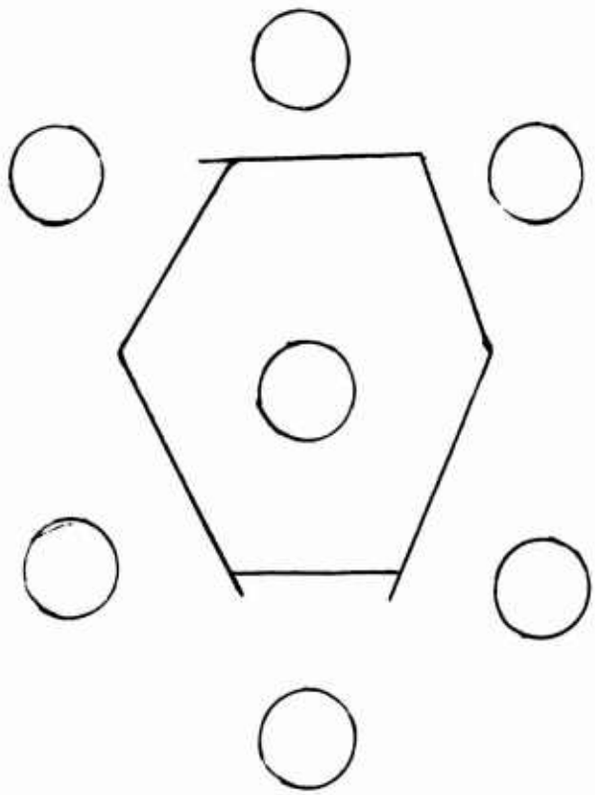
A. E antigen

Apart from the antigenic determinants previously characterized on the surface of HB_S Ag (a, y, d, w, and r), another antigen system always associated with HB_S Ag but not on the same particle as the other determinants, has been described by Magnius (1972). This new antigen has been called e Ag and is found in sera of patients ill with Hepatitis B with HB_S Ag and particularly chronic dialysis patients with HB_S Ag. The nature and biologic significance of this new antigen has yet to be determined. Antibody to e Ag has only been found in asymptomatic HB_S Ag carriers after serum concentration.

In the past year a carrier of HB_S Ag/adw has been identified whose serum also contains e antibody. The discovery was made fortuitously in the course of routinely subtyping his HB_S Ag against WRAIR standard antisera and antigens (Figure 3). The serum sample, EH 421, has been shown to contain at least 2 specificities in agar gel immunodiffusion:

(1) for e Ag as determined by lines of identity between e Ag obtained from the original investigator (Magnius) and an e Ag standard provided by Professor G. L. Le Bouvier of Yale Medical School; (2) a second specificity which has been designated Z antigen and is described below.

Using this antiserum to e and Z antigens, two additional sera with e Ag have been identified. Because these sera containing e antigen in low titer are limited in volume, the search for adequate amounts of e Ag continues so that sufficient quantities will be available for purification and chemical analysis.



1. Standard HB_S Ag/ayw
2. Serum EI4, unknown HB_S Ag
3. Serum EI4, unknown HB_S Ag
4. Standard HB_S Ag/adr
5. Serum EH 421, unknown HB_S Ag (also contains antibody to e & z antigen)
6. Serum EH 421
7. Standard rabbit anti-HB_S/ayw

Figure 3 - Detection of e and z antibody activity serum EH 421; subtyping of HB_S Ag.

B. Z antigen

As described above in the search for reagents in the e Ag-Ab system, a second specificity of antisera obtained from a HB_S Ag/adw carrier was found. This second Ag-Ab system appears to give a reaction comparable to the e Ag-Ab system in agar gel but is not unrelated to either e Ag or HB_S Ag. Indeed the reaction is found often in normal human serum.

Priority was placed on obtaining an antigen with "Z" reactivity that was sufficiently purified to permit animal immunization. During the course of these studies many physical properties of the antigen were ascertained. A few of the more important of these properties are as follows:

1. The antigen can be quantitatively precipitated using 60% saturated ammonium sulfate.

2. A peak of antigenic activity is eluted from G-200 Sephadex slightly behind the void volume which contains the majority of the Au antigen. Fractions containing Z antigen have been shown by precipitin analysis to be contaminated with IgA, IgE, IgM, fibrinogen, transferrin and alpha-2-macroglobulin (Fig. 4).

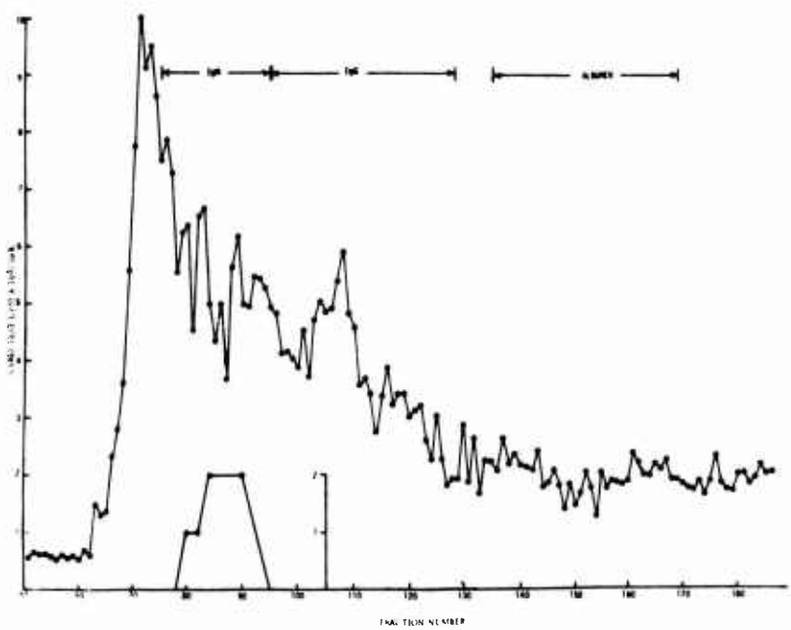


Figure 4. Elution of HB_S Ag from G-200 Sephadex column. HB_S Ag was detected by conventional Ausria test. "Z" Ag was detected by immunodiffusion & the intensity of the precipitin line indicated as $1\text{-}4^+$

3. The antigen can be pelleted by centrifugation at 42,000 RPM for 3 hours. This procedure requires a partially purified starting material such as Sephadex G-200 eluted peak to eliminate the massive pellet obtained by centrifuging plasma.

4. The density of the "Z" antigen appears in the range of 1.26-1.30 following isopycnic banding in cesium chloride.

5. Electron micrographs from density purified Z antigen failed to reveal any characteristic morphology.

6. The isoelectric point of the Z antigen has been shown to be in the range of pH 4.9-5.2. Precise determination is difficult due to the protein precipitation in the column.

Animals are currently under immunization with semi-purified Z antigen. Rabbits have been injected with material that has been separated on G-200 Sephadex, precipitated by ammonium sulfate and isoelectric focused. The only detectable contaminating normal human serum proteins were transferrin and alpha-2-macroglobulin.

Z antigen has been tested against the following specific antisera and found not to react with identity to any: anti-IgA F ab, anti-Ig A, anti-IgG F_C, anti-kappa (L chain), anti-llambda (L-chain), anti-C_4, anti-C_3, anti-CRP, and anti-HB_S.

The relationship of the 2 antigens and antibodies to hepatitis B infection has yet to be determined. The production of animal antisera will hopefully allow for further analysis of these systems.

C. Preparation and distribution of HB_S Ag subtyping reagents

With the discovery of the w, r, HB_S Ag antigenic determinants by LTC Bancroft (now Chief of Virology, Bangkok) many requests for typing antisera and standard antigens have been received. Because of limited supplies and difficulty in obtaining suitable subtype specific precipitating antibody, only small aliquots of these reagents, to be used as reference standards, can be supplied to selected investigators. Table 25 lists the people to whom reagents have been sent in the past year. Investigators from Japan, Spain, Australia, France, Sweden, Hong King, Iran and the USA received typing antiserum during the past year.

Table 25 - Distribution of subtype reagents for w, r, HB_S Ag determinants

Name	University
Yuzo Miyakawa, M. D.	Univ. of Tokyo, Hongo, Tokyo, Japan
Dr. J. Pedreira	Dept de Medicena Interna Paseo Valle Heleron Barcelona 16 Spain
Yorio Hinuma, M. D.	Dept of Microbiology Kumamoto University Medical School Kumamoto, Japan
R. A. Hawkes	Dept of Medical Microbiology University of New South Wales P. O. Box 1 Kensington, N. S. W. - Australia
Professeur Soulier	National Blood Bank Paris, France
Eric Nordenfelt	Institutionen for Medicinsk Mikrobiologi Solvegatan 23 Lund, Sweden
A. Chedid, M. D.	Dept of Pathology University of Cincinnati Cincinnati, Ohio 45221
Anthony K. Y. Lee, M. D.	Department of Medicine Queen Mary Hospital University of Hong Kong Hong Kong
Dr. M. A. Eftekhari	Institu Pasteur Del Iran Teheran

Electronucleonics Lab, Inc. prepared under contract 3 HB_S Ag^+ plasma units, (1 adw, 2 ayw) for use in raising antisera to these HB_S Ag subtypes. Rabbit anti-HB_S Ag/adw was obtained from the HB_S Ag/adw plasma unit. Antisera to HB_S Ag/ayw was not obtained on the first

attempt. Three ultracentrifugation cycles in Cs Cl are routinely used to purify HB_S Ag from plasma and degradation of antigen occurs in the third cycle. Antisera to HB_S Ag/ayw are now being prepared using pools derived from the second cycle centrifugation. This method appears at present to be more satisfactory.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 105 Mechanisms of Transmission of Hepatitis Viruses

Literature Cited.

References:

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6. Cherubin et al (1972) Lancet ii: 149
7. Cossart et al (1973) Lancett ii, 1333
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12. Heathcote et al (1974) Lancet i: 71
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Publications:

1. Irwin, G. R., Allen, A. M., Bancroft, W. H., Karwacki, J. J., Pinkerton, R. H. and Russell, P. K. Hepatitis B antigen and antibody: Occurrence in families of asymptomatic HB Ag carriers. JAMA 227: 1042-1043, 1974

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6477	74 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	H. Termination	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	107
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code): (U)Treatment of pain arising from combat related peripheral nerve injuries by transcutaneous stimulation technique (TST)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 012900 Physiology 016200 Stress Physiology 013400 Psychology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 07	73 07	DA	C. In-House

17. CONTRACT/GRANT: NA
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 73	1.5	25
	CURRENT 74	2.0	30

19a. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL
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TELEPHONE: 202-576-3351

21. GENERAL USE

19b. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Division of Neuropsychiatry
Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Hegge, F.W., Ph.D.
TELEPHONE: 202-576-5521
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Jennings, CPT J.R.
NAME: Tahmoush, MAJ A.J. DA

PII Redacted

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Pain; (U) Peripheral Nerve Injury; (U) Analygesics; (U) Human Volunteer; (U) Psychophysiology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Peripheral nerve injury, frequently resulting from a high velocity missle, may result in causalgia - a state of local hypersensitivity causing chronic pain. Transcutaneous stimulation techniques may relieve this pain and other forms of currently intractable pain. The validity of this analgesic method and the nature of its physiological mechanism are being identified.

24. (U) Using psychophysical and psychophysiological techniques, the amount of pain an individual experiences is measured before and after low level electrical transcutaneous stimulation. The individual's verbal reports of pain and his peripheral physiological reactions are both analyzed. Methods are designed to insure that the volunteer experiences the minimum possible of pain and discomfort.

25. (U)73 07-74 06 Effort in this work unit has been transferred to 91C 0B 6508 WU 116. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 73 - 30 JUN 74.

* Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6502	74 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	H. Term.	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	112
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) System Study of Military Health Care Delivery

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 004200 Computers 016400 Subsystems 080400 Admin and Mgmt

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 03	74 06	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	.75	20
FISCAL YEAR CURRENT 74	2	42

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Div of Biometrics and Med Info Proc

ADDRESS: Washington, DC 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Hannegan, Michael W., LTC, MC

TELEPHONE: 202-576-2011

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Wilburn, James, LTC, MSC

NAME: Levy, James, MAJ, MC DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Computers in Medicine; (U) Health Care Delivery; (U) Military Medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To develop a systematic approach to implementation of computers to assist military health care providers to deliver care. These studies are of importance to military medicine in terms of more efficient care for patients.

24. (U) A multi-disciplinary group of physicians, nurses, pharmacists, dieticians, administrators, bioengineers, and computer specialists was formed to identify problem areas in information transfer in the delivery of health care in the military. This group has systematically addressed the solutions of the identified problems and has formulated a plan for implementation.

25. (U) 73 07 - 74 06 The multi-disciplinary group has met with other professionals to ascertain the requirements for military health care delivery. From this a systems study has been initiated, to formulate implementation plans for solving the identifier problems. The objectives of this research study have been completed and transferred to TRIMIS-Army

*Available to contractors upon originator's approval

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6504	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	114
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Neurophysiological Control of Antibody Response

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002400 Bioengineering 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 04	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	2	44
FISCAL YEAR CURRENT 75	2	44

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
Washington, D. C. 20012

ADDRESS:

RESPONSIBLE INDIVIDUAL

NAME: Buescher, E. L., COL

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Neuropsychiatry

ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)

NAME: Spector, N. H., Ph.D.

TELEPHONE: 202-576-3457

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Koob, G. F., CPT, MSC

NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code) (U) Neurophysiology; (U) Neuropsychiatry; (U) Disease; (U) Trauma; (U) Stress

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) The objective of this research is to study the role of central nervous system in the body's defenses against infectious diseases of military importance.

24. (U) Various parts of the brain seem to influence the general bodily response to systemic infections, thus stimulation or ablation of these brain areas provide a way of obtaining an understanding of how CNS mediated events influence resistance to infection or the course of the disease.

25. (U) 73 07 - 74 06 Experiments examining the influence of brain lesions on response to malaria infection continued. Analysis of data from these groups of experiments revealed that antibody levels at the end of the period of parasitemia were higher in both hypothalamic-lesioned and sham-operated animals as compared with non-operated controls. All infected animals showed non-significant changes in rectal temperature, but significantly elevated temperatures for 3 or 4 days following the return of parasitemia to preinfection levels. These experiments are being conducted in collaboration with the Division of Communicable Diseases and Immunology at WRAIR. Similar experiments utilizing diencephalic-lesioned mice infected with Newcastle's Disease Virus are in progress, in collaboration with Dr. Samuel Baron's laboratory at the National Institutes of Health. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

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PII Redacted

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 114 Neurophysiological control of antibody response

Investigators.
Principal: N. H. Spector, Ph.D.
Associate: LTC Carter L. Diggs, MC; CPT George F. Koob, MSC; MAJ Larry K. Martin, MSC

DESCRIPTION.

This research was undertaken to study the role of diencephalic "centers," particularly in the hypothalamus, in the control and regulation of antibody production, the immune responses and other mechanisms in the body's defenses against disease.

The resources of the Division of Communicable Diseases and Immunology and of the National Institutes of Health were utilized in several collaborative experiments.

PROGRESS.

Thirty-six male Sprague-Dawley rats were given intraveneous injections of Plasmodium berghei (NYU-2 strain). Twenty-four of these rats had small bilateral electrolytic lesions of the anterior, dorsomedial or posterior nuclei of the hypothalamus prior to induced parasitemia. Six sham operated animals received bilateral electrode penetration of the hypothalamus. Six animals served as unoperated controls. Food and water intake were carefully measured. Rectal temperature and levels of parasitemia were recorded over a six-week period. Antibody levels were measured by the indirect hemagglutination method before and after the administration of the parasite. All rats survived. As in our previous experiments, in the ensuing parasitemia there was, statistically, a significantly lower peak level for the unoperated controls as compared to each of the other four groups. The control group also had a significantly lower antibody titre as compared with all other groups. These results were unpredicted in view of the results of Korneva and Khai (1963) and of Tyrey and Nalbandov (1972) and others, but are not in conflict with the data reported by Ado and Goldshtein (1972). However, all of these authors dealt with antibody production in lesioned rodents after horse serum or albumin challenge, and we have employed a specific parasite leading to a disease state; hence, the results may not be comparable.

An analysis of the temperature responses of all rats used in these studies reveals a statistically significant elevation of rectal temperature lasting several days, but only following the reduction of parasitemia to pre-innoculation levels. A more detailed study of twenty-four-hour deep body temperature is under way, in which we will compare the rats' temperature responses to those of humans, during and after the course of the malarial infection.

Further studies on antibody responses and of the relationship between diencephalon and interferon response to viral injection are in progress. These experiments are being conducted in collaboration with the Departments of Medical Zoology and Immunology at WRAIR, and in the laboratories of Dr. Samuel Baron of the National Institutes of Health.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 114 Neurophysiological control of antibody response

Literature Cited.

References:

1. Ado, A. D. and Goldshtein, M. M.: The effect of bilateral destruction of certain structures of the medial hypothalamus on the formation of complement-binding antibodies. Biull. Eksper. Biologii i Med. 174 : 57, 1972.

2. Korneva, E. A. and Khai, L. M.: Effect of destruction of hypothalamic areas on immunogenesis. Fiziol. Zhur. SSSR. 49: 42, 1963; translation in Fed. Proc. Trans. Suppl. 23: 188, 1964.

3. Tyrey, L. and Nalbandov, A.: Influence of anterior hypothalamic lesions on circulating antibody titers in the rat. Amer. J. Physiol. 222: 179-185, 1972.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6506	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D.CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	115
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Behavioral Sc`eduling in Psychosomatic Disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002500 Clinical Medicine 013400 Psychology 012900 Physiology 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	Cont	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	1.5	62
FISCAL YEAR CURRENT 75	1.0	52

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS
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NAME: Hegge, F. W., Ph.D. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Operant Conditioning; (U) Stress (U) Psychosomatic Disease; (U) Autonomic Dysfunction;

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) This work unit is to systematically define functional relationships between behavioral variables and the pathegenesis of psychosomatic disorders, e.g., hypertension and duodenal ulcer, and autonomic dysfunction, to identify potentially effective measures for prevention and treatment of this class of disorders in military personnel.

24. (U) Empirically derived behavior principles are applied in studies of non-human primates to describe the functional relationship between behavioral variables such as, reinforcement, punishment, conditioned reinforcers and conditioned aversive stimuli and activity in a variety of physiological systems implicated in psychosomatic disorders. The focus is on arrangement of consequences for behavior and the parallel control such consequences maintain over physiologic variables. Techniques include the development of effective means of chronically monitoring physiologic status of the organisms as well as the continuous updating of appropriate operant technology as a prerequisite for the exploration of potentially productive methods of treatment.

25. (U) 73 07 - 74 06 Non-human primates are being exposed to increments in the aversiveness of performance demands required of them. Each was surgically prepared to permit direct observation of the gastric and duodenal wall and are being monitored for the development of chronic peptic ulcer. Exposure to this procedure produced superficial lesions in the region of the angulus. Lesions developed before the subjects had learned to respond appropriately to the task. Lesions were self-limited and, after disappearing, did not usually return, except for an acute duodenal lesion which never fully disappeared. Further research is being directed at changes in gastric physiology correlated with this behaviorally-induced pathology. For a technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 73 - 30 June 74.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 115 Behavioral scheduling in psychosomatic disease

Investigators.

Principal: CPT Frank J. Sodetz, MSC
Associate: MAJ Benjamin H. Natelson, MC;
Frederick W. Hegge, Ph.D.

Description.

The modification of autonomic function by perturbations in the environment has been well recognized for many years. However, only recently has the concept been extended to control by conditioned stimuli, that is, stimuli which acquire their controlling properties by virtue of repeated pairing with stimuli already effective in eliciting changes in autonomic activity. While appealing as a potential factor in the etiology of certain psychosomatic disorders, pathological responses to conditioned stimuli are known to be of limited duration occurring only in response to, and in the presence of, eliciting stimuli. It remained difficult to conceptualize long-term pathogenic changes in autonomic function resulting from brief exposures to such stimuli. In situations in which stressful stimuli were known to persist for extended periods, there was usually evidence of adaption to stress. However, within the past three years, evidence has begun to accumulate that the autonomic nervous system responds not only to eliciting stimuli antecedent to responses, but also to stimuli presented following changes in autonomic activity. It has been determined that autonomic activity can be brought under the control of environmental stimuli presented contingent upon a change in autonomic activity. This is precisely the arrangement of response and consequences that produces stable alterations in more conventional behaviors. To date, little has been done to systematically explore the control of autonomic function by manipulating the consequences of alterations in autonomic activity. The purpose of this work unit is to take advantage of these recent findings to examine the relationship of various arrangements of stimuli and responses to determine their role in the production and maintenance of the kind of chronic change in autonomic function thought to be significant in the etiology of psychosomatic disease and to develop models for the study of this class of disorders.

Progress.

Analysis of the behavioral variables contributing to the development of gastro-intestinal ulcers. This study was designed to use the principles developed from behavioral research to pursue development of a sub-human primate model for psychosomatic peptic ulcer disease. To do this, unpredictability of shock presentation and conflict, i.e., punishing previously appropriate responses, were manipulated in a series of sequential experiments during which each monkey's gastric-duodenal mucosa was examined biweekly using a fiberoptic gastroscope.

Acute, superficial gastric lesions developed in 4 of 5 monkeys with gastric cannulae and in 2 of 2 monkeys with esophagostomy. Lesions usually developed before monkeys developed stable performance on the particular task demanded of them, i.e., they were in the acquisition phase of learning. Two of the lesions, occurring at the end of the study, were subjected to histological analysis. Esophagostomies were used in place of gastric cannulae to rule out the possibility that lesion development was due to the presence of the cannula. In support of this study, parallel studies of gastric function, i.e., secretion of water and ions and emptying, were also undertaken. These studies indicated that gastric hydrogen ion secretion as measured by this technique is three-fold greater than studies of gastric secretion using simple drainage through a gastric fistula. To date, gastric function has been measured in 2 esophagotomized monkeys during the stress procedures. An animal that developed acute, prepyloric erosions showed a marked reduction in gastric emptying with a small decrease in hydrogen ion secretion when compared to data collected before the initiation of the stress procedure. The other animal developed an acute duodenal lesion and showed a significantly increased rate of gastric emptying and normal and hydrogen ion secretion when compared to control studies.

Psychosomatic gastric-duodenal disease in the form of stress erosions has been produced in the sub-human primate, but, despite increased behavioral demands on the organism, chronic peptic ulcer disease has not developed. The behavioral pathogenesis of the stress lesions may be mediated by changes in both gastric emptying and gastric hydrogen ion secretion. When the stomach secretes normal or increased amounts of acid and empties rapidly, the duodenum is bathed with a high volume flow of acid juice. This combination may have produced the duodenal lesion. When the stomach secretes less acid, but empties slowly, the stomach is bathed with an acid fluid for a long period of time; this may have culminated in the production of the gastric erosion. These studies are being continued to collect further examples of these potentially important correlations.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6508	74 Ø7 Ø1	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 Ø7 Ø1	D.Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	ØØ	116
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Autoregulation of autonomic response

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

Ø12ØØØ Physiology Ø134ØØ Psychology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 Ø7	CONT	DA	C. In-House

17. CONTRACT/GRANT: NA

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 74	2	39
CURRENT FISCAL YEAR 75	2.5	39

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Tahmoush, MAJ A. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U)Autonomic Dysfunction; (U)Psychosomatic Disease; (U) Human Volunteer; (U)Conditioning; (U)Non-Invasive Monitoring;(U)Cardiovascular Function

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Refinement of behavioral techniques for outpatient management of psychosomatic dysfunctions in military personnel through extension of operant and respondent conditioning principles. Nonpharmacologic management of wound related intractible pain syndromes.

24. (U) Knowledge of operant and respondent conditioning principles, autonomic nervous system functioning, and sophisticated non-invasive bioinstrumentation are applied to normal and target populations to effect clinically relevant changes in biological functions. Procedures developed are extended, simplified, and standardized to facilitate application on an outpatient basis.

25. (U) 73 Ø7 - 74 Ø6 Hardware has been developed to automatically detect salient features of central and peripheral cardiovascular events. Time and amplitude relations between events are measured and computed to establish indirect estimates of cardiovascular function. A minature infra-red hemodensitometer has been developed that permits limb to limb comparisons of relative blood pulse volume and vasoconstriction. Unit is currently employed in study of autonomic function asymmetries in patients with causalgic pain syndrome. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 1973 - 3Ø JUN 1974.

* Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 116 Autoregulation of autonomic response

Investigators.
Principal: Frederick W. Hegge, Ph.D.
Associate: MAJ Daniel P. Redmond, MC; MAJ Albert Tahmoush, MC; CPT John R. Jennings, MSC; Stanley Hall, Jr., B.A.

Description

The applicability of operant and respondent conditioning principles to clinical syndromes involving autonomic dysfunction, such as pain states, hypertensive cardiovascular disease, and cardiac arrhythmia is systematically investigated. Such research leads to an understanding of the pathogenesis of and techniques for intervention in the clinical course of psychosomatic diseases. Transcutaneous stimulation techniques (TST) have been found clinically effective in the treatment of chronic pain, particularly causalgia (Meyer & Fields, 1973). Current work involves laboratory assessment of counter-stimulation techniques, such as TST and acupuncture, as well as investigation of autonomic involvement in the causalgia pain syndrome. Continuing technical developments include sophisticated and non-invasive techniques for monitoring cardiovascular variables that will allow detailed assay of conditioning methods in terms of both mechanisms and efficacy.

Progress

1. Causalgia: Study of Sympathetic Activity

The study of sympathetic activity in patients with causalgia enters its second year. Initial 24-hour recording sessions revealed asymmetries in vascular activity and skin conductance that remained relatively constant over time. Based on these results and in order to investigate day-to-day variations, recording times were changed to a ninety-minute recording period on each of two consecutive days before and two consecutive days after treatment. Four patients and three matched controls have been studied using the two-day sessions. The qualitative asymmetry in skin conductance seen in the preliminary study has been confirmed. However, the pattern of asymmetry is quite variable. Three patients have had consistently higher basal skin conductance levels (SCL) in the affected extremity, whereas two patients have had a consistently lower SCL with marked suppression of spontaneous skin conductance responses (SCR). A more consistent finding has been reduced blood volume pulse (BVP) amplitude in the affected

extremity. This result is strong evidence for greater arterial vasoconstriction in the affected extremity and is consistent with the reduced skin temperature also noted. Finally, the blood volume signal tends to be larger in the affected extremity, indicating a venous dilation of the cutaneous vascular bed. These results are necessarily preliminary; definitive interpretation must await completion of the study.

2. TST Effects on Pain Estimates and Skin Conductance in Normal Volunteers

A within-subject study of decrements in pain intensity with TST was completed this year. The study continues the work discussed in previous reports (Krasnegor, Jennings, and Fields, 1972; Hall and Jennings, 1973). The within-subject study was an attempt to replicate the effectiveness of high-frequency TST applied over the cutaneous peripheral nerve. As in previous work, the experiment consisted of bilateral magnitude estimation before and after a stimulation condition. Stimulation conditions were: a) over the cutaneous peripheral nerve; b) over the median nerve; or c) control condition. Pain was induced with a Hardy-Wolff dolorimeter and both pain judgments and skin conductance reactions were obtained.

The results failed to show significant pain reduction due solely to peripheral cutaneous stimulation. All stimulation conditions produced equal pain decrements. The results were significantly influenced by the order in which a subject received the three stimulation conditions. This effect could not be explained.

Current effort is directed at the exploration of acupuncture as a different counterstimulation technique for the relief of pain. Acupuncture may share the same theoretical mechanism for pain relief as TST. Thus, it should be directly relevant to the relief of causalgia-type pain. Initial observations using acupuncture suggest that it produces larger decrements in scaled experimental pain than TST. A proposal has been developed for the evaluation of acupuncture utilizing the basic design employed in all the previous counterstimulation (TST) studies.

3. Technical Progress -- Monitoring of Cardiovascular Responses

The development of an experimental chamber equipped with sophisticated, reliable, and non-invasive cardiovascular monitoring devices is nearly complete. Present technology is inadequate for reliable and non-invasive measurement of systolic and diastolic blood pressure in the temporal framework of acute psychological experimentation. Concentration on blood pressure and/or heart rate conditioning leads to oversimplified concepts of cardiovascular system behavior and perhaps to erroneous conclusions regarding that system's responses to

independently varied stimuli (Peterson, 1963). Consequently, instrumentation has been developed which will provide a multidimensional perspective consistent with a view defining the cardiovascular system as a multi-loop, homeostatic feedback system. Several measurements are made simultaneously on a heartbeat-to-heartbeat basis, each of which has been shown by various investigators to have empirical meaning (Weissler et al., 1961). By combined measurement of these several parameters, it is anticipated that behavior of the whole system will be better described enabling the analysis of mechanisms of change and specificity of responses. Such analysis is relevant to current understanding of the pathogenesis of certain disease states, and ultimately, to the question of efficacy of autocontrol therapy.

A system for processing signals from electrocardiogram (EKG) and phonocardiogram (PCG) has been devised and constructed. Several significant events are identified electronically on the EKG and PCG, and on a low frequency component of the PCG (i.e., the vibrocardiogram or VCG). These events define various intervals in the cardiac cycle, which are timed to provide systolic intervals (pre-ejection period, isovolumic contraction time, and ejection time) that are closely related to such direct measures of cardiac function as stroke volume and ejection fraction. An initial study, using normal volunteers, to demonstrate reliability and accuracy of these circuits has begun.

Use of electrical impedance plethysmography has continued along the lines described in the last annual report. This technique provides an indirect measure of basal volume and pulsatile flow within the segment studied. The major inconvenience of this method, the use of circumferential electrodes, has apparently been eliminated by recent technology, a manufacturer's claim as yet untested in this laboratory. When applied to the lower limb, data indicating distal blood flow and peripheral pulse propagation time (PPT) are derived. Previous studies in this laboratory have indicated a relationship of these measures to blood pressure (see Walter Reed Army Institute of Research Annual Progress Report Ø1 Ø7 72 - 3Ø Ø6 73 WU 1Ø4). Similar application of impedance plethysmography to the thoracic segment yields data closely related to stroke volume, and the pulsatile waveform closely approximates that of directly measured aortic root pressure and flow curves, according to other researchers (Sova, 1970). A radial pulse tonometer, an air-filled diaphragm which responds to pulsatile movement of the radial arterial wall, has been placed on-line. Its output reflects relative changes in radial pulse pressure, and is utilized as well as an index of PPT in the upper limb. Finally, an automated ultrasonic brachial cuff blood pressure recorder will measure systolic and diastolic blood pressures at 30-second intervals.

Design has also been initiated for innovative circuitry which will process the first and second derivatives of pulsatile waveforms.

These measures assess quantitative changes in peak velocity and acceleration phenomena, as well as the differential timing of the propagation of different portions of the pulse wave from the central to the peripheral vascular bed. Such waveform analysis will in theory be responsive to variations in vascular compliance, although little empirical work has been reported along these lines.

4. Technical Progress - Photoplethysmograph

A light emitting diode-phototransistor plethysmograph has been developed in-house for the study of peripheral circulation asymmetries associated with causalgia. The transducer combines a gallium arsenide infrared emitting diode (LED) and a silicon NPN phototransistor in a compact package weighing 0.4 gms. Experimental trials using standard signals revealed the output to be stable for periods up to 12 hours. With the device applied to the finger, human volunteers experienced no discomfort or heating. Consistently good records with minimal movement artifact were generally obtained.

A calibration procedure was designed for the photoplethysmograph that should permit comparison of the blood volume pulse and blood volume signals between subjects. Munsell color chips of known infrared reflectance are attached to rotating wheel of a DC motor, and a plethysmograph is positioned at a fixed distance from the wheel to measure reflectance. Variations in voltage can then be related to variations in reflectance. When the transducer is placed on a subject's finger, the offset voltage is entered into a linear equation obtained from the calibration procedure and an infrared reflectance value is obtained. This procedure represents a major advancement in the field of photoplethysmography.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 116 Autoregulation of autonomic response

Literature Cited.

References:

1. Krasnegor, N. A., Jennings, J. R., Orr, W. C., and Fields, H. L.: The aftereffects of peripheral nerve stimulation: Changes in psychophysiological judgements and autonomic reactions during the scaling of cutaneous pain. Psychon. Sci. 26: 74-76, 1972.

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3. Peterson, Lysle H.: Systems behavior, feedback loops, and high blood pressure research. Cir. Res. 12: 585-596, 1963.

4. Sova, Josef.: Cardiac rheometry: Impedance plethysmograph of the human trunk as a method for measurement of stroke volume and cardiac output. Ann. N.Y. Acad. Sci. 170:2: 577-593, July 30, 1970.

5. Weissler, A. M., Peeler, R. G., and Rochill, W. H. Jr.: Relationships between left ventricular ejection time, stroke volume and heart rate in cardiovascular disease. Am. Heart J. 62: 367-378, 1961.

Publications:

1. Hall, S., and Jennings, J. R.: Peripheral nerve stimulation after-effects on pain judgements and skin conductance: A double-blind study. Psychophysiol. 11: 222, 1974.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0B 6512	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D.CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	120
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)*
(U) Antigenic Components of the Cell Wall of Neisseria meningitidis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:*
c. TYPE: d. AMOUNT:
e. KIND OF AWARD* f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	3	37
	CURRENT 75	3	42

19. RESPONSIBLE DOD ORGANIZATION
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NAME: DA

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Cell wall; (U) Protein; (U) Lipopolysaccharide; (U) Polysaccharide; (U) Antibodies; (U) Meningitis; (U) Human Volunteers

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) - To analyze the cell surface structure of the meningococcus with emphasis on extracting and purifying the dominant antigens. The goal is to develop candidate vaccines for this disease of military importance.

24 (U) - Protein, lipopolysaccharide and polysaccharide antigens will be characterized biophysically and immunochemically. Antibody responses of animals and humans will be measured by a variety of serologic methods. Antibodies will be purified by immunoabsorbent techniques.

25 (U) - 73 07 - 74 06 Cell wall protein antigens from meningococci of serogroups B and C have been prepared as potential vaccines for human use. In immunized rabbits four vaccine lots induced hemagglutinating and bactericidal antibodies. Sera from rabbits immunized with a group C protein vaccine were capable of opsonizing group C organisms. The phagocytic killing was inhibited by purified cell wall proteins from the homologous organism and also by protein antigen from a group B strain. The group C and B protein vaccines all protected mice from lethal challenge with a group B strain. These vaccines are now ready for initial studies in human volunteers. Serum immunoglobulin A purified from convalescent case sera was shown to inhibit bacteriolysis mediated by IgG and IgM antibodies. Several lots of group A polysaccharide vaccines have been found to contain trace amounts of group C polysaccharide. Serologic studies of volunteers given these preparations demonstrated low level antibody responses to group C polysaccharide. Subsequent injection of a full dose of group C vaccine resulted in mean antibody levels significantly lower than those found in volunteers who received previous group A vaccines free of contaminating C polysaccharide. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*Available to contractors upon originator's approval

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 120 Antigenic components of the cell wall of Neisseria meningitidis

Investigators.

Principal: Malcolm S. Artenstein, M.D.

Associate: CPT Wendell D. Zollinger, MSC: MAJ J. McLeod Griffiss, MC; Brenda L. Brandt, Robert E. Mandrell; SP6 Dennis D. Broud; SP5 Charles L. Pennington; Mary Ann Bertram; MSG Adam D. Druzd.

1. Studies of a meningococcal cell wall protein vaccine.

The outer cell wall of Neisseria meningitidis contains three classes of antigens: capsular polysaccharide (sss), lipopolysaccharide (LPS) and protein [1]. Earlier studies at Walter Reed Army Institute of Research have elucidated the immunochemistry of the capsular sss of serogroups A, B and C and have shown the efficacy of group C polysaccharide vaccines in preventing meningococcal disease [2]. More recently, studies sponsored by the World Health Organization have shown that group A sss vaccine prevented group A disease [3]. The failure of B sss to induce antibody responses in humans [4] has led to studies of other cell wall antigens as potential immunizing products. Meningococci have been shown to possess non-sss antigens which are shared among the specific serogroups [5,6], although all strains do not contain the same complement of antigens. Based upon the serum bactericidal reaction a subtyping scheme has been described for serogroup B and C strains [6,7]. Although the major subtype antigens have been found to be surface proteins [8], LPS antigens are also active in the bactericidal reaction.

Human antibody response to both the protein and LPS antigens of the outer membrane has been demonstrated by specific hemagglutination assays following both systemic disease and nasopharyngeal carriage. Protein antigens from both group B and group C strains gave equivalent response whether the infecting organism was serogroup B or C. Because of the toxicity associated with the LPS antigens we have considered the proteins to be the most promising antigens for a vaccine against group B meningococci. The protein antigens might be prepared from either a group B or group C strain and be expected to cross react.

In the past two years studies of three group B protein vaccines (Lots A, K and Z) were tested in animals and, upon approval of the Army Investigational Drug Review Board, in eight human volunteers.

Results were summarized in the WRAIR Annual Report 1973, Work Unit 181. Briefly, rabbits responded with HA and bactericidal antibodies. Human volunteers occasionally showed HA response, but in all cases no stimulation of bactericidal titers above prevaccination levels was observed.

During the past year new lots of protein vaccines were prepared. They differed from those previously used in several respects: (a) a group C strain (138I) was used for two of the lots; (b) milder procedures were used in their preparation; (c) analysis by polyacrylamide gel electrophoresis showed fewer protein bands and lack of degraded proteins.

Vaccine lots 138I-0 and 138I-M-1 were derived from isolated outer membrane of the group C strain 138I, which is the serotype II reference strain. Lot 138I-0 is the purified protein fraction of the membrane and lot 138I-M-1 contains both the protein and group polysaccharide components of the membrane. LPS was specifically removed from these two lots by Sephadex G-100 chromatography. Lots B-60-SOAP and B-59-TCA were derived from the group B strain B11. The proteins contained in these lots were very similar to those in lots 138I-M-1 and 138I-0 as judged by polyacrylamide gel electrophoresis, but they were less highly purified. Lot B-60-SOAP is rich in group B polysaccharide. A fifth lot, B-59-AG, was considered too toxic for trials in humans since two of three rabbits immunized with 50 mcg of this lot died within 24 hrs.

The antibody responses of rabbits immunized with the different vaccine lots were as follows: All four lots produced high titers of hemagglutinating antibody directed against the protein and high titers of bactericidal antibody against the test strain B11 (Table 1). Only three rabbits had bactericidal titers less than 1:100 at four weeks. Low HA antibody titers against the LPS and polysaccharide antigens suggested that the bulk of the bactericidal activity was due to antiprotein antibody. Only lot B-59-TCA resulted in a consistent antibody rise against the LPS. Group C polysaccharide present in lot 138I-M-1 did not induce antibody unless the rabbits were hyperimmunized. Antibody specific for the protein was purified from the four week serum of one rabbit using an immunoadsorbent and was found to possess bactericidal activities.

Opsonic antibodies of rabbit antisera were studied in a standard phagocytic assay which used human polymorphonuclear cells and human complement. Significant phagocytic killing was observed (Table 2) in antiserum raised against whole live homologous bacteria. Whole serum from a rabbit immunized with purified 138I protein was phagocytic in a dilution as high as 1:100. Purified antiprotein antibodies from this latter serum were also capable of opsonizing for

Table 1. Bactericidal antibody responses of rabbits to meningococcal protein vaccines*.

Vaccine lot no.	Rabbit no.	Radioactive bactericidal titer**			
		0 wk	1 wk	4 wk	7 wk
138I-0	726	40	160	2560	
	737	0	20	5120	
	727	0	0	80	
	70	0	0	10	160
138I-M-1	803	10	0	40	1280
	806	40	20	640	2560
	810	5	10	160	2560
	73	20	80	320	1280
	74	20	40	160	1280
B-59-TCA	724	40	80	10240	
	725	10	40	10240	
	733	0	40	5120	
B-60-SOAP	732	0	160	2560	
	734	5	40	320	
	735	0	20	5120	
	67	20	20	640	640
	68	40	40	1280	1280

* Rabbits were injected with 50 mcg protein, i.v., at 0, 3 and, in some cases, 6 wks.

** Reciprocal of serum dilution causing 25% of maximum release of radioactivity from strain B11.

0 indicates less than 5.

Table 2. Opsonization of N. meningitidis 138I (group C) by immune rabbit antiserum (#737)*.

Rabbit antiserum raised to:	Serum dilution	Phagocytic reduction**
138I whole organism		
preimmunization	1:10	-0.46
postimmunization	1:10	2.81
138I protein		
preimmunization	1:10	-0.52
postimmunization	1:5	0.92
	1:10	1.57
	1:50	1.19
	1:100	1.20
	1:500	-0.41
138I protein	1:80 (no PMN's)	-0.54
(column purified antibodies)	1:80	1.48
	1:160	0.67
	1:320	0.28

* Phagocytic assay consisted of human polymorphonuclear leukocytes, human serum absorbed with 138I served as source of complement.

** Bacterial colony count at 0 time - count after 90 minute incubation. Negative value indicates growth of bacteria rather than kill. Reduction in counts expressed as $\log_{10}$.

phagocytosis. The specificity of the antiprotein opsonins was tested by absorbing the serum with whole bacteria or purified proteins from three group C strains and one group B strain (Table 3).

Table 3. Specificity of rabbit serum opsonins following immunization with 138I purified protein antigen.*

Absorbing antigen	Phagocytic index** (log 10)
None	0.82
Group C bacteria 138I	-0.82
protein 138I	-0.79
protein 35E	0.94
protein 118V	0.60
Group B bacteria B11	-0.62
protein B11	-0.51

* Phagocytic assay consisted of human polymorphonuclear leukocytes, human serum absorbed with 138I served as source of complement. (Rabbit #726).

** Bacterial colony count at 0 time - count after 90 minute incubation. Negative value indicates growth of bacteria rather than killing.

As expected, absorption of 138I antiprotein antiserum with 138I whole organisms and 138I protein removed all opsonic activity. However, purified cell wall protein antigens from group C strains 35E and 118V failed to significantly affect the phagocytic killing. These two strains have previously been shown to lack cross reacting antigens with 138I (except for group C polysaccharide). Both the whole organism and purified protein from group B strain B11 removed opsonic antibody from the 138I serum. Thus, the known bactericidal cross reaction (factor II) of B11 with 138I appears to be due to a common surface protein.

The four vaccine lots were all found to be effective in protecting mice against a lethal challenge of viable meningococci (strain B11). The ED_{50} ranged from 2.3 mcg to 11.6 mcg.

Toxicity tests have shown that when the same standards are applied to these preparations as were applied to the polysaccharide vaccines they were nontoxic. Lots B-60-SOAP and B-59-TCA, however, were pyrogenic in rabbits in concentrations of 0.25 mcg or greater.

Tests in a small number of human volunteers are now required to establish the immunogenicity and safety of these vaccines.

2. Bacteriolytic and inhibitory activities of immunoglobulin classes of human meningococcal antisera.

A deficiency of serum bactericidal activity against pathogenic strains of N. meningitidis has been correlated with susceptibility to systemic disease [9]. Therefore, the complement mediated "cidal" reaction has been of considerable interest in the role of bacterial antigens and antibodies in immunity. Previous studies have shown that the IgG class of immunoglobulins plays a major role in meningococcal immunity [9] and serum cidal reactions [10], although IgA, IgG and IgM antibodies develop following infection [9,11] and polysaccharide vaccination [10,11]. Immunofluorescent and passive hemagglutination assays were used in these latter studies. Because of reports of blocking of bactericidal antibodies by IgA in brucellosis [12], studies of human meningococcal antisera were undertaken to determine whether a similar phenomenon was operative in this disease.

Convalescent sera from four cases of systemic meningococcal infection were studied using the radioactive bactericidal test (RBT) [13]. Immunoglobulins were separated by Sephadex G-200 gel filtration and were further purified by passage over immunoabsorbent columns (containing goat antihuman IgM and IgG covalently coupled to Sepharose 4B). Whole sera and purified immunoglobulins were tested against homologous and heterologous meningococcal strains. Inhibition tests were performed by adding varying concentrations of serum IgA to the reaction mixtures. Quantitative radial immunodiffusion and immunoelectrophoresis were used to determine the purity of the Ig fractions.

Serum IgA purified from convalescent sera of patients infected with serogroup B, C or Y meningococci was shown to inhibit complement mediated bacteriolysis by IgG and IgM from the same sera. Inhibition was dependent on the ratio of lytic to blocking antibodies. Inhibition was strain specific and was greater for IgG than IgM. IgM was more active than IgG in the cidal test.

Blocking activity of purified IgA appeared unrelated to the presence of a prozone in the whole sera in that IgA from the individual with group B disease was a highly efficient inhibitor even though the whole serum had no prozone.

3. Development of chemical and immunologic standards for meningococcal polysaccharide vaccines.

Group C meningococcal polysaccharide vaccines have been shown to be safe and effective in preventing group C disease. They are currently used routinely in military recruits. Standards of potency which have been established are rather imprecise in the absence of an experimental animal assay and, therefore, a continuing effort has been underway to establish an acceptable physico-chemical definition of potency. Arbitrary specifications for these vaccines have included:

(1) Chemical assays for purity.

(2) Immunologic tests for specificity (immunodiffusion and hemagglutination against meningococcal antisera of serogroups A, B and C).

(3) Molecular size determination (chromatography using Sephadex G200 and Sepharose 4B).

(4) Immunization of human volunteers and determination of antibody responses.

In addition, standard NIH animal toxicity and bacteriologic sterility tests have been required.

The chemical assays and specificity tests have proven satisfactory and will not be discussed further. Molecular size (MW) determination has been difficult to standardize among the few laboratories performing this work. It is known that a large MW is necessary to insure antibody responses in humans injected with polysaccharide antigens. However, the exact MW threshold is unknown. Studies at WRAIR of 11 lots of group C polysaccharide vaccine have shown that antibody responses in volunteers were not significantly different with vaccines ranging in MW from 5.2×10^5 to 2.2×10^6 [14]. Data in Table 4 compares the Sepharose 4B elution characteristics (Kav) of further vaccine lots which have been submitted by Merck, Sharp & Dohme to the FDA as part of the licensing application. For reference, Kav 0.416 = MW 5.6×10^5 and Kav 0.228 = MW 2.2×10^6. Lots C-6, C-7 and C-8 were used in the successful field trials of efficacy [15]. It is apparent from the table that later lots (Merck) have larger Kav (smaller MW), yet antibody responses of

Table 4. Group C meningococcal polysaccharide vaccines: Molecular size and antibody responses*.

Vaccine Lot No.	Sepharose 4B (Kav)**	Human antibody responses: Hemagglutination No. tested	Hemagglutination % rise	RABA***
Merck				
487/C-A881	0.354	37	100	
490/C-A884	0.354	39	100	
491/C-A885	0.380	38	97	309
512/C-B047	0.416	40	98	230,291,400
513/C-B048	0.354	40	95	
95937/32415/C-B840	0.425	-		-
Merrill-National				
1228	0.228	27	100	471
1487	-			-
WRAIR				
C-6	0.266	119	96	-
Squibb				
C-7	0.228	123	93	378
C-8	0.241	29	100	-

* Antibody responses at 14 days after 50 microgram dose.

** $Kav = \frac{Ve-Vo}{Vt-Vo}$

*** Radioactive of binding assay. Geometric mean titer increase measured as nanograms of antigen bound by 0.05 ml serum [16].

volunteers were not significantly different. Furthermore, these lots have been used in recruit populations with no indication of failure of protection (although not as a controlled study).

Since the only antibody assay which has been shown to correlate with immunity is the bactericidal test [9] it was of interest to observe that there was not a perfect correlation between bactericidal responses and the other assays (Table 5). The sera from volunteers who received lot 512 was also tested at Merck Laboratories in a standard bactericidal test and the same percentage (84%) of significant responses was found.

These data have been provided to the Bureau of Biologics of the FDA to assist them in selecting specifications to be required of manufacturers who apply for licensing. The ultimate goal of these studies is to develop physico-chemical standards of potency so that human volunteer testing can be discontinued.

4. Paradoxical effect of suboptimal doses of meningococcal polysaccharide vaccines.

The original studies of group A meningococcal polysaccharides showed them to be group specific; ie, subjects injected with group A vaccine developed antibodies to A antigen only, no antibody rises were noted when these sera were tested with group C antigen. These immunizations had been performed using lot A-5 prepared at WRAIR by Dr. E. C. Gotschlich. Very recently, when testing the serological specificity of several new antibody assays, it was noted that several individuals who had received group A vaccine developed anti-C antibodies. A more thorough analysis of the problem was undertaken to determine whether this cross reactivity was due to a true antigenic crossing or was the result of contamination of the culture from which the A vaccine was prepared.

Sera from volunteers immunized with different lots of group A vaccine were tested for anti-C antibodies by hemagglutination test. Lots A-5, A-6 and A-7 were tested at Fort Dix with throat cultures obtained at two week intervals and serum specimens at 0, 2 and 6 weeks. Lot A-8 was tested at Fort Lewis where only prevaccination and two week postvaccination specimens were collected. Data in Table 6 are summarized on the basis of carrier status.

These results suggest that all lots of group A vaccine induce group C antibodies in recipients, lot A-8 being most potent, producing rises in 70-100% of recipients within two weeks. It should be noted, however, that the two rises from lot A-5 were both late, six weeks after injection and conceivably could have

Table 5. Summary of antibody studies on group C vaccines.

Lot No. (Merck)	HA test*		RABA**		Bactericidal+	
	No. pos†/No. tested	%	No. pos/No. tested	%	No. pos/No. tested	%
419	17/17	100	11/11	100	15/17	88.2
420	19/19	100	17/17	100	16/19	84.2
489	28/28	100	28/28	100	25/28	89.3
512	25/25	100	25/25	100	21/25	84.0

* HA = Hemagglutination test, data of LTC C. D. Smith, 6th USAML.

** RABA = Radioactive antigen binding assay [16].

\+ Bactericidal test = Radioactive release assay [13].

† Pos. refers to significant rise in titer.

Table 6. Group C antibodies induced by group A vaccines.

Vaccine group and dose		Acquisition of carrier state	Group C rises** No. pos./No. tested	Comments
A-5	50γ	Neg	2/10	Both late rises
		Mgc*	0/6	
		Group C	3/4	
A-6	50γ	Neg	4/12	One late rise
		Ngc	2/6	
		Group C	4/5	
A-7	50γ	Neg	9/19	
		Mgc	0/4	
		Group C	4/5	
A-8	50γ	Neg	7/10	
		Mgc	1/3	
		Group C	2/4	
A-8	100γ	Neg	8/8	
		Mgc	7/8	
		Group C	0/1	

* Meningococcus groupable or nongroupable but not group C.

** Passive hemagglutination test.

been related to carrier infections not identified by culturing at two week intervals.

Group A vaccine preparations were tested for group C antigen content by means of the sensitive hemagglutination-inhibition (HI) test. Table 7 shows that lot A-5 had no polysaccharide activity at the concentration tested.

Table 7. C polysaccharide content of group A vaccines as determined by HI test.

Vaccine	Source	Concentration γ/ml	Group content γ/ml
A-5	Gotschlich-WRAIR	250	<0.1
A-6	Forest Glen-WRAIR	250	0.1-0.2
A-7	Squibb	250	0.2
A-7		2500	1.6
A-8	Squibb	250	0.1-0.2
A-8		2500	1.6-3.2

Lots A-6, A-7 and A-8 all had approximately the same amount of group C activity. This corresponds to between 0.02 and 0.03 micrograms C polysaccharide per 50 microgram dose of A vaccine.

Lot A-8 vaccine was also tested for presence of sialic acid by the resorcinol method. Using a concentration of A polysaccharide of 2500 micrograms approximately 1.5 μgm sialic acid was detected. This is in very close agreement with the HI assay.

Four lots of group B polysaccharide (B-40S-30K, B-40-SFP, B-30, FP-IAD and B-29 Sevag) were tested at 1000γ/ml and had no group C activity. Two lots of A polysaccharide produced by National Drug Co. were also tested: Lot #3 had no group C activity; Lot #6 had approximately 0.2γ C per 1000γ A polysaccharide.

To test the assumption that the seed culture of group A meningococcus had been contaminated with C organisms, a new A culture was prepared by picking a single colony of the A-1 strain. After transfer and testing serogroup and carbohydrate reactions a new seed culture (A-1a) was lyophilized. Two batches of group A polysaccharide were prepared in the usual fashion from two ampules of A-1 and two batches

from separate ampules of A-1a. These were carried through the Sevag step (short of final purification) and were tested in a concentration of 3000γ/ml for C-HI activity. No C activity was found indicating that less than 0.1γ was present.

These results suggest that if the seed culture of A-1 was contaminated not every ampule had C organisms. An alternative explanation might be that culture A-1 may have been contaminated but that C organisms did not always grow to sufficient numbers to produce recoverable amounts of group C polysaccharide.

In this regard it should be pointed out that each lot of A vaccine was prepared by different personnel or under different conditions. Lot A-5 was prepared by Dr. Gotschlich in four liter flasks (100 ml of culture); lot A-6 was produced by the Department of Biologics Research, WRAIR using 12 liters of culture in each 20 liter bottle; lot A-7 was prepared by Squibb using one liter flasks; lot A-8 was produced by Squibb in 100 liter fermentation volumes.

Sialic acid does not appear to be a constituent of the group A meningococcal endotoxin. Dr. F. A. Wyle prepared group A endotoxin by the phenol-water method and found no sialic acid in it while endotoxin of both groups B and C were found to contain sialic acid.

The source of the group C polysaccharide in three of four lots of group A vaccine is unclear. It seems unlikely that the A organism contains sialic acid or that the mannosamine subunits of the A polysaccharide could become converted to sialic acid during the extraction procedures. It is possible that some vials of the A-1 seed culture were contaminated with group C organisms and that by chance these were used in the production cultures of lots A-6, A-7 and A-8. To prove this would require testing many more ampules of A-1, perhaps in an anti-A bactericidal assay. Another source of contamination might have been the fermentors, flasks or other equipment used in the production of cultures or extraction of polysaccharide since the highly charged polysaccharide molecule may have attached to the surfaces and somehow not been removed by cleaning and sterilization. Each laboratory had made group C cultures prior to the group A.

The fact that most of the available group A vaccines contain traces of group C antigen has unknown clinical significance. No toxicity or interference with group A immunogenicity is apparent. In fact, it is of considerable interest that such small amounts of group C polysaccharide are so highly immunogenic; ie, 0.02-0.03 μgms in lots A-7 and A-8 produced group C antibody rises in 50-75% of recipients, and double this quantity (lot A-8 100 μgm) resulted in group C rises in 100% of recipients tested.

All vaccine producers have been provided with the new single colony derived A-1a culture and all investigators using the present stocks of A vaccines have been notified that group C antibody conversions may be expected in recipients.

In July of 1972 a number of lots of group A vaccine were tested in military recruits to determine stability of the product after prolonged storage. Since two of these group A vaccine lots had been previously determined to have 0.05-0.08% contamination with group C polysacchride (Squibb A-7 and A-8), group C antibody responses were tested. Volunteers received 50 microgram doses of group A vaccine at day 0, and for purposes of preventing group C disease were given the routine group C vaccine at day 14. Each vaccine was administered to groups of 27 to 35 men. As usual in these studies nasopharyngeal carrier surveys were carried out at two week intervals. Group C carriers were excluded from the antibody studies; no group A strains were identified.

Results of antibody assays are shown in Table 8. Since only a few individuals receiving the Merck and Merrell-National vaccines developed group C antibodies at 14 days, these were considered to be free of C polysaccharide and served as controls for the Squibb vaccines. Group C antibody rises at 14 days were found in a substantial proportion of men receiving Squibb lots A-7 (20%) and A-8 (55.6%). The mean titers at 14 days with these two lots were quite low (7.04 and 14.3) when compared to group C responses to 50 µg doses of group C vaccine (200-400) [14]. Following the group C immunizations at day 14 the responses were measured again on day 42. The results showed that group C antipolysaccharide responses were significantly impaired in men who had initially received lots A-7 and A-8. Both mean titers and percent of men with antibody rises were low.

The significance of these findings is currently unknown. The suboptimal response to full doses of group C vaccine following an initial small dose of C polysaccharide cannot be evaluated in terms of immunity since the radioactive binding assay titer has not as yet been correlated with protection. It is conceivable that a longer period between the two doses of C polysacchride would allow a more normal response. Full doses of A and C vaccines have been administered to small numbers of volunteers with no evidence of interference [4], although the RABA was not performed on those sera. In any event, the data suggest that caution must be exercised in using monovalent polysaccharide vaccines containing small amounts of contaminating heterologous polysaccharides.

Table 8. Meningococcal group C polysaccharide responses in volunteers receiving group A vaccine*.

Vaccine lot	Mean antibody binding activity** (95% confidence interval)			Average fold increase		% men with 2-fold or greater increase	
	0 day	14 day	42 day	14 day	42 day	14 day	42 day
Merck 429	4.98 (3.56-6.94)	5.37 (3.71-7.76)	233 (126-470)	1.14	94.5	6.1	96.8
Merck 440	5.0 (3.23-7.74)	5.81 (4.17-8.09)	223 (125-398)	1.4	69.9	0	100
Merrell-Nat'l. 1227	5.46 (3.53-8.65)	5.89 (3.65-9.50)	197 (97.8-398)	1.4	74.3	6.5	96.7
Squibb A-7	3.72 (2.42-5.72)	7.04 (4.68-10.6)	48.4 (23.7-98.7)	2.6	30.9	20.0	68.8
Squibb A-8	3.54 (2.47-5.06)	14.3 (9.54-21.4)	43.4 (24.3-77.7)	6.64	17.23	55.6	80.0

* 50 µg group A vaccine administered day 0; 50 µg group C vaccine administered day 14; 27-35 individuals in each group.

** Radioactive antigen binding assay. Titer expressed as nanograms antigen bound.

5. Epidemiology of meningococcal disease.

Cases of meningococcal meningitis in Army active duty personnel have continued to occur at a very low level in calendar year 1973. Table 9 shows the number of reported cases of disease since 1971, as well as the tabulation of serogroups identified (based upon strains submitted to WRAIR).

Table 9. Meningococcal disease - reported cases and serogroups, 1971-1973, Army active duty.

Year	No. cases reported	B	C	Y	Other	Total
1971	123	8	57	11	0	76
1972	32	1	0	20	0	21
1973	18	1	0	9	1*	11

* Serogroup 135

Incomplete data for 1974 (1 Jan through 4 May) have shown 15 reported cases with group Y strains predominating and an occasional B and C strain identified. Thus, meningococcal disease in the continental U. S. is at a very low level. Since institution of group C routine vaccination of recruits in Oct 1971 group C disease has been virtually nonexistent in this population.

Information from a variety of sources indicates that epidemic group C disease continues in Sao Paulo, Brazil; group A disease has been identified in Western Canada, Finland, Eastern Europe, as well as sub-sahara region of Africa and South Africa. Many group A strains are resistant to sulfonamides and a recent report [17] suggests the possibility of penicillin resistant group A strains.

Summary and recommendations.

Cell wall protein antigens from meningococci of serogroups B and C have been prepared as potential vaccines for human use. In immunized rabbits four vaccine lots induced hemagglutinating and bactericidal antibodies. Sera from rabbits immunized with a group C protein vaccine were capable of opsonizing group C organisms. The phagocytic killing was inhibited by purified cell wall proteins from the homologous organism and also by protein antigen from a group B strain. The group C and B protein vaccines all protected mice from lethal challenge with a group B strain. These vaccines are now ready

for initial studies in human volunteers. Serum immunoglobulin A purified from convalescent case sera was shown to inhibit meningococcal bacteriolysis mediated by IgG and IgM antibodies. Efforts to develop physico-chemical and immunologic standards for polysaccharide vaccines have suggested the utility of molecular sizing by Sepharose 4B column chromatography. Bactericidal tests carried out on sera collected from volunteers immunized with different lots of group C vaccine showed significant responses in 84 to 89% of vaccinees. Several lots of group A polysaccharide vaccines have been found to contain trace amounts of group C polysaccharide. Serologic studies of volunteers given these preparations demonstrated low level antibody responses to group C polysaccharide. Subsequent injection of a full dose of group C vaccine resulted in mean antibody levels significantly lower than those found in volunteers who received previous group A vaccines free of contaminating C polysaccharide. Meningococcal group C vaccine has been used routinely in recruit training centers since 1971. Only 18 cases of meningococcal disease in active duty personnel were reported in calendar year 1973. Serogroup C was not identified among the 11 case strains submitted, whereas nine were serogroup Y.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 120 Antigenic components of the cell wall of Neisseria meningitidis

Literature Cited.

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(U) Radioimmunoassay Methodology for Drugs of Abuse

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13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Radioimmunoassay; (U) Morphine; (U) Antibody; (U) Drug addiction

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Objective is to develop a relatively specific and quantitative radioimmunoassay for the measurement of serum morphine concentrations which will be utilized in studies of drug addiction. There is a military requirement for better procedures to assay drugs of abuse in body fluids.

24. (U) A critical feature of the method is the production of suitable antibodies by immunization with appropriate morphine derivatives which serve as haptene. Preparation of high specific activity morphine I^{-125} is also essential to the method.

25. (U) 73 07 - 74 06 Two distinct radioimmunoassays for the measurement of heroin and similar drugs of abuse have been developed and validated. One system utilizes (Morphine-6-RIA) antibodies obtained by immunization with dihydromorphinone-6-oxime-bovine serum albumin and tritiated dihydromorphine as tracer. The other system (Morphine-3-RIA) uses antibodies from rabbits immunized with 3-carboxymethylmorphine-rabbit serum albumin and morphine-I^{125} for the radioactive label. Both systems are very sensitive and can quantitate as little as 20 nicograms of heroin. These radioimmunoassay methods are now being used to assay plasma and urine samples from experimental studies. The work unit has been terminated due to loss of the investigator, however the basic objectives of the unit have been met. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*Available to contractors upon originator's approval

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 122 Radioimmunoassay methodology for drugs of abuse

Investigators.
Principal: MAJ Scott E. Monroe, MD
Associate: Clarence E. Emery, B.S.

DESCRIPTION

The purpose of this project is to develop sensitive, precise, and specific radioimmunoassay procedures for the quantitation of heroin and related compounds in both plasma and urine. These techniques, when fully validated, will be used to measure plasma and urinary concentrations of heroin and some of its metabolites in support of various in-house research programs focused on drug abuse.

PROGRESS

Description of Radioimmunoassay.

Two distinct radioimmunoassay systems have been validated and are presently supporting various research projects throughout the WRAIR. Details of antibody production and preparation of morphine-I^{125} were described in a previous annual report (FY 73). In brief, one system (referred to as morphine-6-RIA) employs antibodies from rabbits immunized with dihydromorphinone-6-oxime-bovine serum albumin and tritiated dihydromorphine as the radioactive tracer. The second radioimmunoassay system (referred to as morphine-3-RIA) uses antiserum from animals immunized with 3-carboxymethylmorphine-rabbit serum albumin and morphine-I^{125} as tracer.

Both systems are sufficiently sensitive to reliably quantitate as little as 20 picograms of heroin or morphine. Plasma or urine, per se, at the volumes employed in the radioimmunoassays have no effect on the measurement of morphine or heroin. Both the intra-assay and inter-assay coefficients of variation are less than 10 per cent. The use of morphine-I^{125} in the morphine-3-RIA, instead of tritiated dihydromorphine, is advantageous because of the ease of gamma radiation counting, and results in a reduction of technician time, simplification

of the overall procedure and reduced reagent costs. In the morphine-3-RIA, heroin, morphine and morphine monoglucuronide have similar potencies. Morphine monoglucuronide, however, has only about one per cent of the potency of heroin and morphine in the morphine-6-RIA. Compounds with other structural changes such as nalorphine, nalloxone, meperidine, dextrophan, and methadone are less than 0.1 per cent as active as heroin in either system.

Applications.

During the past year, the morphine-3-RIA has been used in laboratory studies. Plasma and urinary concentrations of heroin and its metabolites have been measured by the morphine-3-RIA in a neuroendocrine study of heroin withdrawal in young men. (This study is described in detail under work unit 111). Plasma heroin concentrations in this group of men, which were within four hours of their last reported exposure to the drug, ranged from 95 to 1500 nanogram equivalents per ml and in most subjects gradually fell to levels of less than one nanogram equivalent per ml during the ensuing 72 hours. Peak urinary concentrations of heroin and its metabolites in these subjects ranged from 6 to 400 ug per ml. Metabolites of heroin could be detected in the urine of these men for at least twelve days after their last exposure.

Plasma heroin concentrations in a group of baboons, which are able to obtain heroin by self administration, have also been determined by the morphine-3-RIA (See work unit 102 for details of this study). In general, the plasma heroin concentrations correlated very well with the animals' known intake. In some animals, plasma concentrations of heroin in excess of 20 ug per ml were observed. This work unit has been terminated due to the imminent departure of the principal investigator; however, all of the basic objectives of the unit were accomplished. The work remaining consists of two modifications. The first will be to utilize existing reagents to develop a solid phase radioimmunoassay for heroin. This change will simplify the method even further and will result in radioimmunoassay data being available more rapidly. The second improvement will be the development of a chromatographic procedure for the separation of morphine, heroin, 6-monoacetylmorphine and morphine glucuronic. This procedure, used in conjunction with the existing morphine-3-RIA, should allow the specific quantitation of these compounds in both plasma and urine.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6486	74 06 30	DD-DR&E,AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	H. Term.	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	126
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Development of Capillary Blood-Flow Sensors

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 09	74 06	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE d. AMOUNT:

e. KIND OF AWARD f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	2.25	56
FISCAL YEAR CURRENT 74	2.25	56

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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ASSOCIATE INVESTIGATORS

NAME: Khouri, E. M.

NAME: Bass, B.G. DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Blood Flow; (U) Shock; (U) Trauma; (U) Capillary; (U) Washout

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To develop equipment for the measurement, separately and simultaneously, of capillary blood flow in various organs, e.g., liver, kidney, heart, skeletal muscle, in conscious unanesthetized animals in studies of the physiologic response to exercise, trauma, shock and other stresses of military importance.

24. (U) Beta radiation detectors encapsulated in special configurations are implanted surgically in trained animals and are used to measure the rate of washout of radioactive gases, thus providing an estimate of capillary blood flow rate. Diodes which are at least 10 times more sensitive than those used in previous studies have recently become commercially available.

25. (U) 73 07 - 74 06 The design fabrication and testing of an avalanche diode detector suitable for short-term physiological studies is complete. The high internal resistance of the diodes employed precludes implantation for periods more than 2 weeks. However, other diodes are suitable for this purpose. Having met its technical objectives, this project is terminated. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

* (Applies to contractors only; explain any deletions)

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 126 Development of capillary blood-flow sensors

Investigators.

Principal: COL Ray A. Olsson, MC
Associate: Edward M. Khouri; Billy G. Bass

Description.

Development and application of specially encapsulated beta radiation detectors for long-term studies of capillary blood flow in conscious trained animals under the influence of physiological and pathological stresses.

Progress and Results.

The clearance of an indicator is widely used to estimate local capillary blood flow. The deposition of indicators which are either particulate (microspheres) or which are metabolically concentrated in cells (^{42}K, ^{86}Rb), and also the rate of disappearance of diffusible indicators (^{85}Kr, D_2O) are standard methods for estimating local blood flow. The research program of the Dept. of Cardiorespiratory Diseases requires the means for estimating capillary blood flow rapidly, accurately, with a high degree of anatomical precision, and repeatedly in the same animal with minimal perturbation of physiological and metabolic function. Although the disappearance of an inert gaseous indicator meets most of these criteria, the usual method applied to capillary blood flow in the heart, washout of ^{85}Kr or ^{133}Xe by means of a collimated scintillation crystal located at a distance of several centimeters, gives very poor anatomical localization and considerable averaging due to the heterogeneity of blood flow within the various types of tissues in this organ.

In order to circumvent these technical limitations to the inert gas washout technique, preliminary work on surgically implantable miniature blood flow detectors was begun in 1968. Lithium-drifted silicon semiconductor diodes were used to fabricate detectors which had acceptable biocompatibility and durability but which were only marginally useful because of a low signal-to-noise ratio. When avalanche diodes became commercially available in 1972, several units were bench-tested and were found to be at least 4 times more sensitive than the older lithium-drifted diodes. On the basis of this testing with unmounted diodes, it was decided to undertake the present project.

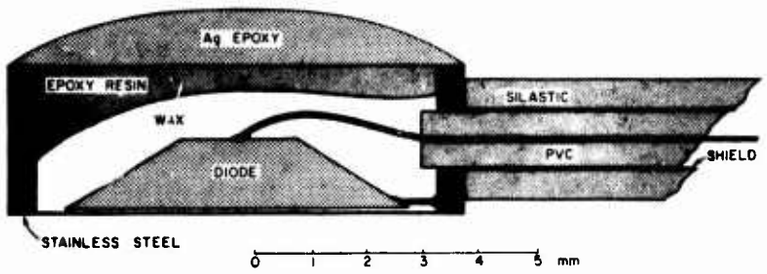

Figure 1. Schematic diagram of semiconductor radiation detector

Contoured-surface silicon tunnel diodes with gallium-diffused semiconductor junctions were obtained from Space Technology Products, a subsidiary of the General Electric Company, Philadelphia, PA. A schematic of an assembled unit is shown in Fig. 1. These devices are in the shape of a shallow truncated pyramid measuring 5 mm on a side and having an active surface area of approximately 8 mm^2. Although the manufacturer recommends that these units be encapsulated in RTV silicone rubber, this was found to increase noise levels prohibitively. This problem was solved by first dipping the units in beeswax heated to 80 C, after which they were compatible with either silicone rubber or epoxy resins. The beeswax-coated diode was then mounted in an 8 mm diameter cylindrical stainless steel case and encapsulated in No. 2795 epoxy resin obtained from the Union Carbide Co. The front window of the case was constructed of 1 mil stainless steel shim stock which was found to be 2 times more electron-lucent than the 0.5 mm epoxy sheet previously employed, and was completely waterproof. Three small metal rings were attached to the case to facilitate suture to the epicardium. A shielded 2 mm diameter coaxial cable terminated in a specially-designed fully-shielded coaxial connector.

The devices have been used to measure capillary blood flow in the heart of pentobarbital-anesthetized and consicious post-operative dogs. The units are high-resistance devices (100 megohm), operated at bias voltages of 250-300 V. Because of their high resistance the sensors are extremely sensitive to traces of moisture, and this has limited their useful life in chronic implantation to 2-3 weeks.

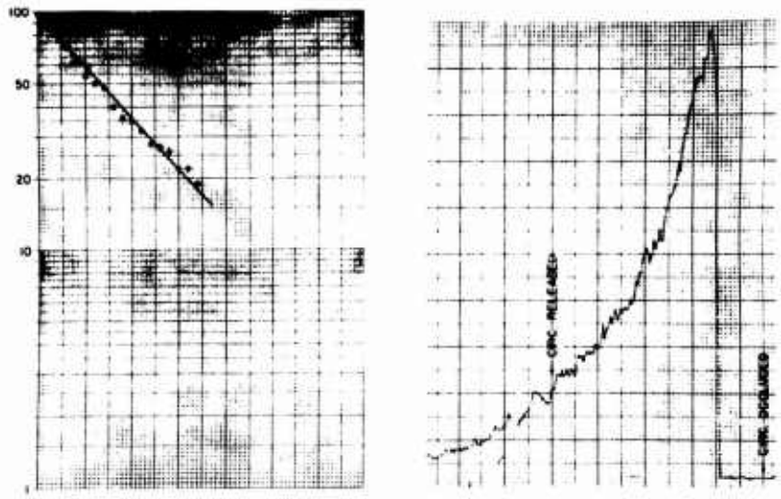

Figure 2. Typical ^{85}Kr washout curve obtained with an epicardial radiation detector.

Units failing at this time consistently have moisture inside the cable and marked by decreased resistance. Several units have been restored to operating condition by drying in a vacuum dessicator for 1-2 weeks. Substitution of polyvinyl chloride for silicone rubber as the cable insulator did not solve this problem, and polyvinylidene chloride, though less water-permeable, was too stiff for chronic implantations. The lack of a flexible waterproof cable insulating material limits the usefulness of this device in chronic experiments. However, these devices are entirely suitable for acute experiments, and the devices have been used repeatedly to measure clearance of ^{85}Kr from cardiac muscle after injection of this isotope into a coronary artery. The clearance curves thus obtained are superior to those obtained by precordial counting in that they are monoexpoential to 10-20% of peak (Fig. 2), whereas clearance curves obtained by precordial counting can be approximated by a single expoential only to about 50% of peak. The high sensitivity of the detectors, coupled with the delivery of radioisotopic gas by close-arterial injection minimizes the amount of isotope needed (2 mCi per determination) and reduces radiation hazard.

This project is terminated, having achieved its goal of the design, fabrication, testing and application of miniature semiconductor radiation detectors for physiological experiments in animals. Although the avalanche diodes employed in this study are extremely sensitive to low levels of radiation, their high internal resistance and resulting dependence on near-ideal insulation makes the detectors

unsuited to chronic animal studies more than 2 weeks in duration. The improved efficiency of the stainless steel window developed in this project enables one to use lithium-drifted silicone diodes, whose resistance is 100 times lower, in detectors for long-term implantation in conscious animals.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6500	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Chg	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A822	00	130
b. CONTRIBUTING	61101A	3A161101A91C	00	
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Gastrointestinal Diseases of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 008800 Life Support 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	6	75
FISCAL YEAR CURRENT 75	7	99

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Marshall D. Kinsey, MAJ, MC NA

21. GENERAL USE

Foreign intelligence not red

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Diarrheal Diseases; (U) Salmonellosis; (U) Intestinal Absorption; (U) Intestinal Adenyl Cyclase; (U) Intestinal Prostaglandins

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Research efforts in this Department will continue to be directed towards gastrointestinal diseases of military importance. In particular, the focus is on the enteropathogenic diarrheal diseases, Salmonellosis and Shigellosis. These have critical military relevance since they represent a major factor in troop mobility.

24. (U) Studies will continue to employ several in vivo and in vitro models. These include profusion models using rhesus monkeys and rats and in vivo rabbit ileal loop models. In vitro studies will be performed on homogenates of intestinal mucosa from rabbits, rats and monkeys. They will involve enzymatic studies, tissue prostaglandin levels and studies of metabolic and respiratory activity.

25. (U) 73 07 - 74 06 Bacterial invasion of intestinal mucosa is required for the development of clinical salmonellosis and shigellosis. However, invasion is not sufficient since certain invasive non-diarrheagenic mutants have been identified. Salmonella and shigella both activate adenyl cyclase and cause increased intracellular cyclic AMP accumulation. This is directly related to the absorptive and secretory defect. Adenyl cyclase activation by salmonella and shigellae can be inhibited with the anti-inflammatory drug, Indomethacin, an inhibitor of prostaglandin synthesis, whereas the activation with cholera and cholera toxin cannot be. It is quite probable, therefore, that there is a prostaglandin intermediate in the pathway from salmonella and shigellae invasion to adenyl cyclase activation. This is currently being investigated. For technical report, see Walter Reed Army Institute of Research, Annual Progress Report, 1 Jul 73 to 30 Jun 74.

* Available to contractors upon originator's approval

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Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 130, Gastrointestinal Diseases of Military Importance

Investigators
Principal: Ralph A. Giannella, LTC, MC
Associates: Ronald E. Gots, MAJ, MC, Alan N. Charney, MAJ, MC, Marshall D. Kinsey, MAJ, MC, Dennis P. Collin, CPT, MSC, Esther P. Jorolan, Ph.D., Alton A. Reeder, LTC, MC (Part time Research Training Fellow)

Description.

The research activities in this Department have focused on problems of intestinal fluid and electrolyte secretion, especially the biochemical and physiological mechanisms of secretion associated with the enteropathogenic diarrheal diseases. To further understand those disease processes and to delineate underlying mechanisms, we have also considered physiologic controls of intestinal fluid secretion and absorption. The aim is to develop a firm grasp on the physiology and biochemistry of intestinal fluid transport so that we might firmly understand diarrheal disease processes. Several limited related studies dealt with absorption of other nutrients such as folic acid and iron in other intestinal disease states.

Progress and Results.

A manuscript detailing the occurrence and probable mechanism of folic acid deficiency associated with iron deficiency has been published (1). It has been found that folic acid deficiency results from abnormal erythropoiesis, increased folic acid utilization, and hemolysis rather than from a disturbance of intestinal function. One group of studies have dealt with the effects of bacterial overgrowth, the Blind Loop Syndrome, on small intestinal structure and function. In the first study, it was shown that bacterial overgrowth resulted in alterations in active transport of sugars and amino acids by causing biochemical abnormalities in the brush border of small intestinal cells (11).

Additional studies are being completed which have considered the mechanism of iron deficiency in the blind loop syndrome (10). Other studies in experimental blind loop syndrome considered the effects of small intestinal bacterial overgrowth. Studies showed

that those bacteria caused morphologic damage to the brush border, mitochondria and endoplasmic reticulum. A manuscript has been published (9).

A large review article is in press which deals with general considerations and specific insights into pathogenic mechanisms of infectious diarrheal diseases (12).

To define the probable sights of small intestinal secretion and absorption, villus cells were isolated sequentially from the villus tip to the small intestinal crypt. One enzyme known to be associted with electrolyte and probably water transport, Na^+-K^+-ATPase, was studied in isolated villus tip and crypt cells. A significant gradient of decreasing activity from the villus tip to crypt was found for Na^+-K^+-ATPase. This was true in both the jejunum and the ileum and the jejunal villus tip activity was considerably higher than that in the ileum. An abstract of this study has been published (2) and a full manuscript has been submitted for publication (13).

To facilitate the study of bacterial invasion of the intestine, the HeLa cell tissue culture line was studied as an *in vitro* model for invasion of cells by *Salmonella typhimurium*. It was shown that various strains of *Salmonella typhimurium* could invade HeLa cells and that the susceptibility of these cells correlated well with the susceptibility of the *in vivo* intestine to these bacteria. HeLa cells, therefore, should prove an effective *in vitro* model for studying invasive enteropathogenic infections. This manuscript has been published (3).

A study detailing the pathophysiology of Salmonella diarrhea in rhesus monkeys has been completed. The study included observations of intestinal transport of water and electrolytes and morphological and bacteriological studies. It was shown that the transport abnormalities in Salmonella diarrhea occur in both the large and small intestine. The degree of transport abnormalities was not necessarily associated with direct observation of bacterial overgrowth or invasion. This suggests that additional factors indirectly arising from the infectious process, such as undefined humoral messengers, may participate in secretory events. Current studies are being undertaken to find these processes. The paper describing the pathophysiology of Salmonella diarrhea in rhesus monkeys has been accepted for publication (14).

Similar studies of the pathophysiology of Shigella diarrhea and dysentery were carried out in rhesus monkeys. It was again found that the diarrhea resulted from small and large intestinal secretory defects. Once again the possiblity of a humoral messenger was suggested by the fact that the intensity of transport abnormalities did not necessarily correlate with bacterial overgrowth of the affected site. Two abstracts were published this year on this subject and one manuscript has been submitted for publication (4,5,15).

Flux studies were performed which detailed the unidirectional movement of electrolytes across isolated ileal mucosa invaded by Salmonella typhimurium. This study demonstrated that the electrolyte transport abnormalities were very similar to those associated with cholera infections and known to be related to changes in intracellular cyclic AMP. That paper has been published (8).

Additional studies designed to further delineate the biochemical mechanisms of Salmonella and Shigella mediated secretion were performed. These examined the activation of adenyl cyclase and the stimulation of intracellular cyclic AMP mediated by experimental salmonellosis and shigellosis. We found that the invasive enteropathogens, like the toxigenic cholera, stimulated adenyl cyclase. We were able to inhibit that stimulation with Indomethacin. This strongly suggested the involvement of prostaglandins in the activation of adenyl cyclase. An abstract of this work has been published and two manuscripts are in preparation (7,17,18).

To further probe the mechanism of intestinal secretion in the enteropathogenic diarrheal diseases, Indomethacin, an inhibitor of prostaglandin synthesis, was used. Indomethacin completely abolished Salmonella-mediated fluid secretion in the rabbit ileal loop model and markedly reduced the secretion secondary to shigellae, cholera toxin and cholera organisms. An abstract of these findings was published and a manuscript has been accepted for publication (6,16).

The preparation of a manuscript from a previously performed study was concluded here in 1973. This manuscript dealt with the functional association of hexokinase to rat liver mitochondria. It considered for the first time a kinetic relationship between an associated enzyme and the supply of substrate by respiring mitochondria. It is one of the few demonstrations of an important

functional association between a substrate provider and a substrate acceptor (19).

To better understand the control of intestinal secretion and absorption and to elaborate on our finding that prostaglandins may be involved, we undertook an in vitro study of prostaglandin synthesis. Full thickness colonic and jejunal segments were compared with mucosal scrapings for prostaglandin synthetic capacity. Full thickness fractions were considerably more active suggesting the intestinal smooth muscle may have a higher prostaglandin turnover rate than intestinal mucosa (20). Further studies will examine prostaglandin synthesis in infected mucosa.

Conclusions and Recommendations.

We have now learned that the biochemical response to salmonella and shigella infections in the intestinal tract involves the activation of adenyl cyclase. This activation leads to fluid secretion in experimental cholera. The mechanism of adenyl cyclase activation by these invasive organisms seems to be different, however, from that of cholera. This was suggested by the observation that Indomethacin blocked the adenyl cyclase activation mediated by salmonella and shigella but had no effect on the activation of adenyl cyclase mediated by cholera. It is likely that a messenger such as prostaglandin play a role in the activation of adenyl cyclase by salmonella and shigella. Studies are currently underway to identify the presence of such an intermediate. If prostaglandins or another intermediate can be found, it is likely that one will be able to interrupt the activation of adenyl cyclase and hence the diarrhea. Preliminary studies in the rabbit ileal loop model have indicated that Indomethacin can markedly reduce the secretion mediated by Salmonella typhimurium.

The aim of this Department is to elucidate the fundamental mechanisms of physiologic and pathologic intestinal fluid electrolyte transport. By so doing, therapeutically useful information should be generated. Proposed studies include an examination of bile salts and other naturally occurring agents on intestinal fluid electrolyte transport, examination of specific transport alterations engendered by Indomethacin and aspirin, observation of specific enzymes associated with fluid electrolyte transport such as Na^+-K^+-ATPase and adenyl cyclase and attempted elucidation of the role of prostaglandins in the intestinal secretory processes.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 130, Gastrointestinal Diseases of Military Importance

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2. Giannella, R. A., Rout, R., and Toskes, P. P.: Jejunal brush border injury and impaired sugar and amino acid uptake in the blind loop syndrome. In press, Gastroenterology, 1974.

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9. Giannella, R. A., Gots, R. E., Formal, S. B., Charney, A. N.: Pathogenesis of Shigella-mediated diarrhea activation of adenyl cyclase. Manuscript in preparation.

10. Gots, R. E. and Bessman, S. P.: The functional compartmentation of mitochondrial hexokinase. In press, Archs. Biochemistry and Biophysics, 1974.

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Publications

12. Toskes, P. P., Smith, G., Bensinger, T., Giannella, R. A. and Conrad, M.: Folic acid abnormalities in iron deficiency: mechanism of decreased serum folate levels. Amer. J. Clin. Nutr. 27: 355-361, 1974.

13. Charney, A. N., Gots, R. E. and Giannella, R. A.: Sodium Potassium ATPase in isolated intestinal villus tip and crypt cells. Gastroenterology 66: 673, 1974.

14. Giannella, R. A., Washington, O., Gemski, P., Formal, S.: Invasion of HeLa cells by Salmonella typhimurium: A model for the study of salmonella invasiveness. J. Infect. Dis. 128: 69-75, 1973.

15. Rout, R., Giannella, R. A., Formal, S., Dammin, G.: The pathophysiology of shigella diarrhea and dysentery. Clin. Res. 21: 964, 1973.

16. Giannella, R. A., Rout, R., Formal, S. B., Dammin, G. J.: The pathophysiology of shigella diarrhea and dysentery in the rhesus monkey. Gastroenterology 66: 698, 1974.

17. Gots, R. E., Formal, S., Giannella, R.: Indomethacin inhibition of salmonella, shigella, and cholera toxin mediated rabbit ileal fluid secretion. Clin. Res. 21: 975, 1973.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6532	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A822	01	128
b. CONTRIBUTING	61101A	3A161101A91C	00	CH FK 170
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Biochemical Methodology and Laboratory Automation

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

008300 Inorganic Chemistry 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	c. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	2	64
FISCAL YEAR CURRENT 75	6	65

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Automation; (U) ESR; (U) Toxicology; (U) Drugs of Abuse; (U) Gas Chromatography

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The technical objective of this work unit is to develop and establish modern automated methods for qualitative and quantitative chemical analyses of military importance in medical laboratories.

24. (U) Automated analytical instruments, on-line digital computers, electronic digital or analog processors and semi-automated manual techniques will be developed and adapted to identify and quantitate various compounds for clinical and research use. Emphasis will be placed on precise, accurate, and fast analytical systems, on simultaneous identification of a variety of compounds, on analytical standardization and on practical systems for laboratory and intensive care facilities use. Efforts will be focused on fast identification and quantitation of drugs of abuse and their principal metabolites, the utilization of simplified automated analytical systems for data collection and on standardization of techniques and equipment for automated laboratory systems.

25. (U) 73 07 - 74 06 Analytical chemistry methodology development and application was continued in this work unit. A micro-analytical continuous flow analysis system has been developed, demonstrated and reported. Development of a four channel model is complete. Use of desk top programmable electronic calculators as analytical system processors has been demonstrated and reported. A variation on high performance liquid chromatography using one third the column pressure previously required without sacrifice of resolution has been developed and demonstrated. Application to analysis of a variety of metabolic products in body fluids has been initiated. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 170 Biochemical methodology and laboratory automation

Investigators.

Principal: LTC Douglas J. Beach, MSC

Associate: MSG Vaughn G. Ayer; PFC Kenneth P. Arnold, B.S.; Billy G. Bass, M.S.; Ann R. Berman, B.S.; Betty J. Boone, Ph.D.; Nesbitt D. Brown, B.S.; SFC James R. Carr; SP5 Gary L. Catchen, B.S.; CPT James A. Cella, MSC; John I. Davis, B.S.; LTC Gale E. Demaree, MSC; James E. Doolittle; Laurence R. Hilpert, B.S.; SP5 John H. Ibbotson; Leo Kazyak, B.S.; CPT James A. Kelley, MSC; SP4 Aubrey L. Kiser, B.S.; SP4 Edward F. Kenehan, B.S.; Robert T. Lofberg, Ph.D.; PVT Leighton C. Makanui; SP4 Burton L. Markham, B.S.; Edward J. Matusik, B.S.; Jean E. Matusik, B.S.; SP5 Michael E. McKay; SP4 James P. McGrath, B.S.; MAJ William E. Neeley, MC; SP4 Howard Nelson, B.S.; Dominic F. O'Donnell; Robert C. Permisohn, M.S.; SP5 Lloyd B. Salt, B.A.; Helen C. Sing, M.S.; SP5 Steven J. Weise, B.S.

The objectives of this work unit are to develop and exploit new laboratory methods for application to automation in order to provide rapid, precise and accurate qualitative and quantitative chemical analyses in support of military medicine. During the reporting period, efforts have been focused on the following areas:

1. Automated miniaturized continuous flow microanalytical systems.

2. Application of electronic programmable calculators to on-line clinical laboratory analyses.

3. High performance liquid chromatography applications.

4. Computer controlled gas chromatography applications.

1. Automated miniaturized continuous flow microanalytical systems.

a. Design and operation of a signal comparator to increase efficiency of continuous flow analyzers. A signal comparator has been designed and developed that allows the bubbled stream of continuous flow analysis to remain intact as it passes through the colorimeter flow cell. This bypassing of the debubbling stage in the analysis

stream improves the efficiency of these analyzers. The signal comparator eliminates the bubble artifact from the photodetector signal while allowing the signal produced from the fluid segment to remain intact.

Parallel glucose analyses on 94 randomly selected sera were performed using a miniaturized continuous flow system and the signal comparator (System I) vs a continuous flow method employing a debubbler (System II). The regression line for the comparison is:

$$Y \text{ (System II)} = 0.9840 \text{ X (System I)} + (-1.3211) \text{ mg/100 ml}$$

with

$$R = 0.9984 \text{ (correlation coefficient)}$$

The magnitude of the intercept point was negligible and did not indicate a significant difference between the two systems.

b. Design and performance of a miniaturized high speed continuous flow analyzer. Design and testing of a high speed miniaturized continuous flow analyzer has been completed. The analyzer, using the comparator described above, is capable of analyzing samples 3 to 25 µl in volume with a reagent consumption of 180 µl/sample at a rate of 150 samples/hour. In a series of determinations for serum glucose and total protein (n = 15), the miniaturized system had a coefficient of variation of about 1%. The series was also run on an AutoAnalyzer system with almost identical results. The comparison of system performance with two other continuous flow analyzers is given in the summary below.

Comparison of System Performance

Instrument	Analysis Rate	Sample Size (µl)	Reagent Cost (Cents/Test)
AA I	60/hr	67	2.8
AA II	60/hr	67	0.8
Mini-System	150/hr	22	0.14

The marked reduction in sample size makes the system ideal for microanalyses, especially in the pediatric clinical laboratory, and in any other situation where small sample volume is important.

c. Design and operation of a four-channel miniaturized system. Using a modification of the above described analyzer system, a four-channel miniaturized continuous flow system for simultaneous analysis of serum Na, K, Cl and CO_2 was developed. A Corning Flame Photometer

(Model 450) was interfaced with a miniaturized manifold for Na and K determinations. The bubbled stream flows directly into the flame photometer with no disruption in flame stability. Cl and CO_2 were analyzed using a newly designed colorimeter employing the signal comparator described above eliminating the need for a debubbler.

Analysis can be performed under steady state conditions at a rate of 150 samples/hr. The sample volume is 56 µl. The total volume of reagent consumed by all four channels was less than 500 µl/sample. The coefficient of variation ranged from 0.4% to 2%.

2. Application of electronic programmable calculators to on-line clinical laboratory analyses.

a. Use of programmable desk top calculators for on-line acquisition, computing and printing of results from single- and multi-channel laboratory instruments. The use of a low cost programmable desk top calculator for on-line processing of data from a variety of laboratory instruments has been successfully demonstrated. The calculator, a Hewlett-Packard Model 9810 A, was connected to the instrument through a digital voltmeter that served as an A to D converter and interface. The calculator was used to compute and plot on an X-Y plotter a standard curve, quality control values, confidence limits and the values of the sample analyses, for single-channel continuous flow analyzers operating at rates up to 300 samples/hr. For multi-channel (1-4 channels) continuous flow analyzers at speeds up to 120 samples/hour/channel, the calculator was used on-line to provide real time printed results by sample number. A four-channel multiplexer was designed to allow a single calculator to handle all four channels of information. Optionally, a tape cassette may be used to record all results for transfer to a larger processing system.

The on-line use of these calculators with laboratory instruments provides numerous advantages: reduction of time between receipt of a specimen and reporting of final results, elimination of the laborious task of calculating results, and the immediate indication of analyzer malfunction through the availability of quality control results as they are analyzed. The calculator system requires minimal space, is economical, reliable, easy to use and portable.

b. A modularized, automated data acquisition, processing and reporting system for the clinical laboratory. A comprehensive modularized, automated system has been developed for handling test requests, on-line data acquisition, processing, reporting and patient file storage for the clinical laboratory. Numerical data from patient requisitions were entered into a programmable calculator (Hewlett-Packard 9810 A) by use of a hand-held optical scanner. Programmable

calculators were used on-line with multi-channel analyzers for real time data processing. Results were recorded on tape cassettes and then transferred to a larger programmable calculator (Hewlett-Packard Model 9830) to merge the test results with patient requisitions. The merged data were transmitted directly from the larger calculator to a remote time-sharing computer for storage and recall. Patient data can be recalled for display on CRT terminals in hospital wards and may be permanently listed by a high speed printer. The total system was demonstrated and the parts of the system not requiring the large computer are presently undergoing operational testing by the staff of the Clinical Pathology Laboratory of the Walter Reed Army Medical Center.

3. High performance liquid chromatography in the study of p-aminobenzoic acid metabolism.

A simple, rapid high performance liquid chromatographic method has been developed for determining p-aminobenzoic acid (PABA) and some of its metabolites in physiological fluids. The glycine conjugated and acetylated metabolites of PABA, p-aminohippuric acid (PAHA), N-acetyl-p-aminobenzoic acid (N-A-PABA) and N-acetyl-p-aminohippuric acid (N-A-PAHA) were quantified with a lower detectable limit of 5 nanograms. The method is applicable to urine and serum without solvent extraction or pretreatment.

A commercially available high performance liquid chromatograph was modified to operate at a 7-fold decrease in column pressure with no noticeable degradation in resolution of the compounds of interest. A one meter stainless steel column, packed with an anion exchange resin was used. Compounds were detected with the standard 254 nm detector. Elution was accomplished with a sodium formate buffer within a 20-min period.

The system was evaluated for clinical application. Human volunteers ingested 2.5 gms of PABA orally; urine and serum specimens were collected and analyzed. A definite excretion pattern was observed for the aromatic metabolites in the urine specimens. PABA was completely conjugated or excreted as the unchanged compound during the first hour. PAHA was detected in the first specimen (15 min post-administration) and did not disappear until 5 hrs post-administration (p.a.). N-A-PABA began to form 30 min p.a. and remained at a constant level for 4 hours. N-A-PAHA was not detected until 90 min p.a. and was present for about one hour.

Similar patterns were observed in the serum specimens and in urine specimens following intravenous injection of PABA. Additional studies of the excretion patterns of PABA and metabolites are planned using patients in various stages of renal disease.

4. Computer controlled gas chromatography studies.

Four automated gas chromatographs have been interfaced to an on-line real-time computer system and are functioning satisfactorily. The analyses of over 150 plasma specimens for valium (diazepam) were performed on the system. The system is now being programmed to operate in a multi-programmed mode to allow other analytical equipment such as the mass spectrometer and ultraviolet spectrophotometer to operate under control of the computer simultaneously with the gas chromatographs.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 170 Biochemical methodology and laboratory automation

Literature Cited.

Publications:

1. Neeley, W. E., Wardlaw, S., and Swinnen, M. E. T.: Design and operation of a signal comparator to increase efficiency of continuous flow analyzers. Clin. Chem. 20: 78, 1974.

2. Neeley, W. E., Wardlaw, S. C., and Sing, H. C.: Design and performance of a miniaturized high speed continuous flow analyzer. Clin. Chem. 20: 424, 1974.

3. Neeley, W. E.: Design, development, and operation of a miniaturized continuous flow system. Clin. Chem. 19: 1973.

4. Kazyak, L.: Information exchange and computerized data retrieval for toxicology. J. Forensic Sci. 19: No. 1, 1974.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6517	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	☑ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	185
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Speciation of Biomolecules by Mass Spectrometry

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 008300 Inorganic Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	1	47
FISCAL YEAR CURRENT 75	4	78

19a. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Mass Spectrometry; (U) Drug Metabolism; (U) Gas Chromatography; (U)Analytical Chem.

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The technical objective of this work unit is to develop and establish methodology and analytical techniques for the detection, identification and characterization of important biochemical compounds and their principal metabolites using coupled gas chromatography-mass spectrometry (GC-MS) for use in military medicine.

24. (U) Computer controlled GC-MS systems will be used to definitively characterize and quantitate compounds of biochemical importance for both developmental and research uses. Efforts will be focused on rapid, accurate identification of a variety of compounds and on feasibility of the implementation of these systems for research and practical general laboratory use. Efforts will be concentrated on metabolites of compounds of interest in drug abuse programs and in the study of metabolism of biomolecules both natural and foreign to mammalian biosystems. A library of mass spectra of drugs of abuse of military significance will be developed.

25. (U) 73 07 - 74 06 Operational debugging and reliability testing of the computerized GC-MS system was performed. The principal metabolites of methaqualone have been characterized. Quantitative correlation of the alpha-hydroxy methaqualone and methaqualone has been determined in urine specimens from over 30 users. A 100:1 ratio of metabolite to parent drug was found. Other drugs of abuse such as cocaine and tetrahydrocannabinol are being characterized. A library of mass spectra of drugs of abuse is being developed. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval.

DD FORM 1498 MAR 68 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 185 Speciation of biomolecules by mass spectrometry

Investigators.
Principal: LTC Douglas J. Beach, MSC
Associate: Billy G. Bass, M.S.; LTC Gale E. Demaree, MSC;
Laurence R. Hilpert, B.S.; Leo Kazyak, B.S.;
CPT James A. Kelley, MSC; Robert C. Permisohn, M.S.;
SP4 Steven J. Weise, B.S.

The objective of this work unit is to develop and establish methods and analytical techniques for the detection, identification, characterization and quantitation of important biochemical compounds and their principal metabolites using coupled gas chromatography-mass spectrometry systems. The major effort during the past year was in the following areas.

1. Characterization of 11-hydroxy-Δ9-tetrahydrocannabinol.

2. Methaqualone metabolite characterization.

3. Collaborative mass spectrometer research activities.

4. Equipment performance evaluation.

1. 11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-Δ9-THC).

The metabolic fate of tetrahydrocannabinol (THC) and, more specifically, its major active component Δ9-THC, is at best incompletely misunderstood. A study was initiated to characterize and quantify THC metabolites and to describe the time dependency of their appearance in human body fluids obtained from marihuana smokers. Marihuana contains a complex mixture of closely related natural products. Only a small dose (less than 3 mg) of the active component is required to produce psychotropic effects, and all the presently known metabolites are excreted as glucuronides.

Cannabidiol, cannabinol, and 11-OH-Δ9-THC were characterized by gas chromatography and mass spectrometry as the free compounds and their respective trimethylsilyl (TMS) and heptafluorobutyro (HFB) derivatives. Gas chromatographic studies with the HFB derivatives of THC and 11-OH-Δ9-THC indicated that 0.5 to 10 nanograms can be detected in standards by electron capture. TMS derivatives produced too many extraneous substances for reliable detection at anticipated urinary levels. An

extensive study of various extraction procedures was made to determine the optimum conditions for recovery of 11-OH-Δ9-THC from urine. Aqueous standards at a concentration of 1 µg/ml gave recoveries of 75-85% using 2% isobutanol in chloroform at pH 5. However, urines to which 11-OH-Δ9-THC had been added gave lower extraction efficiencies indicating possible adsorption or binding phenomena. Some interference with the derivatization reaction necessary for electron capture with gas chromatography may have contributed to recovery difficulties. This aspect of the problem is still under investigation.

Twenty ml of a pooled urine of a marihuana smoker, collected 3-4 hrs after a reefer had been smoked, were hydrolyzed, extracted and analyzed by GC-MS. Cannabinol was identified in relatively large amounts compared to a small concentration of 11-OH-Δ9-THC. A carboxylated metabolite was detected that is presumably 11-carboxymethyltetrahydrocannabinol. This compound was esterified by ethereal diazomethane to further confirm its carboxylic characteristics. These cannabinoid components of the urine extracts were determined by mass fragmentography of characteristic ions, correlated with standardized retention data, and confirmed by comparison of the complete mass spectra of each gas chromatographic peak in question with standardized library spectra.

2. Methaqualone.

In collaboration with the U.S. Air Force Europe Drug Abuse Detection Laboratory, urine specimens from suspected methaqualone users were studied for methaqualone metabolites, in order to test possible correlation in excretion of methaqualone and its metabolites. Gas chromatography and combined GC-MS were used to resolve the complex mixtures of metabolites that were present in the samples analyzed. Because of the wide range of polarity, two packed columns with somewhat different liquid phases (OV-1 and OV-17) were necessary to separate the mono- and di-hydroxylated metabolites of methaqualone in the chromatograph. Except for two of the metabolites, all others were extremely polar compounds, and resolution, as well as sensitivity, could be achieved only after trimethylsilyl ether derivatization prior to analysis by gas chromatography.

Quantitative data have been obtained on methaqualone and four of its metabolites. The principle metabolites are 3'-hydroxy methaqualone, 4'-hydroxy methaqualone, α-hydroxy methaqualone, and 2-methyl-hydroxy methaqualone. 6-hydroxy methaqualone and several other metabolites were identified, but since the concentrations were very low, no significance was attached to these compounds, and no attempt at quantification was made. The concentration of methaqualone was in a range of 0.28-10.00 µg/ml of urine in those specimens that were processed. Concentrations of the major metabolites were from 15 to over 200 times that of the methaqualone in a given specimen. With the exception of

two urine specimens, there were considerably high concentrations of the ring-hydroxylated metabolites (3'- and 4'- hydroxy methaqualone) than the side chain substituted analogues.

The distinctive metabolite pattern of the gas chromatogram produced by the relative differences in concentration appears to have some significance perhaps in relation to dose and time between ingestion and specimen collection. The pattern is certainly important for identification purposes by gas chromatography, especially when low levels of methaqualone cannot be distinguished from interferences by normal urinary constituents. Further interpretation of these data will be possible after the next phase of the study has been completed. This study will attempt to relate the quantity of each metabolite to ingestion time and dose.

3. Collaborative mass spectrometer research activities.

During the past year the initial drug library of 90 compounds has been expanded to include over 150 drugs, metabolites, derivatives, and substances commonly encountered in extracts of body fluids. The additional compounds were characterized by gas chromatography as well as by mass spectrometry. Computer programs have been devised to convert retention data to indices, and to compare these reference data with data from specimens to identify suspect compounds.

4. Equipment performance evaluation.

Mass spectrometry is rapidly becoming a valuable new tool in the biomedical and organic chemical analysis armamentarium. The highly complex and sophisticated instruments used in the research laboratory are becoming simpler to operate, less cumbersome and less expensive. They are also providing exciting new possibilities for the study of drug metabolism and pharmacokinetics. However, little is known about the long term reliability and usefulness of the instruments in the clinical milieu. To this end, a brief review of the operating experience of this laboratory seems appropriate.

The coupled gas chromatograph-mass spectrometer employed is the Hewlett-Packard Model 5930 A system. The system consists of a GC-mass spectrometer and a 2100 A minicomputer data system with auxiliary tape cassette storage and a computer controlled data plotter. The system has been operational for approximately one year.

During the first year's operation, 473 samples were analyzed. The sources of these samples are given below:

Source	% of Total
GC-MS laboratory	44.0
System tests and calibration	11.8
Division of Biochemistry	26.4
Extra-divisional (WRAIR)	17.8

The time devoted to system tests is also divided into two categories:

Optimization	7.4%
Trouble-shooting	4.4%

The operational reliability of the system is summarized below:

Fully operational	128 days	63.0%
Partially operational	37 days	18.0%
Inoperative	38 days	19.0%
Total working time	203 days	

Inoperative time was considered time during which a major component of the system malfunctioned. Weekends and holidays were not included in the working time unless samples were actually analyzed. Twenty major component failures occurred during this period with only one consistent pattern discerned. The electron source filament was replaced three times. This problem appears to be common in all instruments of this type manufactured by Hewlett-Packard.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6519	74 06 30	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DOD'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	K. Comp.	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	190
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Effects of Antimalarial Drugs on Dental Plaque

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	74 06	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	0.5	20
FISCAL YEAR CURRENT 74	0.5	39

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: DA

NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Test System; (U) In vitro; (U) Dental Plaque; (U) Periodontal Disease

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To design and develop in vitro and in vivo test systems for the evaluation of chemical compounds as prophylactic and therapeutic agents against diseases of major military significance. Infections such as malaria, which render troops ineffective for extended periods, receive principal emphasis. Complete characterization of candidate antimalarial drugs is an essential part of the Army Drug Development Program.

24. (U) Appropriate in vitro and animal pilot models are designed to meet a specific need for testing chemical compounds. These models are expanded into operating test systems in-house and utilized to provide guidance to contractors when testing is to be done under contract. When necessary, modifications to existing test systems are designed and developed.

25. (U) 73 07 - 74 06 Approximately one-third of sixty-seven known antimalarial compounds used from several chemical classes were found to inhibit the growth of cultures of microorganisms causing dental plaque. None, however, were effective in preventing the development of plaque in an in vivo hamster model. The apparent ineffectiveness against dental plaque formation in the hamsters occurred whether the test agents were applied topically to the teeth or administered via the diet. Included in the studies were three chloroquine-primaquine drug diet regimens. One of the C:P ratios used was 20:3, i.e. that ratio used in the tablets employed for malaria prophylaxis among troops in Viet Nam. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

* Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 65 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 190 Effect of antimalarial drugs on Dental Plaque

Investigators.
Principal: LTC Kenneth E. Kinnamon, VC
Associate: 2LT Charles R. Wolf

It was noted that the incidence of dental plaque and related oral diseases in patients which had recently returned from Viet Nam seemed to be much lower than in patients with an otherwise similar history. Although these were subjective observations that had not been confirmed by definitive measuring techniques, it led to the speculation that such a reduced incidence of disease might have resulted from the regimen for prophylaxis of malaria (300 mg chloroquine and 45 mg primaquine per week) required in Viet Nam.

Sixty-eight compounds that exhibited antimalarial activity in one or more tests employed by us were assessed in an in vitro system. Thirty-seven percent of all compounds tested were active, i.e. demonstrated inhibition of bacterial growth. Except for sulfonamides no more than half of the compounds in each class were active. Primaquine was active; chloroquine was not. Folic acid antagonists used were three 2,4 diaminopyrimidines; three 2,4 diaminotriazines; six 2,4 diamino, 6 sulfur-substituted quinazolines; proguanil and nitroguanil. All six quinazolines and one each of the pyrimidines and triazines were inactive. The quinoline-methanol designated WR 142,490 [α-(2-piperidyl)-2,8-bis(trifluromethyl)-4-quinoline-methanol] and 4-4' diaminodiphenyl sulfone (Dapsone), both of which have been tested under an Investigational New Drug Application, were found to be active in the in vitro system described. Among active compounds in the miscellaneous class were quinacrine and the compound designated RC 12. The latter is 5-bromo-4-bis-(2-diethylaminoethyl)amino-1,2-dimethoxy benezene.

Of the sixty-eight compounds tested in vitro, ten (seven active; three inactive) were tested by topical application to the teeth of the test animals and six (four active; two inactive) by adding them to the hamster diet. Four of the latter were added as previously described. Chloroquine was used alone at levels of 100-, 500- and 1000 mg/kg of diet; primaquine alone was used at 15-, 75- and 150 mg/kg of diet; chloroquine plus primaquine was used respectively at 100- plus 15-, 500- plus 75- and 1000- plus 150 mg/kg of diet. With the exception of those receiving the positive control compound, tetracycline, no compound was found to inhibit the in vivo development of dental plaque.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6520	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	191
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Immunosuppressive Activity of Medicinal Compounds

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA — EXPIRATION:

b. NUMBER:

c. TYPE: — d. AMOUNT:

e. KIND OF AWARD: — f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING 74	.2	38
	CURRENT 75	.3	36

19a. RESPONSIBLE DOD ORGANIZATION

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TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

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Div of Medicinal Chemistry

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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

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TELEPHONE: 202-576-2292

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence not considered

DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Immunosuppression; (U) CFUs; (U) Spleen Colony Assay; (U) Simonsen Assay; (U) Bone Marrow; (U) Allogeneic

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To conduct testing to evaluate the immunosuppressive activity of various medicinal compounds. Knowledge re immunosuppressive activities of new drugs is essential to the Army Drug Development Program.

24. (U) Certain chemical agents which are being tested primarily for certain properties, e.g., antimalarial properties, may also have immunosuppressive properties. These immunosuppressive properties in some cases may serve to guide the development of potential drugs.

25. (U) 73 07 - 74 06 Adult spleen cells, if injected into a suitable host, will be stimulated by antigens of the host to react in the latter in a truly immune or hypersensitive way. This response depends upon the absence of host type antigens in the donor. Contrariwise, the presence of donor antigens which are absent in the host does not lead to an immune reaction under the condition of the assay system. In the system the recipient host is young enough at the time of transplantation that it is not yet immunologically competent and is therefore tolerant of the foreign cells. This system has been employed to assess the immunosuppressive properties of the antimalarials chloroquine (WR 1544), WR 30,090, WR 33,063 and WR 122,455. None of these compounds demonstrate immunosuppressive properties. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 191 Immunosuppressive activity of medicinal compounds

Investigators.
Principal: LTC Kenneth E. Kinnamon, VC
Associate: CPT Lyle L. Ketterling, MSC

Adult spleen cells, if injected into a suitable host, will be stimulated by antigens of the host to react in the latter in a truly immune or hypersensitive way. This response depends upon the absence of host antigens in the donor. Contrariwise, the presence of donor antigens which are absent in the host does not lead to an immune reaction under the condition of the assay system. In the system the recipient host is young enough at the time of transplantation that it is not yet immunologically competent and is therefore tolerant of the foreign cells.

The assay for immunosuppressive activity consists of recording the spleen and liver enlargement which results in the recipients after transplantation of adult spleen cells to recipients that are less than 10 days old. From the spleen and liver enlargements, organ indices are computed. Recipient mice are first generation hybrids between the donor strain and the other strain against which the donor is expected to react, i.e. (C57BL/6 x CBA) F_1.

The system has been used to assess the immunosuppressive properties of the antimalarial compounds, Chloroquine (WR 1544), WR 30,090, WR 33,063, and WR 122,455. None of these compounds demonstrate immunosuppressive properties.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6521	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	192
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Antiarrhythmic Effects of Aliphatic Amines

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	1.0	41
FISCAL YEAR CURRENT 75	1.0	39

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
Washington, DC 20012
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RESPONSIBLE INDIVIDUAL
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20. PERFORMING ORGANIZATION

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NAME: DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Pharmacology; (U) Drugs; (U) Medicinals; (U) Hypotension; (U) Shock

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Research is directed toward investigating the pharmacology of promising medicinal aliphatic amines, drug interactions, developing and refining animal models for the study of arrhythmias and associated cardiovascular effects produced by drugs or injury. Candidate drugs will be tested in these model systems and compared with standard drugs. The goal of this research is to develop a highly effective, non-toxic drug which would be useful in the treatment or prevention of arrhythmias occurring spontaneously or as a complication of therapy or injuries involving military personnel.

24. (U) Drugs are tested in animal models for efficacy in preventing or treating experimental arrhythmias and associated problems. These may be induced by exogenous catacholamines or by injury.

25. (U) 73 07 - 74 06 The mechanism of bradycardia induced by intravenously injected WR 2823 was investigated in anestnetized cats; results of drug pretreatment and of spinal transection indicate that the effect may be mediated by an action on central vasomotor centers. WR 2823 promotes marked reduction in mortality in mice subjected to rolling trauma or to endotoxin challenge; no protection was afforded by WR 2823 in tourniquet-induced injury. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 192 Antiarrhythmic effects of aliphatic amines

Investigators.
Principal: Melvin H. Heiffer, Ph.D.
Associate: Dr. A. Einheber

1. Introduction.

Simple aliphatic amines have a number of potential medical uses. Some of the series that we have studied have important cardiac and autonomic system effects while closely related structures may not exhibit these. In addition to affording some protection against ionizing radiation, some of these compounds protect against lethality of shock and of trauma. Some of these compounds also exhibit important antiarrhythmic action. Studies have been carried out to broaden our understanding of these various pharmacological activities.

2. Protective effects of WR 2823 against lethality in mice.

a. Background:

WR 2823, an aliphatic sulfur-containing compound, produces alpha-adrenergic blockade in several animal species (Heiffer et al, 1969). Drugs which possess alpha-adrenergic blocking properties, such as phenoxybenzamine, have been observed to promote survival of animals subjected to various types of experimental shock. Preliminary observations in anesthetized dogs subjected to hemorrhagic shock (Vick et al, 1971) indicated that WR 2823 improves survival. Thus the following studies were carried out to investigate the possible beneficial effects of this agent in unanesthetized animals subjected to other types of experimental trauma, such as tumbling trauma, tourniquet injury and endotoxin poisoning.

b. Methods:

Female CD-1 mice (Charles River) weighing 23-28 gms were used as experimental animals and were housed in their home cages in groups of 20 with routine laboratory food and water ad lib. The mice were exposed to automatically timed, alternating 12 hr periods of illumination (0730-1930 hr) and darkness. Experimental procedures were always performed in the morning between 0900 and 1200 hr.

Mice in the various groups being compared were matched for body weight and were run in parallel.

During an experiment mice were housed individually in clear plastic compartments for 48 hr and then returned to their home cages. In some short term experiments the procedures were abbreviated. Anesthesia was not employed in any experiment. All injections were intraperitoneal.

The drugs tested were prepared fresh daily in sterile, pyrogen-free water, and the volume of solution injected was 0.01 ml/gm of body weight. Any deviations from the foregoing general procedures are indicated below.

Noble-Collip Drum Trauma (NCDT) was one method used for injuring mice (Noble and Collip, 1942). This procedure involves the repeated tumbling of animals in a revolving drum apparatus and results in a contusive type of injury which may be lethal, depending on the total number of revolutions to which the animals are exposed. The apparatus as modified for use in this laboratory consists of 6 stainless steel drums that are powered to rotate from a single pulley driven by an electric motor. Each drum is 15 inches in diameter and 8 inches wide and has 3 equally spaced shelves on the inside which project 2 inches toward the center of the drum. Five mice are placed in each drum and as it rotates the mice are caught by the shelves and dropped about twice during each revolution so that at a uniform speed of 38 rpm the mice are dropped at least 750 times in a 10 min period and 1100 times in a 15 min period of tumbling. Mortality of the mice is noted at the end of trauma, designated as "0" hr, and at at regular intervals thereafter until 48 hr. Mice dying prior to "0" hr are considered acute deaths, whereas those dying subsequently are delayed deaths. Mice alive at 48 hr virtually all survive thereafter and are designated as permanent survivors.

Tourniquet injury (limb-ischemia shock) was produced by placing rubber bands as high as possible on both hind legs, and the induction of saline-irreversible tourniquet shock was attained by additionally tourniqueting the front legs at the axillae so that all 4 legs were rendered ischemic (Einheber and Wren, 1967; Einheber et al, 1970). Mice were subjected to various periods of occlusion and mortality checked at intervals up to 72 hr since virtually all mice surviving to this time survived indefinitely.

Endotoxin shock was produced by suspending E. coli endotoxin (Difco Lab.) in 0.9% saline solution and administering it i.p. at a constant dose of 600 µg per mouse in a volume of 0.3 ml.

On the basis of preliminary trials this dose of endotoxin was calcluated to be lethal to approximately 50% of control mice within 24 hr after challenge and lethal for 80 to 100% of unprotected mice by 96 hr after administration. Since deaths from endotoxin were observed to occur only rarely after 96 hr, mice surviving at this time were considered permanent survivors.

Blood glucose determinations using a Technicon autoanalyzer were made on blood drawn from the retro-orbital venous plexus into heparinized 50 µl to 100 µl capillary tubes. A complete series of glucose standards was assayed during each run and saline washes were used between blood samples.

The hematocrit value for each mouse was obtained by averaging the values from 2 blood samples drawn into capillary tubes from the retro-orbital venous plexus and centrifuged for 3 min.

The statistical significance of differences between mortality data was assessed by chi-square, using Yates correction. The difference between mean values was assessed by the t-test. Statistical significance was set at a probability level of 5% or less.

c. Results and Discussion:

The data shown in Table 1 indicate that WR 2823 promotes a marked reduction in acute and delayed mortality resulting from a 15 min episode of Noble-Collip Drum Trauma (NCDT) when it is administered i.p. to mice, 50 mg/kg, 15 min before trauma. Thus, mice pretreated with WR 2823, as compared to those pretreated with water only, consistently showed a significantly greater survival at the end of tumbling trauma and during the ensuing 48 hr. A comparison of 3 different lots of WR 2823 showed that there was no significant difference in the relative protective efficacies.

WR 2823 AB was formulated into an injection suitable for intravenous administration to human volunteers in phase I clinical trials. Thus it was of interest to compare this material with the unformulated chemical for its protective efficacy against NCDT. Both the materials were equally effective in promoting survival of mice when given 15 min in advance of the injury.

From Table 2 it is evident that WR 2823 provides significant protection against the lethality of NCDT which endures for at least one hr when treatment is 15 min before trauma. Protection has evanesced by 2 hr and is likewise not demonstrable at 24 hr post treatment.

Phenoxybenzamine was given to mice at a dose of 5 mg/kg one hr before injury. As shown in Table 3 this pretreatment was about equally efficacious to a dose of 50 mg/kg of WR 2823 given 15 min before NCDT. Phenoxybenzamine pretreatment has been found by many investigators to protect animals against various forms of fatal shock. Protection has been ascribed to the well known alpha-adrenergic blocking properties of this agent and the results of this study are consistent with these findings.

To determine the effects of beta-adrenergic blockage, propranolol was employed and the results (Table 4) indicate: this compound markedly reduced the mouse's ability to survive NCDT; WR 2823 given after propranolol negates this deleterious effect; and the mortality after combined treatment is significantly improved over that observed after water alone.

Although preliminary studies showed that WR 2823 had no significant effect on blood glucose levels in uninjured mice, from the data presented in Table 5 it appears that the drug blunts the blood glucose rise that is a consequence of NCDT. The mechanism of this effect is unknown, but in view of the fact that liver metabolism is largely responsible for blood glucose elevation via glycogen breakdown, it would appear that WR 2823 may have some direct effect on liver metabolism.

The effect of WR 2823 on the hematocrit was studied and it was found to have no significant effect on hematocrit values. Thus, a hemodilution signifying possible expansion of plasma volume which could better prepare the animal to withstand NCDT would not appear to be involved in the protective effect of the drug.

WR 2823 did not reduce the mortality of mice subjected to tourniquet shock that is otherwise "saline-reversible" or "saline-irreversible", whether it was given alone or in conjunction with saline therapy.

Table 6 shows the significant degree of protection against the lethal effects of endotoxin when mice are given 50 mg/kg of WR 2823 15 min before endotoxin challenge. Thus, the mortality in endotoxin shock can be expected to be significantly decreased by this drug.

3. Pharmacology of WR 2823 in cats and isolated preparations.

a. Background:

The pharmacological effects of WR 2823 were first described by Heiffer et al, 1969. This aliphatic sulfur-containing compound was found to exert both immediate and prolonged actions on the cardiovascular system. The intravenous administration of WR 2823 produced an immediate hypotension and bradycardia in several animal species (Heiffer et al, 1969, Herman et al, 1971). These initial responses are followed by a long lasting alpha adrenergic blockade (Heiffer et al, 1969). Perhaps because of this latter property, WR 2823 has been shown to have efficacy in the prevention of mortality from shock due to hemorrhage or endotoxin in animals (Vick et al, 1969, Vick and Heiffer, 1970, Vick et al, 1973). The clinical usefulness of WR 2823 in human shock conditions remains to be determined but could be limited because of the initial cardiodepressor responses. The present study was initiated to determine in detail the nature and possible attenuation of the initial hypotension and bradycardia induced by WR 2823.

b. Methods:

45 cats (2.5 to 3.5 kg) were anesthetized with 40 mg/kg pentobarbital sodium. The right femoral vein was cannulated for drug injection. Cannulae were also inserted into the right femoral artery and trachea for monitoring blood pressure and respiration. Needle-tipped electrodes were inserted into the appropriate limbs to record lead II of the electrocardiogram. Heart rate was determined from the interval of the R wave by means of a Cardiotach preamplifier. In some experiments carotid artery blood flow was determined by means of a Biotronex flow probe placed around a carotid artery and connected to an electromagnetic flow meter. All recordings were made on a Hewlett-Packard polygraph.

In 3 experiments the spinal cord was sectioned between the first and second cervical vertebrae. Immediately thereafter, the cats were placed on artificial respiration by means of a Harvard positive pressure respirator. An additional 3 cats were decerebrated at the intercollicular level according to the method of Sherrington (1898). Both types of animal preparations were allowed to stabilize for 30 min prior to the administration of WR 2823.

The drugs utilized in the present experiments, the dosage (calculated as as the free base) and the equilibrium period before WR 2823 administration were as follows: control (5 ml isotonic saline, 10 min); atropine (1.0 mg/kg, 10 min); mecamylamine

(2.5 mg/kg, 15 min); hexamethonium (10.0 mg/kg, 15 min); phenoxybenzamine (5 mg/kg, 60 min); dl-propranolol (0.5 mg/kg, 10 min); reserpine (0.1 mg/kg, 2 injections, 48 and 24 hr); lyergic acid diethylamide (50 µg/kg, 10 min); and diphenhydramine (10 mg/kg, 10 min). These agents were either dissolved or diluted in normal saline so that the amount given was contained in a volume of 5 ml or less. Each compound except for phenoxybenzamine and reserpine was injected over a 3 to 5 min period; phenozybenzamine was injected intraperitoneally while reserpine was given intraperitoneally 48 and 24 hr before the experiments.

Following the equilibrium period, WR 2823 (50 mg/kg), dissolved in 5 ml of isotonic saline, was injected over a 4 min period. The various physiological parameters were monitored for up to 60 min after WR 2823 injection and the experiment then terminated.

Control observations of the various physiological parameters were made just before either surgical or pharmacological pretreatment and again before administration of WR 2823. Changes in these parameters were also determined at 1, 5, 15, 30 and 60 min after injection. The mean and standard errors of the mean were calculated when three or more values were obtained for each parameter.

Three adult mongrel dogs (10-12 kg) were anesthesized with phenobarbital sodium (30 mg/kg) and the heart removed and perfused with autologus blood (Vick & Herman, 1971). The force of contraction was measured with a Walton-Brodie strain gauge sutured to the left ventricle. Coronary perfusion pressure was obtained by means of a needle-tipped catheter inserted into the perfusion circuit and attached to a pressure transducer. The electro-cardiogram and heart rate were determined by means of needle-tipped electrodes inserted into the left and right ventricles. Each was allowed to stabilize for approximately 15 min after which control responses to 1.0 µg epinephrine were obtained. WR 2823, dissolved in 5 ml saline, was injected into the perfusion circuit over a 2 min period at doses of 50 mg (2 hearts) or 100 mg (1 heart). Each heart was rechallenged with epinephrine at 15, 30 and 60 min after WR 2823 injection.

c. Results:

WR 2823, 50 mg/kg intravenously, induced immediate hypotension and bradycardia in all 5 intact control animals. These results, together with the effect of WR 2823 in the surgical pretreatment cats are summarized in Table 7. Changes were noted to begin during the infusion and reached maximum decreases of 32% to 48% in mean arterial pressure and 30% to 35% in heart rate by 1 min

post injection. Although recovery began within 5 min, blood pressure ultimately stabilized below control levels. Heart rate returned to control levels by 60 min. Carotid artery blood flow, recorded in 2 experiments, increased 175% and 200% by 1 min post injection but returned to control levels within 15 to 30 min. In 3 of 5 experiments, WR 2823 also induced brief minor increases in respiratory rate and in the amplitude of the lead II R wave.

The 3 intercollicular decerebrated cats responded in nearly the same way to WR 2823 as did the saline controls except that heart rate did not recover as completely (Table 7). Complete interruption of the spinal cord caused a prolonged decrease in both mean arterial pressure and heart rate. The administration of WR 2823 after 30 min produced a brief hypotensive response during the injection but by 1 min post injection both blood pressure and heart rate were slightly elevated. Within 5 min, and during the remainder of the experiment, blood pressure stabilized at 10% to 20% below control levels, while during the same period heart rate was at or near control levels.

Both of the ganglionic blocking agents (mecamylamine and hexamethonium) produced an almost immediate decrease in heart rate (10% to 41%) and blood pressure (10% to 35%). During the administration of WR 2823 brief episodes of hypotension and bradycardia occurred in 2 experiments while increases in both parameters occurred in 4 other animals. However, in all experiments, by 1 min post injection blood pressure was at or slightly above control levels while heart rate increased to preganglionic-blockage levels or above. Pulse pressure and carotid artery blood flow were also elevated during this period. All parameters declined slightly over the remainder of the 60 min experimental period. The results of these and all drug pretreatment experiments are summarized in Tables 8 and 9.

In 3 experiments phenoxybenzamine produced a 10 to 24 mmHg decrease in mean arterial blood pressure. Heart rate increased slightly in 2 of the 3 cats. Alpha-adrenergic blockade was confirmed before the infusion of WR 2823 by injection of epinephrine. WR 2823 caused a decrease in mean arterial pressure of between 36 to 60 mmHg. The changes in heart rate were much [illegible] than those seen in control animals, and at the end of 60 min were at or above pre-phenoxybenzamine levels in all 3 experiments.

Within 5 min of the injection of 0.5 mg/kg of dl-propranolol heart rate was depressed 5% to 16% and the typical cardiovascular responses to isoproterenol were inhibited. The injection of WR 2823 produced a 28% to 48% decrease in mean arterial pressure. The

magnitude of the depression was similar to that seen in control animals. Heart rate, carotid artery blood flow and pulse pressure remained relatively unchanged during and following WR 2823 administration.

The effect of reserpine pretreatment was determined in 3 experiments. Blood pressure and to a certain extent heart rate were found to be lower in these animals than in the untreated controls. WR 2823 caused a moderate but brief (< 5 min) fall in blood pressure (23%). Heart rate fell but to a lesser extent than in control animals. Carotid artery blood flow was increased the first 5 to 15 min after WR 2823 administration.

In 11 experiments pretreatment with atropine (4), lysergic acid diethylamide (4) or diphenhydramine (3) had little effect on resting blood pressure or heart rate. These animals responded in nearly the same way to WR 2823 as did the saline controls except that heart rate fell to a lesser extent in the atropinized cats.

The injection of either 50 mg or 100 mg WR 2823 into the isolated dog heart caused a brief 5% to 10% decrease in force of contraction with little or no change in heart rate. Coronary perfusion pressure fell slightly following the injection. Both the force of contraction and coronary perfusion pressure had returned to control levels 1 to 2 min after WR 2823. The positive inotropic and chronotropic responses to epinephrine (1 µg) were essentially unchanged after either 50 mg or 100 mg of WR 2823.

d. Discussion:

The intravenous administration of WR 2823 consistently induced hypotension and bradycardia in the anesthesized cat. In the present experiments a dose of 50 mg/kg of WR 2823 was infused over a 4 min period. The cardiodepressant effects appeared before the injection was completed. Doses as low as 12.5 mg/kg have been shown to lower blood pressure and heart rate in the rat (Herman et al, 1971). The magnitude of the fall was similar to that seen after 25 mg/kg. In the dog WR 149,024, a dimer of dephosphorylated WR 2823, produced hypotension and bradycardia over a dosage range of 6.25 to 25.0 mg/kg (Caldwell et al, 1972). It was noted that the 6.25 mg/kg dose depressed blood pressure and heart rate to the same extent as the 25 mg/kg dose. Thus the initial cardiovascular effects are not dose dependent.

A critical dosage might be necessary if WR 2823 exerted direct non-specific depression on the myocardium or vascular smooth muscle or both. However, in the present experiments WR 2823 had little effect on the force of contraction and heart rate in the

isolated dog heart. Likewise the tone of isolated rabbit aortic strips was not altered when WR 2823 was added to the bathing fluid (Demaree et al, 1971). In spite of the lack of direct WR 2823 effect on heart and vascular smooth muscle, the fact that both carotid artery blood flow and pulse pressure increased suggests that the initial hypotension is due to a decrease in total peripheral vascular resistance.

The decrease in peripheral resistance was not the result of parasympathetic nervous system stimulation since atropine failed to prevent the fall in blood pressure. This fact would also tend to rule out stimulation of the Bezold-Jarisch reflex mechanism which can be blocked by atropine. Histamine can also produce a hypotensive response in the intact animal. Although plasma histamine levels were not determined in the present experiments, the fact that pretreatment with the antihistaminic diphenhydramine failed to influence the magnitude of the responses tends to rule out histamine release as a prime factor in the hypotension. Likewise lysergic acid diethylamide pretreatment failed to alter the initial effects of WR 2823, suggesting that the release of 5-hydroxytryptamine is also not involved in the responses. These results tend to suggest that the locus of the hypotensive response resides with the sympathetic nervous system.

WR 2823 has been shown to possess alpha-adrenergic blocking properties (Heiffer et al, 1969). However, this action required 30 to 60 min to develop and thus could not be responsible for the immediate alterations in blood pressure and heart rate. When the peripheral portions of the sympathetic nervous system were blocked with either phenoxybenzamine or propranolol, WR 2823 administration still induced hypotension and bradycardia. However, some components of the response were modified. For example, propranolol prevented the increase in carotid artery blood flow and pulse pressure. Also, the decline in heart rate was less in animals pretreated with either of these agents than in the untreated controls. A more significant attenuation of the initial WR 2823 responses was noted in the reserpinized cats. In these animals both the hypotension and bradycardia were less than in the untreated controls. Reserpine, by biogenic amine depletion, alters sympathetic nervous system activity both peripherally and centrally.

That higher autonomic centers may be involved has been suggested by the demonstration of both blood pressure and heart rate increase after WR 2823 in spinal transected or ganglionically blocked animals. The positive inotropic and chronotropic actions may reflect a peripheral action of WR 2823 which is masked by the intact nervous system. It is not known if reserpinization will

prevent this. These responses are opposite to those occurring in decerebrate or control cats suggesting that the brain stem may be a critical area for WR 2823 activity. The importance of these areas has been emphasized by the demonstration that α-methyldopa (Henning, 1969), L-dopa (Henning and Rubenson, 1970) and clonidine (Kobringer and Walland, 1967) have centrally mediated hypotensive effects. Also, intracisternal injection of phentolamine, an alpha-adrenergic antagonist, caused hypotension and bradycardia in vagotomized rats (Ito and Scharberg, 1974). These responses were found to be elicited from the medullary brain areas. The comparison between cardiovascular responses of these 2 agents is not absolute since alpha-adrenergic blockage appears to be involved in phentolamine action but is not necessarily the case for WR 2823. The areas of the brain stem affected, however, may be similar. In any event, the results of the present experiments tend to indicate that the immediate cardiovascular effects of WR 2823 can be attenuated by agents which alter sympathetic nervous system activity at central levels.

Table 1

Protection of Mice Against the Lethal Effects of Contusive (Tumbling) Trauma in the Noble-Collip Drum by Pretreatment with 50 mg/kg WR 2823[a]

Series	No of Expts	Agent (i.p.)[b]	No. of Mice	% Cumulative Mortality at Hours Post-Injury					P Value[d]
				0[c]	0.16	6	24	48	
I	10	Water	120	50	52	55	61	72	
		WR 2823 B	120	6	7	8	8	8	<.001
II	8	Water	90	72	72	73	77	81	
		WR 2823 AC	85	26	31	33	36	40	<.001
		WR 2823 B	85	24	25	26	28	31	<.001
III	5	Water	74	70	73	76	80	82	
		WR 2823 AC	75	24	31	32	40	45	<.001
		WR 2823 AB	75	17	24	25	29	32	<.001
Total of Series	23	All water	284	62	64	66	71	77	
		All WR 2823	440	18	22	23	26	29	<.001

[a]Duration of trauma was 15 minutes.

[b]Injection volume always 0.01 ml/gm of body weight 15 minutes before trauma.

[c]At termination of tumbling.

[d]Probability (based on chi-square with Yates' correction) that the difference in 48 hour mortality between the water-controls and WR 2823-treated mice is due to chance alone.

Table 2

Duration of Protective Effect of WR 2823 AB
(50 mg/kg) against Lethality of NCDT

Group	Treatment (i.p.)	Time Elapsing Between Rx and NCDT (for 15 minutes)	No of Mice	% Cumulative Mortality Hours post NCDT				
				0	0.16	6	24	48
A,C, E,G	Water	15, 60 or 120 minutes or 24 hr	155	63	67	70	77	80
B	WR 2823 B	15 min	50	12	20	22	26	26
D	WR 2823 P	60 min	50	30	34	34	38	40
F	WR 2823 B	120 min	35	54	60	66	66	74
H	WR 2823 B	24 hr	50	54	54	60	70	74
Group Mortalities				P value				
B vs (A,C,E, & G)				<.001	<.001	<.001	<.001	<.001
D vs (A,C,E, & G)				<.001	<.001	<.001	<.001	<.001
F vs (A,C,E, & G)				n.s.	n.s.	n.s.	n.s.	n.s.
H vs (A,C,E, & G)				n.s.	n.s.	n.s.	n.s.	n.s.

Table 3

Comparison of the Effects of Phenoxybenzamine and WR 2823 B Pretreatment on the Lethality of NCDT[a]

Group	Treatment		No of Mice	% Cumulative Mortality Hours Post NCDT				
	Agent (i.p.)	Rx-Time Pre-NCDT min.		0	0.16	6	24	48
A	Water	60	30	63	70	70	70	70
B	Phenoxybenzamine 5 mg/kg	60	30	23	30	30	30	30
C	WR 2823 B 50 mg/kg	15	20	20	20	20	20	20
Group Mortalities Compared				P value				
B vs A				<.005	<.005	<.005	<.005	<.005
C vs A				<.010	<.005	<.005	<.005	<.005
C vs B				n.s.	n.s.	n.s.	n.s.	n.s.

[a]Duration of trauma was 15 minutes.

Table 4

The Individual and Combined Effects of Propranolol and WR 2823 on the Lethality of NCDT[a]

Group	Treatment: First Rx: 3 min Pre-NCDT (i.p.)	Treatment: Second Rx: 15 min Pre-NCDT (i.p.)	% Cumulative Mortality: Hours Post NCDT: 0	0.16	6	24	48
A	Water	Water	68	70	74	76	76
B	Propranolol 0.5 mg/kg	Water	96	100	100	100	100
C	Propranolol 0.5 mg/kg	WR 2823 B 50 mg/kg	38	42	42	46	52
D	Water	WR 2823 B 50 mg/kg	28	32	32	34	34
Group Mortalities Compared			P value				
	B vs A		<.001	<.001	<.001	<.001	<.001
	C vs A		<.010	<.010	<.005	<.005	<.050
	D vs A		<.001	<.001	<.001	<.001	<.001
	D vs C		n.s.	n.s.	n.s.	n.s.	n.s.

[a]Duration of trauma was 15 minutes. Fifty mice per group.

Table 5

Effect of WR 2823 Pretreatment on the Glycemic Response of Fed Mice After 10 minutes of NCDT

Group	Treatment[a] Agent i.p.	Duration of NCDT, min	Average Time Elapsing From Rx to Blood sampling (min)	Average Time Elapsing From End of NCDT to Blood Sampling min	No of Mice	Blood Glucose (mg %)[b]
A	Water	none	42	11	30	126 ± 2.6
B	Water	10	46	17	35	172 ± 7.2
C	WR 2823 B 50 mg/kg	10	46	17	43	137 ± 3.6

Group Means Compared	P value
B vs A	<0.001
C vs A	<0.025
C vs B	<0.001

[a]Treatment was given 15 minutes before NCDT.

[b]Values are for whole blood, mean ± S.E.M.

Table 6

Effect of WR 2823 on Endotoxin (*E. coli*) Lethality
When Given 15 Minutes Before Endotoxin Challenge

Group	Agent[a] (i.p.)	% Cumulative Mortality Hours after Endotoxin							
		3	6	24	30	48	54	72	96
A	Water	0	0	53	76	93	93	93	93
B	WR 2823 B	0	0	25	33	65	67	71	72
Group Mortalities Compared		P value							
B vs A		--	--	<.001	<.001	<.001	<.001	<.001	<.005

[a]Agent given 15 minutes before endotoxin, 600 µg per mouse. Each group contained 75 mice.

Table 7

Effect of Surgical Pretreatment on Initial Cardiovascular Responses Following a Four Minute Intravenous Infusion of WR 2823 (50 mg/kg) in the Anesthetized Cat

Time Postdose (min)	% of Predosing (Control) Values					
	Mean Arterial Pressure[a]			Mean Heart Rate[a]		
	No Operation	Decerebrate	Spinal	No Operation	Decerebrate	Spinal
1	65±4	52±6	112±6	67±1	71±8	130±14
5	69±5	54±2	91±1	73±6	68±8	119±10
15	63±4	57±4	85±3	69±5	64±7	101±3
30	69±3	67±1	79±2	82±5	71±11	98±3
60	73±3	73±3	79±2	100±5	83±10	97±2
	135±16 mmHg	104±23 mmHg	65±10 mmHg	168±10 bpm	204±12 bpm	161±17 bpm

[a]Values are mean ± S.E.M. and represent at least three cats per group.

Table 8

Effect of Pharmacological Pretreatment on Initial Mean Arterial Pressure Responses Following a Four Minute Intravenous Infusion of WR 2823 (50 mg/kg) in the Anesthetized Cat

	% of Predosing (Control) Values[a]								
	No Pretreatment	Mecamylamine 2.5 mg/kg	Hexamethonium 10.0 mg/kg	Phenoxybenzamine 5.0 mg/kg	dl-Propranolol 0.5 mg/kg	Reserpine 0.1 mg/kg x2	Atropine 1.0 mg/kg	LSD .05 mg/kg	Diphenhydramine 10.0 mg/kg
After Pretreatment		64±1	86±4	83±5	98±3	-	96±3	99±3	109±5
After WR 2823 (min)									
1	65±4	80±4	86±3	46±3	62±6	77±10	55±5	68±5	54±10
5	69±4	70±10	73±1	53±9	68±9	96±3	59±6	63±5	67±7
15	63±4	70±10	72±8	57±8	60±7	93±3	62±6	70±10	72±4
30	69±4	71±11	73±1	66±11	66±7	96±5	66±6	78±12	87±1
60	73±3	70±5	82±7	78±8	68±10	101±6	77±5	86±13	89±3
Predosing Values (mmHg)	135±16	110±9	88±7	118±16	111±16	78±5	111±8	102±6	119±19

[a]Values are mean ± S. E. M. and represent at least three cats per group.

Table 9

Effect of Pharmacological Pretreatment on Initial Mean Heart Rate Responses Following a Four Minute Intravenous Infusion of WR 2823 (50 mg/kg) in the Anesthetized Cat

	% of Predosing (Control) Values[a]								
	No Pretreatment	Mecamylamine 2.5 mg/kg	Hexamethonium 10.0 mg/kg	Phenoxybenzamine 5.0 mg/kg	dl-Propranolol 0.5 mg/kg	Reserpine 0.1 mg/kg x2	Atropine 1.0 mg/kg	LSD .05 mg/kg	Diphenhydramine 10.0 mg/kg
After Pretreatment		68±7	86±3	106±3	90±3	-	102±1	99±1	104±5
After WR 2823 (min)									
1	67±1	100±5	129±5	103±5	90±3	94±1	95±4	82±9	77±2
5	73±6	78±5	114±6	96±9	87±4	90±3	93±3	79±6	73±1
15	69±5	74±16	91±4	89±9	89±5	91±7	85±8	76±6	73±1
30	82±5	70±11	89±1	88±8	91±6	97±12	89±6	86±13	83±6
60	100±5	70±7	93±3	112±6	104±9	92±9	96±1	104±6	115±11
Predosing Values (bpm)	168±10	210±17	152±19	173±15	163±13	141±13	179±20	184±11	180±4

[a]Values are mean ± S. E. M. and represent at least three cats per group.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 192 Antiarrhythmic effects of aliphatic amines

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6522	74 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	H. Term	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	193
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with security classification code)

(U) Synthetic Liposomes and Macrophage Activity

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	74 06	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 73	1	12
	CURRENT 74	1	39

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Mooney, MAJ, J.
NAME: DA

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with security classification code)

(U) Liposome; (U) Macrophage; (U) Virus; (U) Immunology; (U) Red Blood Cell

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security classification code.)

23(U) The liposome which is derived from lipid constituents of red cell membranes has many applications; liposomes offer a simple, reliable and convenient model for red cell membranes and can be used as an antigen of known lipid components. Studies on roles of liposomes in various immunologic events will provide new information on immunology and pathogenesis of infectious diseases which are current or potential problems in the Armed Forces.

24(U) Various biochemical extraction and assay methods, radioactive counting procedures

25(U) 73 07-74 06 Radiolabelled Sindbis virus bound to protein-free liposomal model membranes prepared from chloroform extracts of sheep erythrocytes. The attachment of virus to liposomes was pH-dependent, but not significantly affected by high ionic strength. Maximal virus binding to liposomes occurred over the range pH 3.3-6.0, and declined above pH 6.0 to reach a minimum at pH 6.8. Once formed, the virus-liposome complex was stable to adverse pH conditions. Specific antiviral antibody completely inhibited the attachment of virus to liposomes. Additional studies have shown that the simultaneous presence of phosphatidylcholine and cholesterol markedly enhance binding of this virus to liposome model membranes. Sphinogomyelin, phosphatidylserine, and membrane glycolipids are not required. Other sterols may be substituted for cholesterol such as coprostanol and cholestanol.

This project is being terminated because of the completion of active duty of the associate investigator.

For technical reports see Walter Reed Army Institute of Research Annual Report 1 Jul 73-30 Jun 74.

* Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 193 Synthetic Liposomes and Macrophage Activity

Investigators.
Principal: Akio Takeuchi, M. D.
Associate: LTC John Mooney, MC

A. The Binding of Virus to Liposomes Derived from the Red Blood Cell Membrane

Background

The liposome which is derived from lipid extracts of red blood cells (RBC) has many applications; liposomes offer a simple and convenient yet reliable model of RBC membranes and can be used as an antigen of known lipid components. Various known biochemical compounds can also be incorporated into the liposome. These artificial membranes are protein-free and composed of phospholipids as well as other lipids in a bilayer configuration (1). A variety of liposome-protein interactions and virus interactions with lipid or lipid-protein monolayers have been reported (3-5, 7-9).

Studies on roles of liposomes of different immunologic events will provide new information on immunology and pathogenesis of infectious diseases which are current or potential problems in Armed Forces.

For the last two years the attachment of radiolabelled Sindbis virus to RBC derived liposomes have been studied in order to determine whether lipids play a role in binding the virus to the biological membrane. The results will elucidate the mechanism of the hemoagglutination test which is widely employed throughout virology for the detection of both virus and specific antibodies. (See also WRAIR Annual Report 1 Jul 72 - 30 Jul 74.)

Approach to the Problems

The preparation of intrinsically-labelled Sindbis virus has been previously described in detail (6); infected chick embryo cell cultures were incubated in amino acid-free medium for 4-6 hours post infection followed by the addition of a radioactive amino-acid mixture at a concentration of 10-30 microcuries per ml. Labelled virus supernatant fluids were clarified, precipitated by ammonium sulfate, and virus concentrates purified by rate-zonal sucrose gradient centrifugation.

Antibody preparations consisted of mouse hyperimmune ascitic fluid prepared according to the method of Brandt, et al. (7) and modified to include Sarcoma 180 cells for the induction of ascites (8). Repeated clot formation was prevented by acid treatment to remove residual fibrin (9).

Erythrocyte ghosts were obtained by methods previously described (10, 11); the ghosts were extracted with chloroform, methanol, and water by a modification of the method of Bligh and Dyer (11, 15), and the methanol-water phase discarded. The chloroform phase was protein-free, but contained phospholipids, glycolipids, and cholesterol (13, 14). This phase was used to prepare the liposomes utilizing a Vortex mixer (Scientific Industries, Inc., Springfield, Mass.) as previously described (11). Each assay tube contained 100 µl of liposome dispersion containing 1 µmole of phospholipid phosphate as measured by the phosphate assay of Gerlach and Deuticke (15).

In a typical assay, 600 µl of the appropriate buffer, 100 µl of liposomes dispersed in this same buffer, and 100 µl of virus were incubated for 45 minutes at 37° C. Regardless of the final pH of the incubation medium, the Sindbis virus was always diluted in pH 5.8 buffer. After incubation, the entire mixture was layered over 3.9 ml of 0.308 M sucrose and a cushion of 0.5 ml of 70% sucrose (w/v). The tubes were centrifuged at 50,000 rpm (234,000 x g) and 4° C. for 45 minutes in a SW 50.1 rotor (Beckman Instrument Company, Palo Alto, CA). This centrifugation was sufficient to bring down unattached virus onto the 70% sucrose cushion and to cause the liposomes to float to the top of the tube. Each tube and its contents were frozen in liquid nitrogen and cut into five segments with a jeweler's hacksaw. As soon as the contents had melted, the liquid volumes of the segments were measured, the contents of each segment mixed, and 200 µl samples were removed for radioactivity counting. Approximately 90% of the added volume and associated radioactivity could be recovered routinely from each tube.

Results and Discussion

A typical experiment illustrating the distribution of radiolabelled virus after centrifugation in the presence and absence of sheep erythrocyte liposomes is shown in Figure 1. Virtually all of the recovered virus appeared in either the top fraction (fraction 1) or on the high-density sucrose cushion (fraction 5). Phosphate analyses performed on control tubes which contained only 100 µl liposomal dispersion revealed that at least 96% of the added liposomal phosphate was routinely recovered in fraction 1. Control experiments utilizing each of the other buffers employed in this study gave similar results. The interaction of virus with liposomes was detected by a change in the sedimentation of the virus such that it floated with the liposomes. The term "% Binding" hereinafter refers to the amount of radiolabel in the top fraction (fraction 1) compared to the total counts recovered from all five segments of the centrifuge tube.

Pretreatment of radiolabelled Sindbis virus with antiviral neutralizing antibody completely inhibited the attachment to sheep erythrocyte liposomes at pH 5.8 (Figure 2). The degree of inhibition was proportional to the quantity of antibody added. In tubes containing the highest levels of antibody, all radioactivity quantitatively sedimented to the bottom of the centrifuge tube, despite the presence of liposomes, which, as usual, floated to the top of the tube. This result is consistent with the interpretation that all of the radiolabelled material corresponded to virus and was recognized as such by specific antibody.

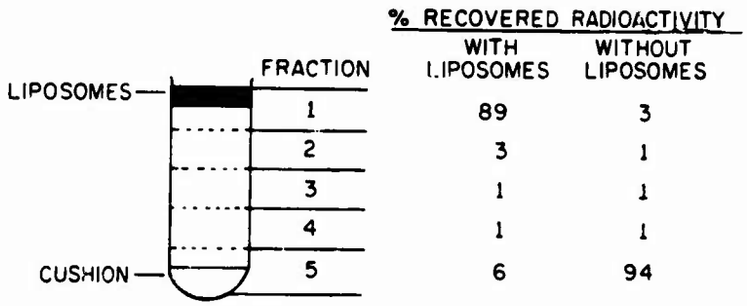
The hemagglutination of erythrocytes by Sindbis virus is pH-dependent and agglutination is maximal below pH 6.0 and minimal above pH 6.8. We have found that the sheep erythrocyte can be substituted for avian cells without significantly altering the hemagglutinating properties of the virus. Liposomes from sheep erythrocytes exhibited a pH-dependence for attachment which was similar to that of erythrocytes in the range of pH 6.0-6.8. Thus, maximal binding occurred at pH 6.0, and minimum binding above pH 6.8 (Figure 3). It should be noted that pH-dependence of virus binding to liposomes was not absolute, and a low level of binding (at least 18%) was still observed above pH 6.8. In contrast to erythrocytes, which cannot be easily tested in a hemagglutination assay below pH 5.8, liposome binding was measured over a more acid range, and near-maximum binding was consistently observed between pH 3.3-6.0. Control experiments demonstrated that, with respect to sedimentation characteristics, the virus itself was not altered by low pH. The above effects of pH suggested the possibility that the interaction of virus and liposomes might be due to electrostatic bonds. However, the influence of pH appeared to be important only during the initial association of the virus with the liposomes. When the pH was raised to pH 6.7 subsequent to the initial interaction, the virus-liposome complex remained stable and did not dissociate (Table I). Furthermore, separate experiments showed that raising the ionic strength eight-fold had little or no effect on the binding of the virus. These results suggest that, although electrostatic forces may be important, this could not fully explain the results, and it is possible that other bonds may also play a role in the phenomenon.

TABLE I

pH stability of the virus-liposome complex

pH of Initial Mixture	Final pH	% Virus Bound
5.8	5.8	94
5.8	6.7	92

100 µl of sheep erythrocyte liposomes, swollen in isotonic phosphate buffered saline, pH 5.8, were incubated with radiolabelled virus for 25 minutes at 37° C. Where indicated, the pH was then raised to pH 6.7 by adding an appropriate volume of buffer of pH 7.4. As a control, an identical volume of pH 5.8 buffer was added to the other tube, and incubation was continued for 20 minutes. Each value represents an average of two experiments.



FRACTION	% RECOVERED RADIOACTIVITY WITH LIPOSOMES	% RECOVERED RADIOACTIVITY WITHOUT LIPOSOMES
1	89	3
2	3	1
3	1	1
4	1	1
5	6	94

Figure 1. The centrifugation characteristics of Sindbis virus and liposomes. Each tube contained 14,000 cpm radiolabelled Sindbis virus with and without 100 µl of liposomes prepared from sheep erythrocyte extracts. The assays were conducted at pH 5.8 in borate-phosphate-saline buffer prepared according to the method of Clarke and Casals. The values shown are the means of either five experiments (with liposomes) or four experiments (without liposomes).

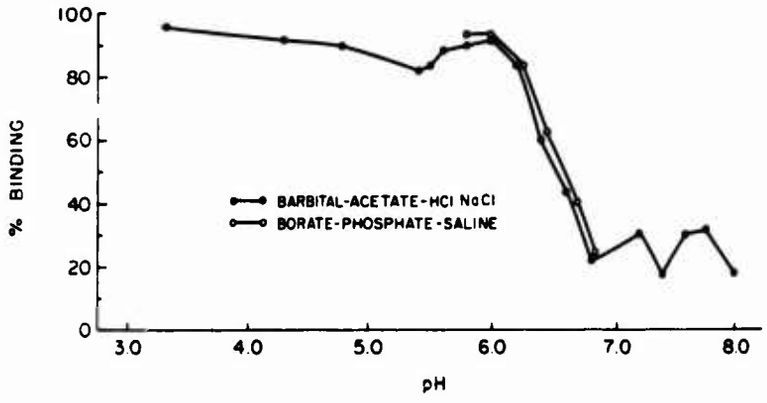


Figure 2. Inhibition of virus attachment following exposure to virus-specific antibody. Radiolabelled Sindbis virus was pre-incubated with concentrations of either normal or immune mouse ascitic fluid for 10 minutes at 37° C. in borate-phosphate-saline buffer at pH 5.8. 100 µl of sheep erythrocyte liposomes were then added and incubation continued for 45 minutes at 37° C. "Inhibition" is defined as 100% minus % virus binding observed in fraction 1 (see Figure 1). Additional control tubes which lacked ascitic fluid showed 95% binding of the virus to liposomes.

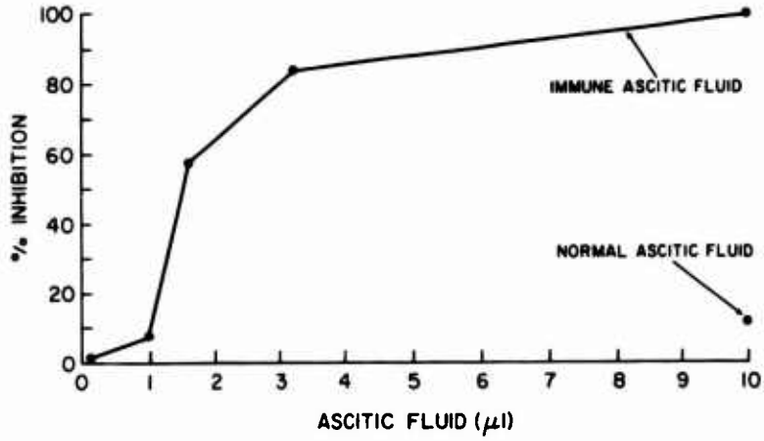


Figure 3. pH-dependence of Sindbis virus attachment to liposomes. Each point represents a separate liposome preparation. The isotonic barbital-acetate-HCl-NaCl buffers were prepared as follows. 10 ml of 0.16 M sodium barbital-0.143 M sodium acetate was mixed with 1.0 N HCl to achieve the desired pH. The solution was made isotonic by adding appropriate quantities of 1.54 M NaCl and water to a final volume of 50 ml. 100 µl sheep erythrocyte liposomes, swollen in the indicated buffer at the appropriate pH, were incubated in the same buffer with radiolabelled Sindbis virus.

CONCLUSION

Additional work with liposomal model membranes from sheep RBC and Sindbis virus have demonstrated the following results:

(1) The binding of radiolabelled virus to the membrane phospholipids is markedly enhanced when cholesterol is present in concentrations normally found in mammalian RBC. The binding increases as the concentration of cholesterol increases in a manner described by a sigmoid-shaped curve, reaching a plateau at a cholesterol/phospholipid rate of 0.75.

(2) The binding of this virus to cholesterol containing liposomes is significantly enhanced in the presence of the phospholipid phosphatidylethanol-amine (PE), while the binding to liposomes which contained either sphingomyelin (SM) or phosphatidylcholine and cholesterol was markedly reduced. Liposomes prepared from SM, PE, and cholesterol gave excellent binding, thus indicating that phosphatidylserine (PS), a normal constituent of sheep RBC, was not required for attachment. Liposomes prepared from PE, PS, and cholesterol also gave excellent binding, demonstrating that the presence of SM was not necessary.

(3) The total absence of glycolipids such as Forssmann antigen in these preparations rules out the possibility that these red cell membrane constituents are required for virus attachment. Other sterols such as cholestanol and coprostanol may be substituted for cholesterol. This suggests that these sterols enhance binding by their well known property of restricting the movement of the phospholipids in these bilayer configurations.

The present project entitled "Synthetic liposomes and macrophage activity" is being terminated because of completion of active duty of the associate investigator.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 193 Synthetic Liposomes and Macrophage Activity

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6523	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8a. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	194
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Development of an Organ Culture Method from Intestinal Biopsies

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 01	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	1.5	42
FISCAL YEAR CURRENT 75	1.5	40

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Richardson, MAJ W.

NAME: DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Organ Culture; (U) Intestine; (U) Phase Microscopy; (U) Electron Microscopy

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To develop a reliable and reproducible method in which organ cultures of small and large intestines of experimental animals and man may be cultured and maintained without producing alterations of function and metabolism. The cultured gut will be employed in studies on interactions between the mucosa and various bacteria, viruses and microbe-derived toxins. These results will provide new information which should clarify the pathogenesis of acute diarrheal diseases in military personnel.

24 (U) Conventional morphologic techniques including phase contrast, light and electron microscopy, and histochemistry and isotope tracer methods are being used. Methods such as interference microscopy and cinematography will also be added as the work progresses.

25 (U) 73 07-74 06 Utilizing a new modified Rose's tissue culture chamber, the viability and growth of epithelial cells of the small and large intestine have been studied in fetal and adult mice and guinea pigs. Fetal intestinal epithelial cells could be grown and remain viable up to 2 weeks while the adult counterparts do not survive more than 2 days. Cinematographic observations on growing intestine demonstrated the characteristic movement of lymphocytes and macrophages in the mucosa and determined that a close relationship was found between villus motility and absorption of macromolecular transport and mucus discharge from goblet cells. Current efforts have been directed toward the establishment of electron microscopic preparation of cultured guts and also methods of sequential determination of protein and immunoglobulin synthesis in the growing gut.

For technical reports, see Walter Reed Army Institute of Research Annual Reports 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 194 Development of an Organ Culture Method from Intestinal Biopsies

Investigators.
Principal: Akio Takeuchi, M. D.
Associate: MAJ Walter Richardson, MC

Description

To develop a reliable and reproducible method for the organ culture of the small and large intestine of experimental animals and man. The cultured gut will be employed in: (1) Studies by various parameters of responses of the gut mucosa and submucosa to various enteric microbes and microbe-derived toxins; special attention will be paid to cinematographic recordings of as well as conventional static observations on various cellular interaction of the gut epithelium mucosa with various microbes and toxins. (2) Studies of replication sites of certain enteroviruses in the cultured gut.

These studies should provide valuable new information which will clarify as yet unsolved problems in pathogenesis of acute infectious diarrheal diseases common in military personnel at home and overseas.

Background

Organ culture techniques have proven to be of considerable value in the study of respiratory tract infections in man and animals. Recently, interest has developed in the organ culture of gut tissues for the cultivation of enteric viruses. Rubenstein and Tyrrell (1971), Dolin and Stenhouse (1970), and Derbyshire and Collins (1971) provided evidence of the multiplication of viruses in organ cultures of the small intestine of the human embryo. Dolin et al. (1972) reported that viral antigens from viruses which belong to different viral groups were successfully detected in human fetal intestinal organ cultures by immunofluorescent techniques. Kagnoff et al. (1972), in their organ culture study of adult rabbits, have reported that the metabolic function and synthesis of macromolecular substances including secretory IgA were still active after 24 hours culture in a Petri dish. Finally, Eastwood and Trier were able to culture the human small intestine of normal subjects and patients with ulcerative colitis up to 24 hours (Eastwood and Trier 1973).

Most of the chambers used by the above investigators appeared adequate for the growth of certain viruses and for evaluation of limited metabolic activities in the cultured bowel. These chambers do not allow for an immediate and accurate and also sequential method of morphologic evaluation of intestines growing in cultures. This shortcoming prompted the development of a new chamber allowing a convenient observation during actual organ culture.

The chamber we have developed by modifying Rose's chamber provides easier handling of tissues and better visualization of growing cells and tissues under the phase contrast microscope than conventional culture chambers (Takeuchi et al. 1974). The viewing unit consists of a phase microscope equipped with a time-lapse cinematographic instrument, enclosed in a plastic housing connected with a thermo-control device which maintains the temperature of the unit at 37° C.

Progress

After numerous attempts to choose appropriate culture medium, we have found Medium 199 with 10% fetal calf or adult horse serum containing both streptomycin and penicillin to be satisfactory. However, the concentration of serum varies from 20% to 5% depending upon the age of fetus. Contrary to the general belief that a constant supply of oxygen is imperative for maintaining an organ in culture, the fetal gut can grow without it for up to 48 hours and shows no structural degeneration. Recently we have discovered that the supply of 100% CO_2 at 37°C gives the best results for the gut in organ in our chamber. With changes of medium every two days, fetal small and large intestines of mouse and guinea pig have been maintained successfully for up to 2 weeks (Fig. 2 and 3). The direct visualization by phase contrast microscopy and continuous recording by time-lapse cinematography of the gut mucosa in the culture chamber have demonstrated occasional rhythmic movements of the muscle and villi for up to 10 days.

Electron microscopy techniques for cultured intestines are being perfected. This technique, when completed, promises the demonstration of details of cellular structures which will be correlated with static observations by phase contrast microscopy and dynamic movements of cells and tissue recorded by time-lapse cinematography.

Functional studies on guts cultured in the organ culture chamber have been initiated. They include study of protein synthesis by radio-tracer methods and of DNA synthesis by autoradiography.

Personnel assigned to this work unit have applied tissue and organ culture methods for electron microscopy to provide assistance in studies of Neisseria gonorrhea infections on tissue culture cells (See Annual Report of Dept. of Bacterial Diseases, WRAIR).

Conclusions and Recommendations

With improved organ culture techniques, fetal guts can be cultured up to 2 weeks without structural and functional alterations. Sequential observations can be successfully made on growing guts in our organ culture chamber by direct visualization by phase contrast microscopy with a magnification of up to 700 times and be recorded sequentially by time-lapse cinematography. With the latter technique, movements of the mucosa and also of individual cells can be analysed. Techniques for the application of biochemical and immunological methods are progressing and will provide important data on the growing bowel. All these techniques are to be utilized in determining the viability of growth of cultured bowel and will form the basis for the study of various experimental infections and injuries of the intestine in vitro.

Fig. 1 Fetal small intestinal mucosa of mouse maintained for 8 days in culture chamber. A growing villus shows no evidence of structural alterations. The brush border (BB) and epithelial cells are well preserved.
Phase contrast microscopy. X 200

Fig. 2 Fetal small intestinal mucosa of mouse maintained for 7 days in culture chamber. The epithelium and lamina propria are unaltered. The brush border (BB) and nucleus (N) of epithelial cells are easily visualized. Occasional epithelial cells are well defined by intercellular borders. The cells are in a process of shedding from the epithelial layer. Numerous free cells represent lymphocytes, shedded epithelial cells and macrophages (M).
Phase contrast microscopy. X 300

Fig. 3 Electron micrograph showing apical portion of epithelial cells, mid-villus, fetal small intestine, maintained for 4 days in culture chamber. All cytoplasmic components including both rough and smooth endoplasmic reticulum, mitochondria and free ribosomes are unaltered. The glycocalyx (Gly), microvilli (M) and intercellular junctional complex (arrows) are well preserved.
X 5,800

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 194 Development of an Organ Culture Method from Intestinal Biopsies

Literature Cited.

References:

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Publications:

1. Takeuchi, A., Asaka, T. and Takeda, T.: Cinematographic observations on the bowel in organ culture. Fed. Proc. 33: 328 Abs., 1974.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6524	74 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	H. Term	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	195
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Sheep Red Cell Model for G6-PD Deficiency

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	74 06	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 73	0.5	4
CURRENT FISCAL YEAR 74	0.5	41

19. RESPONSIBLE DOD ORGANIZATION

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TELEPHONE 202-576-3014

SOCIAL SECURITY ACCOUNT NUMBER [redacted]

ASSOCIATE INVESTIGATORS

NAME: Giles, CPT, R.

NAME: Hildebrandt, LTC, P.

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) G6-PD Deficiency; (U) Sheep; (U) Primaquine; (U) Malaria; (U) Model

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) Studies utilizing in vitro Chromium fifty-one labelled sheep erythrocytes revealed a significantly shorter half-life than would be predicted from life span data obtained from the in vivo cohort Iron fifty-nine and Iron fifty-five studies. A study was conducted which demonstrates that Chromium fifty-one is rapidly eluded from sheep red blood cells and is excreted in the urine, and is not a suitable isotope for blood studies in sheep; these models are used in the military to determine human use.

24(U) Conventional methods and the utilization of isotope tracers such as Chromium fifty-one, Iron fifty-five and Iron fifty-nine are utilized to monitor the hemogram following exposure to primaquine.

25(U) 73 07-74 06 The administration of primaquine to sheep via various routes, did not cause any deviation of hemograms from baseline or controls. However, the erythrocyte survival time and ferrokinetics using the radioisotopes Iron fifty-five and Iron fifty-nine in adult sheep revealed values considerably different from the few previously reported studies in the literature.

This project is being terminated due to the completion of active duty of an associate investigator.

For technical reports see Walter Reed Army Institute of Research Annual Report 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE DD FORMS 1498-1 1 NOV 65 AND 1498-1 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 195 Sheep Red Cell Model for G6-PD Deficiency

Investigators.
Principal: LTC Paul K. Hildebrandt, VC
Associates: MAJ Charles A. Montgomery, VC: CPT Ralph C. Giles, VC

Background

The basis for this study was to determine if sheep may serve as a model for glucose-6-phosphate dehydrogenase deficiency and the hemolytic crisis which occurs with exposure to anti-malarial drugs such as primaquine. It was concluded that sheep, although deficient in G-6-PD, would not develop hemolysis when exposed to drugs such as primaquine. Our work suggested that Cr^{51} was not a suitable isotope tag for sheep red blood cells although the procedure has been established as one of the foremost methods for determining changes in erythrocyte survival rates in man, and certain experimental animals (1-3).

Our initial studies and one previous report revealed biphasic disappearance curves (5). There was very rapid loss of ^{51}Cr activity from the circulation in the first few days followed by a slower decay curve, yielding an apparent red cell half-life which was significantly shorter than would be predicted from work using in vivo cohort labelling with isotopic iron (4,5).

The present study was, therefore, undertaken to further evaluate the ^{51}Cr technique as a method for studying erythrocyte survival in sheep.

Approach to the Problem

^{51}Cr Survival Studies. Under atraumatic, sterile conditions, 30 ml of blood was drawn from the jugular vein of each of five mature ewes and mixed with 10 ml of acid-citrate dextrose solution (UNITAG Centrifuge Bag with A-C-D solution, Abbott Laboratories) (6). One hundred microcuries of sodium chromate (RACHROMATE-51, specific activity 200 μCi/mg Abbott Laboratories) was added and the samples were incubated at 23°C for 30 minutes, with gentle agitation. Following this, 100 mg of ascorbic acid was added to each sample with gentle mixing. The cells were washed 3 times with 0.9% saline at 23°C, and resuspended to the original bag volume with 0.9% saline. Thirty ml of the suspension was injected into the jugular vein of the autologous animal, and the remainder used to calculate dosage administration.

Blood samples (5-6 ml) were collected at 30 minutes, 1, 2, 3, 4, 5, and 6 hours, and then at 8, 10, 12, 16, 20, 24 and 36 hours post-injection. Samples were then collected daily for 1 week and then 3 times weekly for the next 3 weeks. Both whole blood and plasma samples were counted during the first 48 hours post-injection. Thereafter, whole blood was counted. The 24 hour sample was designated as time zero for the survival studies. All samples were counted in a Nuclear-Chicago Auto Gamma Spectrometer with a 3 inch thallium activated well-type sodium iodide crystal. Samples were counted simultaneously at the end of the study to avoid correction for radioactive decay.

Total urine volume was collected from each animal during the first 48 hours after injection of labelled cells. The urines were collected using anchored Foley catheters (Bardex, C.R. Bard, Inc.) and attached plastic bags which were preplaced 24 hours before erythrocyte labelling. The urine samples were collected and volumes recorded at the same intervals as blood samples during the first 48 hours. One milliliter aliquots from each urine sample were saved for counting.

The ^{51}Cr survival study was repeated on sheep #52 and #64 approximately 6 months after the initial study.

"In vitro" ^{51}Cr study. Two 10 ml blood samples were removed from the jugular vein of each of two adult ewes. Using the technique described in the ^{51}Cr survival study, one sample from each animal was mixed with 3 ml acid-citrate-dextrose solution, incubated with 30μ Ci of sodium chromate and washed 3 times. Thirty milligrams of ascrobic acid was added at the end of the labelling procedure. The other sample from each animal was used as an unlabelled control and handled identically otherwise. Under sterile conditions the labelled and nonlabelled samples were then suspended in 30 ml of autologous heparinized plasma to which dextrose had been added to a concentration of 250 mg%. The samples were incubated at 38.5°C for 24 hours using mechanical agitation to mimic circulatory trauma. Samples were removed at 0, 8, 16, and 24 hours for determination of free plasma ^{51}Cr activity, erythrocyte ^{51}Cr activity, free plasma hemoglobulin activity, and total hemoglobulin content (7).

^{59}Fe Survival Study. Isotopic iron (Ferrous citrate-^{59}Fe, Abbott, specific activity of 23 mCi/mg) was diluted in a 0.1% sodium citrate solution to a concentration of 5 μCi/ml. Each of six adult sheep received 50 μCi of ^{59}Fe intravenously in a volume of 10 ml. Blood samples (3-4 ml) were collected at 15 minutes, 1, 2, 3 and 4 hours post-injection. Blood samples were then collected twice weekly for the next 4 weeks. The average life spans were

calculated (8). All samples were counted simultaneously at the end of the study to avoid correction for radioactivity decay in a Nuclear-Chicago Auto Gamma Spectrometer with a 3-inch thallium activated well-type sodium iodide crystal.

Results and Discussion

In the five animals used in the ^{51}Cr study, approximately 21% of the total injected label was excreted in the urine during the first 8 hours post-injection. At the end of 24 hours, 33.6% of the label had been excreted, and at 48 hours, 36.2% had been excreted. All urines were negative for both red cells and free hemoglobin. During this period an equally rapid loss of ^{51}Cr activity from the circulation was noted. Figure 1 shows the urinary appearance of label paralleling the loss of circulating label. The corresponding plasma samples revealed an insignificant amount of radioactivity with counts ranging from 0 to 0.004% of the total injected label.

The mean half-life of ^{51}Cr-tagged autologous erythrocytes in the five sheep was 13.7 (S.E. $\pm$ 1.7) days (Fig. 2). The respective half-life values for the five animals were 14.0, 20.0, 12.0, 10.5, and 12.0 days. The 24 hour sample was used as the time zero sample.

The ^{51}Cr survival time when repeated in sheep #52 (12 days) and #64 (14 days) approximately 6 months after the first study revealed comparable values of 11 days and 13 days for erythrocyte half-life.

The mean erythrocyte survival time as calculated by *in vivo* ^{59}Fe-labelled erythrocytes in six mature sheep was 111.7 (S.E. $\pm$ 8.4) days. The individual life span values were 96.5, 102.4, 102.8, 107.1, 108.6 and 152.9 days.

The *in vitro* incubation of labelled erythrocytes from two adult sheep in glucose-rich plasma revealed a mean loss of 8.9%, 12.5% and 15.5% of the label from the red cell at 8, 16, and 24 hours after labelling. Minimal release of hemoglobin into the plasma occurred during the incubation, with no significant difference observed between the labelled and nonlabelled samples (Fig. 3). The mean hemoglobin concentration in the plasma of all samples at time zero was 3.91 mg.% and the concentration at 24 hours was 19.9 mg.% in the labelled samples and 19.5 mg.% in the nonlabelled samples. In the labelled samples only 0.58% of the total erythrocyte hemoglobin was released into the plasma at the end of 24 hours, whereas, 15.5% of the erythrocyte ^{51}Cr was lost into the plasma. In the unlabelled controls, 0.57% of total erythrocyte hemoglobin was released into the plasma at 24 hours.

This study confirms our earlier observations on the short apparent red cell half-life when ^{51}Cr is used to label sheep erythrocytes. The rapid loss of ^{51}Cr activity from the circulation appears to be due primarily to rapid elution of the isotope from the labelled cells. Approximately one-third of the injected label disappears from the circulation and appears in the urine during the first 24 hours. The urinary ^{51} Cr-activity is not associated with hematuria or hemoglobinuria, suggesting that label elutes rapidly from the erythrocytes and is cleared and excreted by the kidneys. Our in vitro incubation study lends further support to the notion that elution of label from the red cells is the single most important determinant of ^{51}Cr disappearance. In the in vitro incubations we noted elution of 15.5% of label into the incubation medium over 24 hours in the absence of any significant hemolysis.

In man, ^{51} Cr elution from red cells is also a major determinant of the ^{51} Cr-disappearance rate (2). The elution rate is slower than we have observed here in sheep, yielding ^{51}Cr red cell half-lives of 28-32 days (7). Total red cell life span, as determined by cohort isotopic iron labelling, is similar for both humans and sheep, measuring about 110-120 days (4,7). The molecular events which account for the elution of ^{51} Cr from red cell binding sites remain undefined. In human erythrocytes, the major binding of ^{51} Cr is at residue 93 on the β-chain (9). Additional binding also occurs to glutathione and other low molecular weight proteins (10-12). It is not clear from which pool the ^{51}Cr loss occurs. In certain hemolytic states, and with certain hemoglobinopathies, the elution rate is sufficiently variant from normal to yield false estimates of actual red cell life span (13).

In sheep erythrocytes, the ^{51}Cr binding sites remain to be determined. It may be that no β-chain binding occurs, and the rapid elution occurs because of this. We are planning to explore this possibility.

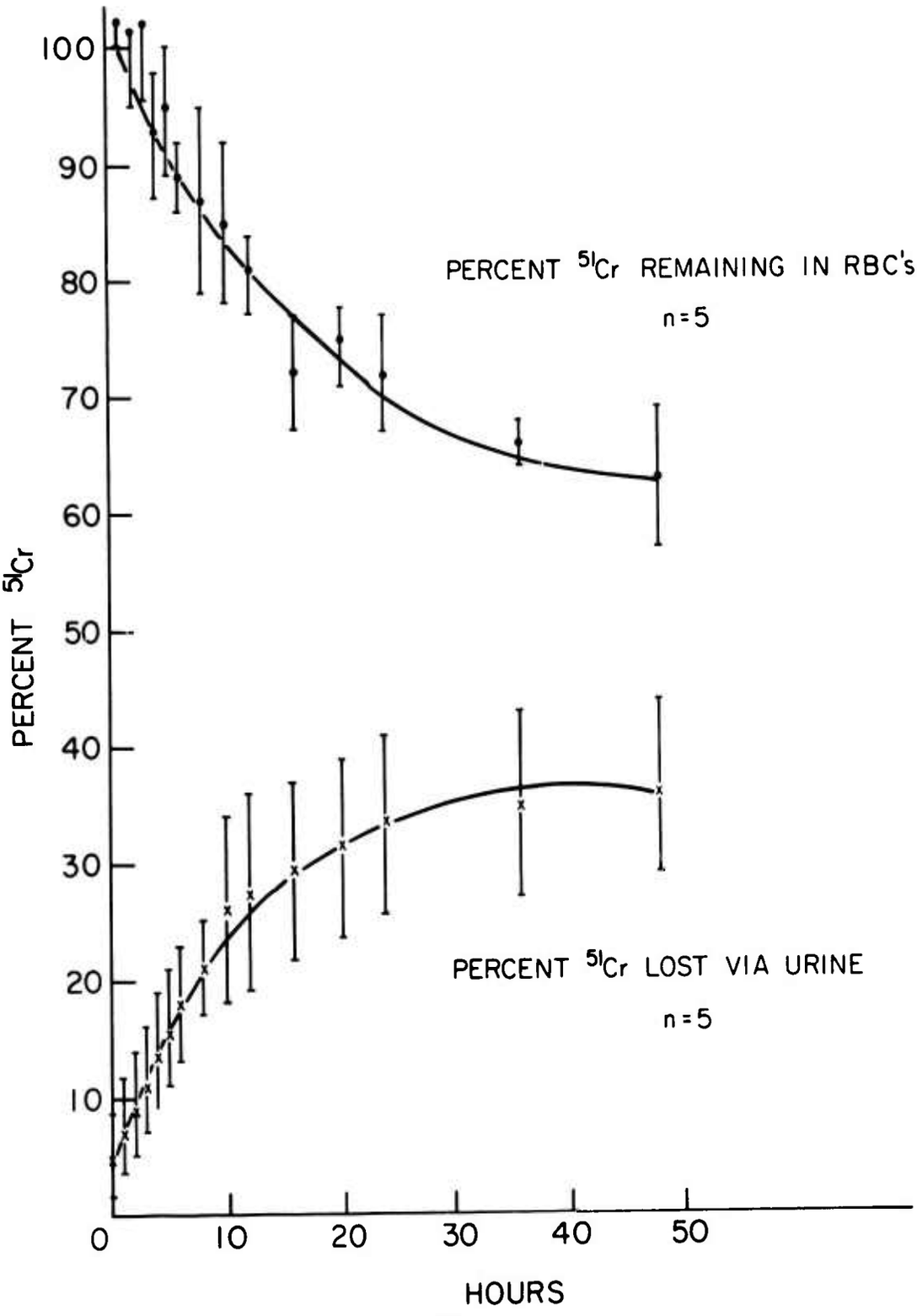
We conclude that there is very rapid elution of ^{51} Cr from sheep red cells. Since it is not clear what the determinants of this elution rate are, the use of ^{51} Cr to evaluate sheep red blood cell life span in experimental or pathological states should be approached with caution.

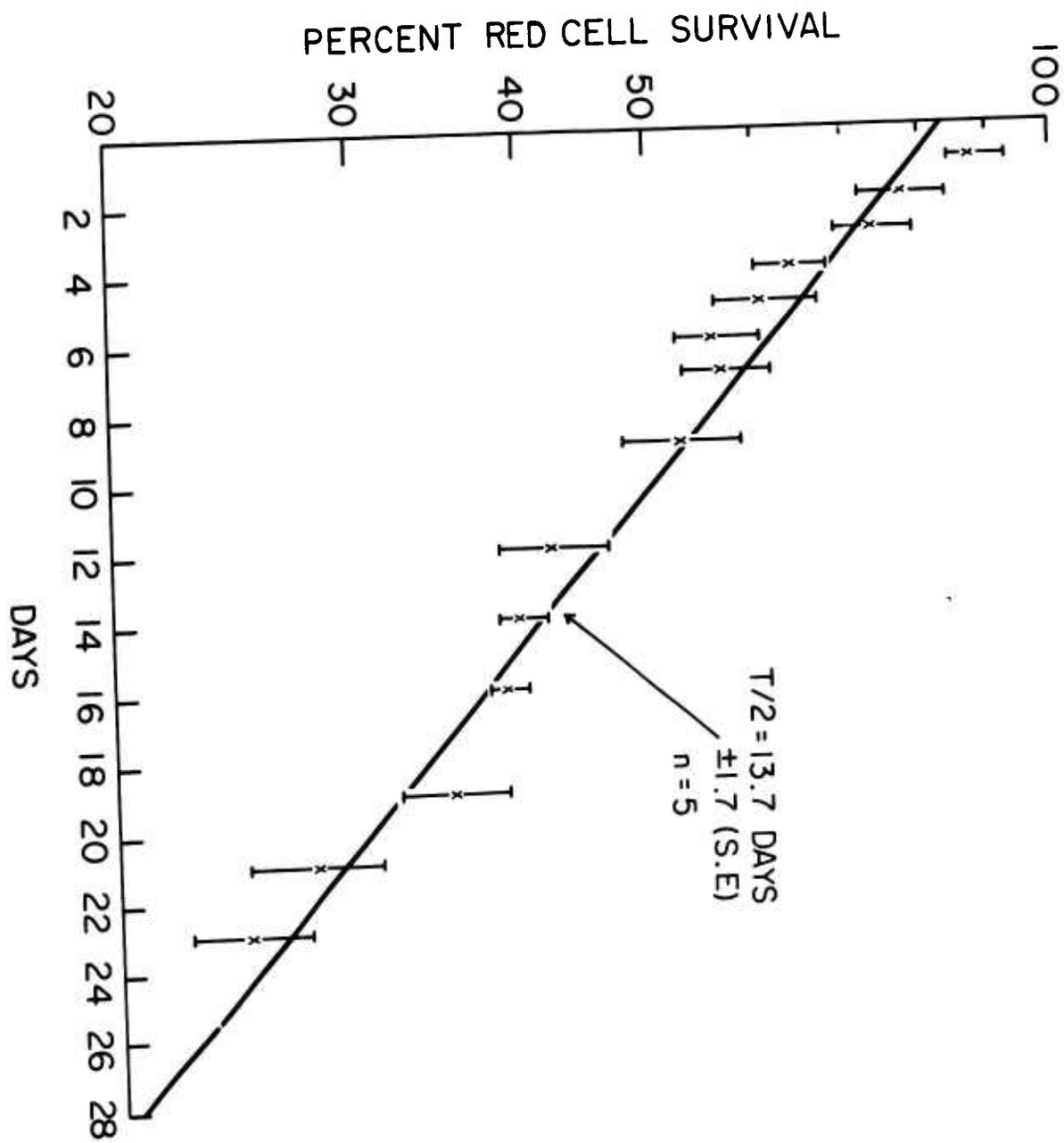
Figures

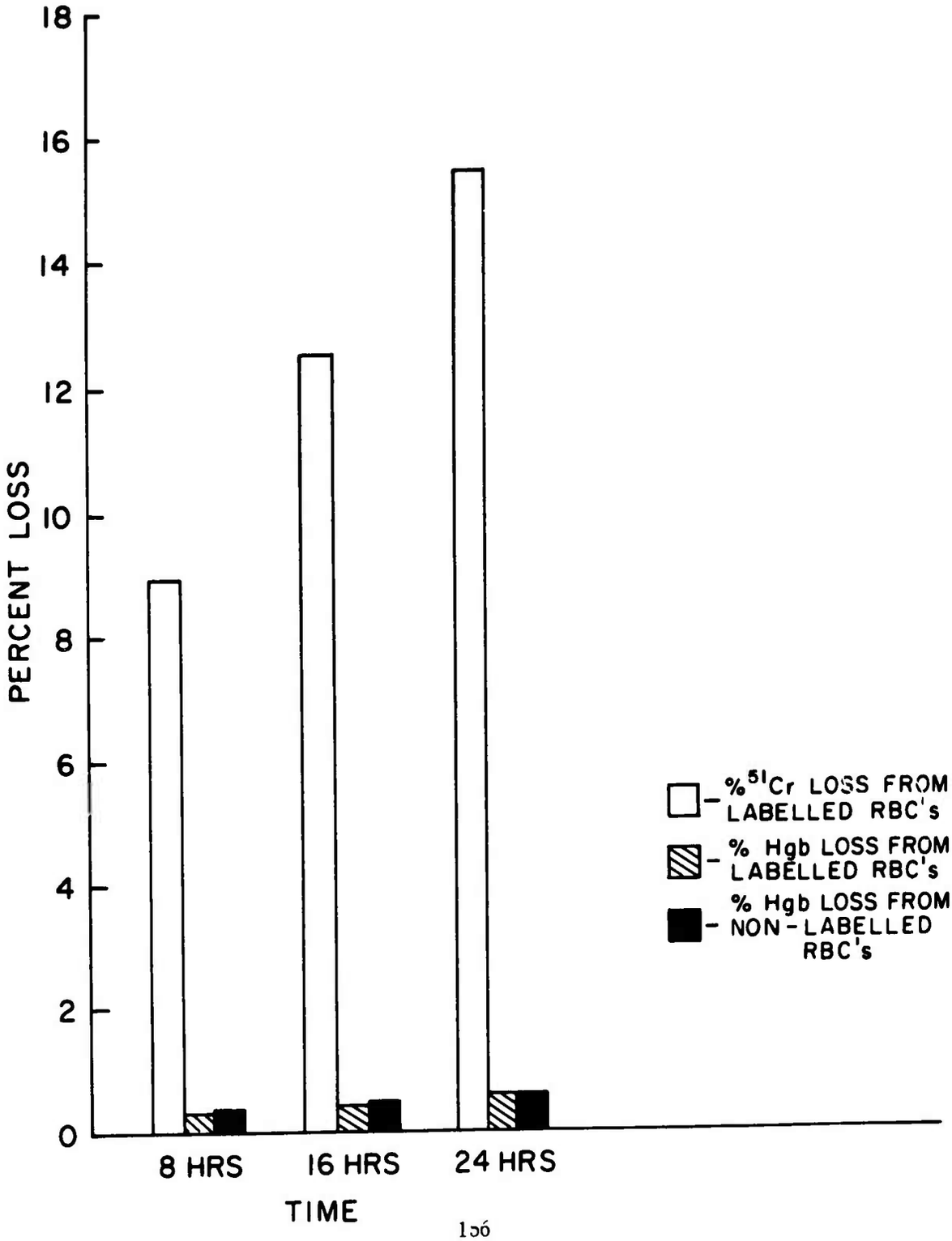
Fig. 1. Approximately 33.6% of the label was lost from the circulation and excreted into the urine 24 hours after injection of labelled cells.

Fig. 2. The mean half-life of ^{51}Cr-tagged autologous erythrocytes in the five sheep was 13.7 days.

Fig. 3. Percent *in vitro* loss of ^{51}Cr and hemoglobin into the plasma from labelled and non-labelled erythrocytes.


100
90
80
70
60
50
40
30
20
10
PERCENT 51Cr
PERCENT 51Cr REMAINING IN RBC's
n=5
PERCENT 51Cr LOST VIA URINE
n=5
0
10
20
30
40
50
HOURS


PERCENT RED CELL SURVIVAL
100
50
40
30
20
DAYS
2 4 6 8 10 12 14 16 18 20 22 24 26 28
T/2 = 13.7 DAYS
±1.7 (S.E)
n = 5


18
16
14
12
10
8
6
4
2
0
PERCENT LOSS
8 HRS
16 HRS
24 HRS
TIME
% 51Cr LOSS FROM LABELLED RBC's
% Hgb LOSS FROM LABELLED RBC's
% Hgb LOSS FROM NON-LABELLED RBC's

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 195 Sheep Red Cell Model for G6-PD Deficiency

Literature Cited.

References:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION* DA 0B 6514	2. DATE OF SUMMARY* 74 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUM'RY 73 07 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING* NA	8. DISP'N INSTR'N NL	9A. SPECIFIC DATA-CONTRACTOR ACCESS ☒ YES ☐ NO	9B. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	198
b. CONTRIBUTING				
c. CONTRIBUTING				CS FB 167

11. TITLE (Precede with Security Classification Code)*

(U) Spin Labeling of Biomolecules

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

002300 Biochemistry 008300 Inorganic Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	1	59
FISCAL YEAR CURRENT 75	3	120

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

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NAME: Bass, B. G., MS; Copeland, E.S. PhD DA

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) ESR; (U) Spin label; (U) Spectrometry; (U) Drugs of Abuse

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The technical objective of this work unit is to develop methods for analysis of important biochemicals and to study mechanisms by use of spin labeling and electron spin resonance (ESR) spectrometry for use in military medicine.

24. (U) ESR and organic synthetic techniques will be used to develop reagents and methods for attachment of spin labels to biomolecules. Commercial reagents for spin labeling will be evaluated. Investigation of spin labels other than nitroxides will continue. Methods will be developed for synthesizing spin-labeled drugs and metabolites for use in studying pharmacokinetics of drugs of military importance, physicochemical characteristics of receptor sites for drugs and enzymes, and binding site characteristics of antibodies. Spin label will be attached to antibiotic molecules to study pharmacokinetics of antibiotic action. Miniaturized high speed continuous flow analysis and antibody detection with application toward detection and analysis of microorganisms of medical interest and for clinical diagnosis will be evaluated.

25. (U) 73 07 - 74 06 Acquisition and installation of minicomputer for the Varian E9 ESR spectrometer was accomplished. The binding sites for spin-labeled morphine were located in rat brain synaptosomes. FRAT antibody to morphine was characterized. Stereoselective gels for detection of drugs of abuse were tested. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 198 Spin labeling of biomolecules

Investigators.
Principal: LTC Douglas J. Beach, MSC; CPT James A. Cella, MSC; Edmund S. Copeland, Ph.D.; Bhupendra P. Doctor, Ph.D.;
Associate: Billy G. Bass, M.S.; Betty J. Boone, Ph.D.; MAJ Michael E. Boykin, MC; LTC Gale E. Demaree, MSC; CPT James A. Kelley, MSC; SP4 Edward F. Kenehan, B.S.

A number of problems of biochemical and clinical importance require complex organic molecules as probes, substrates, indicators, etc. The synthesis of these molecules often constitutes a major obstacle in these types of problems. Hence there is a need, not only for application of synthetic organic chemistry to biomedical problems, but also for preliminary research into the development of new and better methods for synthesizing biologically important molecules. It is this approach which has been applied in this work unit. The principle areas which have been investigated are:

1. Synthesis of spin labeled opiates.

2. Synthesis of new types of stable free radicals which can be used as spin labels.

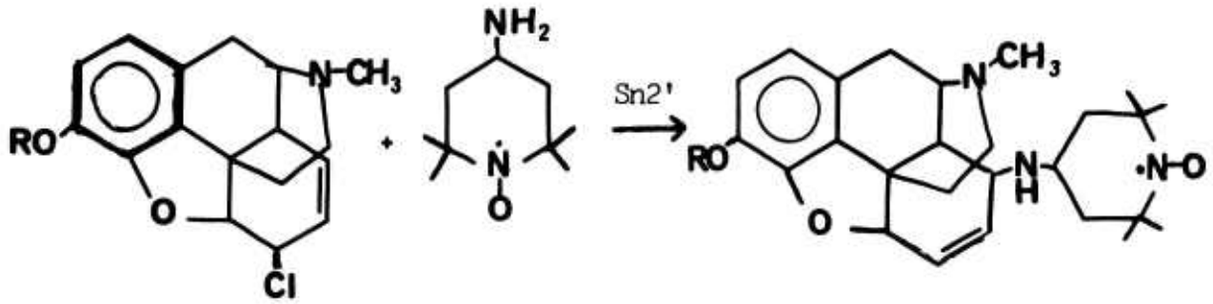
3. Applications of stable free radicals in clinical chemistry, biochemistry and cellular biology.

4. Characterization of the narcotic binding site in the central nervous system using spin labeled morphine (SLM) as a physical probe.

5. The use of ESR flow systems in conjunction with the S-122 computer system to eliminate ESR tuning errors and errors in analysis of ESR spectra from low concentrations of spin labeled molecules.

1. Spin labeled opiates.

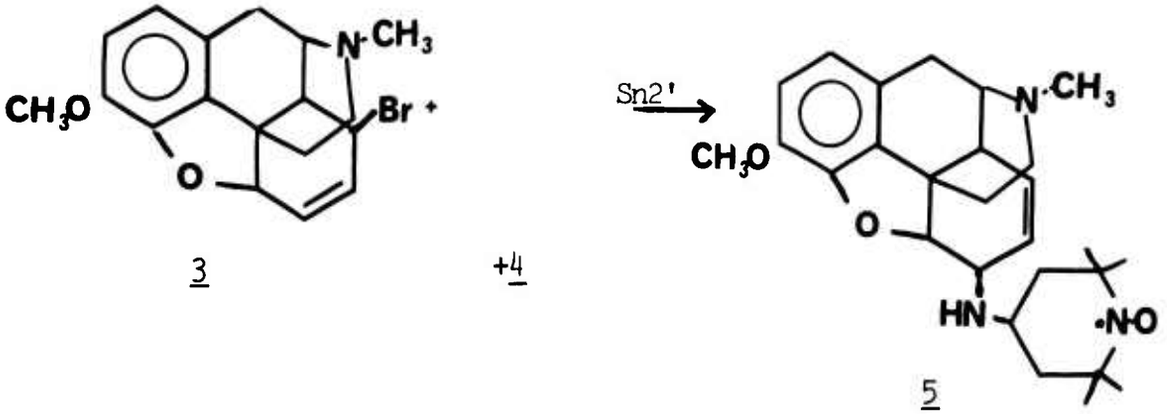
After several unsuccessful attempts at selective alkylation of morphine or codeine at the sterically hindered 6-position with alkylating agents derived from stable nitroxide radicals, the mode of the reaction was reversed. Instead, the opiates were converted to alkylating agents while the nitroxide containing moiety acted as the nucleophile. Treatment of morphine with thionyl chloride afforded α-chloromorphide, 1, which reacted with 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl in diglyme at 100-110° to produce the 8-spin labeled derivative, 2, in ca. 25% isolated yield.



1(R=H)
1a (R=CH_3)

4

2(R=H)
2a(R=CH_3)



3 +4

5

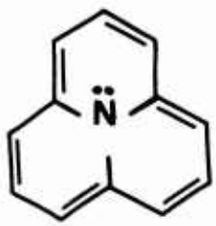
Similar treatment of codeine with thionyl chloride followed by Sn2' displacement of the chloride, 1a, with the same nitroxide reagent afforded 8-spin labeled codeine, 2a. Bromination of codeine with PBr_3 afforded bromocodide, 3, which reacted with radical 4 in an Sn2' fashion to yield 6-spin labeled codeine, 5. The route used to prepare the 6-labeled codeine derivative, 5, has not yet been successful with morphine and several alternate approaches to this derivative are underway. Although these spin-labeled derivatives give satisfactory ESR and mass spectra and have correct elemental analyses, it has not been possible to differentiate the positional isomers, i.e., 2a and 5. At this point, crystalline samples of 2a and 5 are in preparation and these should yield more definitive information about the structure of these isomers.

These spin-labeled compounds are of value for a number of reasons. First, neither of the physiologically important functions, the 3-hydroxyl and the tertiary nitrogen, are altered. Hence, the compounds should possess pharmacological activity not too different from the unlabeled compounds. Second, these derivatives are hydrolytically stable and will not lose the spin-label under ordinary physiological conditions. Third, the method of synthesis of these compounds allows for structural variation not only of the position of attachment of the label to the opiate but also variation of the length of the chain joining the spin label and the opiate.

2. New stable radicals.

Phenalene, 10, is an interesting molecule in that removal of a proton, hydride and hydrogen atom all occur with facility to afford a relatively stable anion, cation and radical respectively. It has been

10



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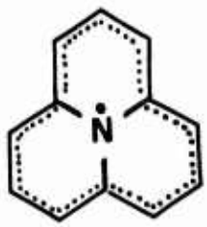

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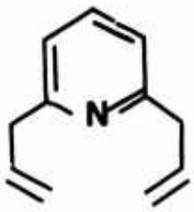

13

proposed that 9-azaphenalene, 11, should be interesting in that it is isoelectronic with the anion derived from 10. Moreover, in view of the remarkable stability of the radical derived from 10 (stable for months in solution), the analogous radical, 12, derived from 9-azaphenalene should be of considerable stability and may prove useful as a free radical-fluorescent probe. Accordingly, the synthesis of this molecule is currently being investigated by a number of routes. The most promising route to 11 is via 2,6-bis-allylpyridine, 13, which can be prepared in several steps from commercially available 2,6-dihydroxylutidine.

3. Clinical applications of stable radicals.

A number of substances interfere with blood glucose determinations, notably L-Dopa and ascorbic acid. The redox potential of nitroxide radicals is such that they are reduced by many of the interfering substances but not by glucose. Conjugation of a nitroxide with a protein should afford a high molecular weight non-dializable compound capable of reacting with the intefering substances in a continuous flow analysis. Accordingly, 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl was conjugated to bovine thyroglobulin using a water soluble carbodiimide. Dialysis of the reaction mixture afforded a solution of the protein containing covalently bound nitroxide. ESR spectroscopy confirmed that the

nitroxide was indeed bound to the protein. Preliminary tests with this protein in the AutoAnalyzer system indicate that interference by ascorbic acid is only partially removed. Further studies will be conducted to attempt to eliminate this interference.

4. Characterization of the narcotic binding site in the central nervous system using spin labeled morphine (SLM) as a physical probe.

It has not been possible to detect bound SLM in studies with CNS subfractions. Until ESR evidence can directly demonstrate morphine immobilization by a brain organelle or membrane, it will not be possible to obtain data leading to the characterization of narcotic binding sites. However, we have obtained some evidence pertaining to the magnitude of the binding phenomenon using whole brain synaptosomes and SLM at 1 μM concentration with 10 min incubation at 23° and ESR observation at 4°. The reduction in concentration of free SLM during various incubation processes showed that a total of 36.1 ± 1.1% of the SLM present was bound; 28.9 ± 2.5% of the SLM was bound during incubation and 21.1 ± 4.8% of the SLM binding was prevented by preincubation of the synaptosomes with 1 mM nalorphine. Maximal binding of SLM by synaptosomes was reached at 50 mg of synaptosomal protein per ml. At higher synaptosomal protein concentrations, SLM binding remained constant.

Further work on this project awaits completion of efforts in progress to 1) increase sensitivity of the ESR spectrometer so as to detect spin labeled preparations in the nanomolar concentration range, 2) prepare different types of spin labeled morphine which retain more biological activity than 2-SLM, and 3) localize and concetrate the narcotic binding site in appropriate brain subfractions.

5. The use of ESR flow systems in conjunction with the S-122 computer system to eliminate ESR tuning errors and errors in analysis of ESR spectra from low concentrations of spin labeled molecules.

Two different types of flow systems coupled to ESR flat cells have indicated the feasibility of obtaining optimal sensitivity while maintaining the sample temperature at fixed values in the 0° to 40° range. Using a specially constructed variable temperature insertion Dewar with an ID of 7 mm (vs 5 mm), it has been possible to insert a modified variable temperature flat cell with .015 inch Tygon tubing leading along each side from the top to the bottom of the flat cell where the sample can be pumped at .64 ml/min into the flat cell. By carefully positioning the tubing along the sides of the flat cell, it has been possible to keep the aqueous sample in regions of minimal microwave electric field concentrations, to allow flow of the sample through the cavity during the ESR scan, to remove traces of samples by air bubbles interspersed with buffer solution washes between samples and to capitalize

on the inherently greater sensitivity of the quartz flat cell for aqueous sample analysis. Using the S-122 computer system, it has been possible to detect and characterize spin label preparations of less than 10^{-7} M. Refinement of the system should permit detection and characterization capability for 10^{-8} and 5×10^{-9} M preparations.

The software for the S-122 computer system has been adapted so that background signals from buffer and other cavity contents can be subtracted from the ESR signal before integration and line width analysis. Developments have been made to cause the S-122 to make the measurements necessary for rotational correlation time analysis. This removes sources of error inherent in manually obtaining the necessary parameters from relatively noisy ESR spectra.

A second flow system was devised which offers promise in eliminating error arising from attempts to reposition a flat cell identically each time that it is loaded with sample. It utilizes a large room temperature flat cell with a cooling bath external to the ESR cavity to maintain constant sample temperature. Because of the markedly larger size of this flat cell in the sensitive volume of the ESR cavity, this flow system is currently an order of magnitude more sensitive than the modified variable flat cell system described above. However, sample volumes of about 5 ml are required with the current design and it is not as amenable to continuous sample analysis as is the smaller system.

3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 198 Spin labeling of biomolecules

Literature Cited.

Publications:

1. Copeland, E. S.: The effect of ambient temperature on spin label immunoassay for morphine. Ann. N.Y. Acad. Sci. 222: 1097, 1973.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6518	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. CHG	U	U	NA	NL	☑ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C		199
b. CONTRIBUTING				
c. CONTRIBUTING				CH FY 169

11. TITLE (Precede with security classification code)

(U) Membrane Transport in Gut

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	1	42
FISCAL YEAR CURRENT 75	1	40

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with security classification code) (U) Membrane Transport; (U) Intestinal Mucosa; (U) Membrane Potential

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security classification code.)

23 (U) To evaluate the effect of adrenergic receptor stimulation on colonic mucosal electrolyte flux and to determine the feasibility of using intestinal mucosal potential difference as an indicator of viability subsequent to ischemia. These studies provide information that relates to gastrointestinal mucosal changes that occur in severely wounded soldiers.

24 (U) An in vitro system was used to measure short circuit current, membrane potential and electrolyte fluxes in order to determine adrenergic effects on flux. In vivo measurement of transmural electrical potential was performed in both control and ischemic intestinal segments to evaluate viability.

25 (U) 73 07 - 74 06. Epinephrine, an alpha adrenergic stimulant, when administered over a broad dose range decreased membrane short circuit current and electrolyte flux. This response was abolished by pretreating the tissues with phenoxybenzamine, an alpha adrenergic antagonist. Isoproterenol, a beta adrenergic stimulant, only had an effect at high concentration. The data demonstrate alpha adrenergic receptor effects on membrane electrolyte flux. The effect may be of significance in the gastrointestinal response to stress since plasma epinephrine levels become elevated. Transmural potential difference decreased during intestinal ischemia. The decrease and return of potential upon revascularization correlated well with survival of the intestinal segments. Measurement of transmural potential may provide an intraoperative test for intestinal viability subsequent to mesenteric ischemia.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

* Available to contractors upon originator's approval

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 199 Membrane transport in gut

Investigator.
Principal: MAJ Nelson J. Gurll, MC

Background and Problem. Much has been learned about the basic mechanisms of electrolyte and water secretion and absorption in the intestine. However, alterations of these transport processes have not been elucidated in a variety of clinically important states such as shock, mesenteric ischemia, and strangulation obstruction. Studies have been conducted to investigate the effect of catecholamines on electrolyte transport by the gut and the effect of mesenteric vascular obstruction on the mucosal membrane potential.

Experimental Approach. In vitro studies of a rabbit's large intestinal mucosa were performed using modified Ussing chambers. The effects of catecholamines on ion transport as determined with radioisotopes were investigated under short circuit current conditions. Mucosal potential difference (a function of the electrogenic sodium pump) was also measured in vivo in a rabbit's small intestine and the responses to mesenteric ischemia and strangulation bowel obstruction were determined by comparing control and ischemic loops of intestine.

Results and Discussion. Epinephrine and norepinephrine caused a dose dependent decrease in short circuit current (net ionic fluxes) associated with an increase in coupled sodium absorption and a decrease in bicarbonate secretion. This effect was independent of substrate but was dependent upon the presence of CO_2 and bicarbonate bathing the mucosal surface of the tissue. Since the effect was blocked by the alpha adrenergic antagonist phenoxybenzamine, it would appear there exists an alpha adrenergic mechanism that influences the absorption of sodium and bicarbonate in the colon. This mechanism may be important in the mucosal response to shock characterized by a high circulating level of catecholamines.

The transmural small intestinal potential difference fell when the blood supply to a loop of intestine was compromised. There was a significantly lower potential difference in ischemic versus control intestinal loops in rabbits later developing intestinal infarction. In rabbits that did not develop intestinal infarction no difference in potential difference was found between ischemic and control loops. Thus potential difference can be used as an indicator of future intestinal viability; but its clinical usefulness is limited by the necessity of performing an enterotomy in order to measure the electrical potential.

(Two manuscripts are in preparation on these areas of research.)

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00 In-House Laboratory Independent Research

Work Unit 199 Membrane transport in gut

Literature Cited.

Publication:

1. Gurll, N.: Colonic mucosal adrenergic receptors for electrolyte fluxes. Gastroenterology 66: 705, 1974.

PROJECT 3A161102B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01
Biomedical Sciences

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6432	74 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	H Term	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71P	01	070
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114(f)			CH FR 015

11. TITLE (Precede with Security Classification Code)

(U) Antigen-Antibody Reactions in vivo and in vitro

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	74 06	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	6	160
FISCAL YEAR CURRENT 74	6	142

19. RESPONSIBLE DOD ORGANIZATION

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NAME:

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Allergy; (U) Enzymes; (U) Immunology; (U) Antigen; (U) Antibody; (U) Hypersensitivity

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) This work unit is concerned with the study of basic mechanisms of the immediate type allergies, and the development of methods for the isolation and characterization of antibodies and enzymes involved in hypersensitivity reactions. The ultimate goal is the control of hypersensitivity reactions and the control of the immune response in parasitic and other infectious diseases in military personnel.

24. (U) The production and characterization of homocytotropic antibodies, the mechanism of leukocyte dependent histamine release, the characterization of cellular receptor sites through attachment of immunoglobulins to well defined artificial membranes and the role of cellular and humoral immunity in various parasitic infections are being studied.

25. (U) 7307-7406 The response of guinea pig skin in passive cutaneous anaphylactic reactions has been studied with respect to the dose response relationship. Studies on schistosomiasis have revealed a role for both antibody and cellular effectors. Lipid antigens have been shown to be involved in the reaction of antibody with trypanosomes and schistosomes. Methods have been developed for in vitro studies of malaria parasites and trypanosomes. This work unit is being combined with Project 71Q DAOA6449, work unit 172. For technical report see Walter Reed Army Institute of Research Annual Report, 1 July 1973 to 30 June 1974.

*Available to contractors upon originator's approval

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Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 070 Antigen-antibody reactions in vivo and in vitro

Investigators.

Principal: LTC Carter L. Diggs, MC, M.D.

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The mission of this work unit is to perform basic research directed towards a better understanding of the immunology of infectious diseases. These studies involve the use of model systems both in vitro and in vivo. A major effort is in the application of approaches used in other areas of immunological research to infectious disease processes. These projects are described below.

1. Quantitative passive cutaneous anaphylactic (PCA) reaction

Description. The most commonly used method for the titration of skin sensitizing antibodies by the passive cutaneous anaphylaxis reaction (PCA) is the end-point titration. This consists of a determination of the highest dilution of antiserum which provokes a positive reaction when challenged with antigen (1). Treadwell et al. (2), utilizing the end-point titration, expressed their results as PCA units/ml, which are determined by dividing the reciprocal of the highest dilution of antiserum giving a minimal reaction by the volume of antiserum injected.

Although these methods of quantitation are useful, they are prone to large error. In addition, there is a wide range of animal responsiveness that is not controlled, necessitating the use of a large number of test animals.

This investigation was initiated to explore methods which might allow more reliable estimates of the potency of antisera and to eliminate or minimize the contribution of individual animal variation to such estimates.

It has been demonstrated that the diameter of the blue spot obtained when skin sensitizing antibody reacts with antigen in skin is proportional, within limites, to the amount of antibody injected (3),

and that the diameter obtained with various vascular permeable substances is directly proportional to the logarithm of the dose injected (4). In the present experiments, antisera were titrated and the diameter of the response plotted against the logarithm of the antiserum dilution injected intradermally. The regression line obtained was extrapolated to the 5 mm intercept and the titer defined as the dilution required to give a diameter of 5 mm.

Results and Discussion

Over 250 antisera were titrated and the data analyzed as described above. The lesion diameters plotted against the logarithm of the dose generally resulted in parallel regression lines. The titers obtained by the slope method were compared to the usual end point titration method. Table 1 lists the results obtained with selected sera. Out of 112 sera compared, 82 had slope titers that fell between the last positive reaction and the first negative value and the remainder had a slope titer greater than the first negative reaction. These results establish that the slope method generally agrees with the end titer. More importantly, there are several advantages that the slope method has over end point titrations. First, with the slope method, all the values are involved in the determination of the titer, in contrast to the end point titration where only the last value is used. Second, there is no theoretical limitation as to the extent of differences needed to determine the effects of various treatments whereas the end point method requires at least a 50 percent difference. Third the slope method offers a visible indication of changes in mechanism (i.e. change in slope) when comparing various treatments which may not be apparent by end point titration.

Table 1

Comparison of Slope and End Point Titration

	PCA Titer*	
Antisera	End Point Titration**	Slope Titration***
F1	320	377
F2	160	246
F5	320	483
A300	640	769
A400	80	120
F300	640	1540
S20	640	2000
F10	640	885
F40	320	476
A50	160	704
A20	640	1087

*Reciprocal of the dilution.
**The last dilution giving a positive reaction.
***Extrapolation of slope to obtain dilution giving a 5mm diameter.

The data from over 30 titrations of various guinea pig antisera were analyzed to determine what equation best described the PCA reaction. The results obtained suggest that the PCA reaction can be interpreted by the Michaelis-Menten equation, described as follows:

$$b = \frac{Bmax}{1 + \frac{Km}{Ab}}$$

where b = diameter of lesion

Bmax = maximal diameter of attainable

Ab = antibody concentration

Km = concentration of antibody at which lesion diameter attains half its limiting value (Bmax).

Tentative acceptance of this application of the data allows the comparison of various parameters derived from this equation. Table 2 lists the Bmax, Km and the slope obtained using the linear portion of the Michaelis-Menten plot of some of the antisera analyzed. It is apparent that the Bmax, that is, the maximal diameter attainable, is within the range of 15-20 mm, that the Km varies considerably as would be expected and that the slope of the line for all the antisera analyzed fall either between -2.4 to -3.1 or -1.3 to -2.0. Table 3 demonstrates the inverse relationship between the Km and the titer of various antisera. The significance of this relationship between Km and titer is being studied.

Table 2

Parameters Obtained From
Michaelis-Menten Equation

Antisera	Bmax	Km	Slope
F1	19.07	31.36	-3.1
A300	19.41	75.65	-3.1
S10	18.78	106.50	-3.1
F20	15.85	39.36	-2.4
F30	17.97	77.68	-2.7
A20	19.37	6.78	-3.1
S50	15.72	69.24	-2.5
F50	16.01	39.82	-2.7
A40	15.62	1.86	-1.3
A10	15.87	5.96	-2.0
S40	15.98	24.89	-1.7
S30	17.87	15.22	-1.9
F200	15.43	15.33	-1.7
F100	15.15	56.92	-1.6
F504	17.59	32.59	-1.7
S401	17.24	88.04	-1.4

Table 3

Relationship Between Km and Titer

Sera	Boost	Titer	Km
A300	3B	400	75.65
	4B	833	34.64
	5B	769	28.00
S10	4B	313	106.50
	5B	690	41.68
	6B	1170	34.13
F50	3B	862	51.86
	4B	1587	32.59
	5B	7042	4.59

It should be noted that there are a number of unanswered questions regarding the application of the Michealis-Menten equation to the PCA reaction. The main question to be resolved is whether or not the parameters derived from this analysis of the data relate reasonably close to what is found experimentally. Preliminary results suggest the Bmax calculated by the Michaelis-Menten equation does not reflect the true Bmax that can be obtained experimentally. Work is planned to determine the Bmax with various pharmacologically active substances. It is felt that the Bmax is not only influenced by the particular antibody (substrate) used but also by the individual test animal used for the assay. In other words, the Bmax reflects the individual animal variation. If this is true, then by utilizing the Bmax the data can be expressed as percent response at a given antibody concentration, which is suitable for probit analysis. Expression of the data in this manner will minimize the effect of individual animal variation and should make titrations among different animals comparable.

2. Characterization of guinea pig homocytotropic antibody

Objective: This investigation was undertaken with the aim of determining an optimal antigen-adjuvant combination and immunization schedule for the production of homocytotropic antibodies in guinea pigs.

Description: The production of antibody and indeed, various classes of immunoglobulins is influenced by the adjuvant as well as the antigen used for immunization (5). The antigen used throughout this investigation was p-amino benzoate diazotized to guinea pig serum albumin (PABA-GPSA). This hapten conjugated homologous protein antigen was selected for immunization so as to limit the production of antibody and thus to augment the effects of the various adjuvants with regard to antibody potency and specificity. The results of this report will deal exclusively with IgG production.

The antigen used for immunization was p-aminobenzoate conjugated by a modification of the method of Nisinoff (6) by coupling the diazonium salt derivative to guinea pig serum albumin. Guinea pigs were primed and boosted with 10 μg antigen either in Freund's complete adjuvant or adsorbed to alum or diluted in saline. Bleedings by puncture of the orbital plexus were obtained every 7 days post injection and the individual sera were frozen at -50°C until assayed for antibody activity by the passive cutaneous anaphylactic (PCA) reaction. The technique used was essentially the same as reported by Ovary (7) with some modification. Estimation of the titer of antiserum was determined by plotting the diameter of the blued spots against the logarithm of the antibody concentration injected as described in the preceding section. The titer of the antiserum was expressed as the antibody dilution required to evoke a 5 mm reaction diameter as determined from the plotted values.

Results: Figure 1 illustrates the percentage of guinea pigs producing IgG1 homocytotropic antibody after each booster injection with different antigen-adjuvant combinations for priming and booster injections. The results demonstrate that all guinea pigs primed with antigen emulsified in complete Freund's adjuvant (Ag-CFA) produced antibody after 4 or 5 booster injections regardless of the antigen-adjuvant combination used for the booster injection; a substantial number of these animals produced homocytotropic antibodies after the first booster injection. This is contrasted with guinea pigs primed with antigen diluted in saline (Ag) where only the group boosted with the combination of Ag-CFA responded maximally after 6 booster injections whereas only 25% of the guinea pigs boosted with the combination of antigen adsorbed to alum (Ag-A) were capable of homocytotropic antibody production and none of the animals boosted with Ag without adjuvant produced antibody. Further, the appearance of antibody was delayed in comparison to the group primed with the Ag-CFA combination in that antibody was not present in those animals capable of antibody production until the third or fourth booster injections. Those guinea pigs primed with Ag-A combination were intermediate between the group primed with Ag-CFA and Ag. Although most of the animals produced antibody, the response was never maximal and with the group boosted with Ag there was a decrease in the number of guinea pigs producing antibody. The time of appearance of antibody was also intermediate in this group in that at least 2 or 3 boosts were required to demonstrate the presence of antibody.

Table 4 demonstrates the importance of the combination of antigen-adjuvant used for the primary antigenic stimulus as well as the interaction between the primary injection and different antigen-adjuvant combination used for subsequent booster injections. It is apparent that when the group of guinea pigs primed with Ag-CFA is compared to the group primed with either Ag-A or A that there are a greater number of positive sera with the combination of Ag-CFA used for priming regardless of the antigen-adjuvant combination used for boosting. This is most pronounced in the animals boosted with Ag (no adjuvant) where 86%

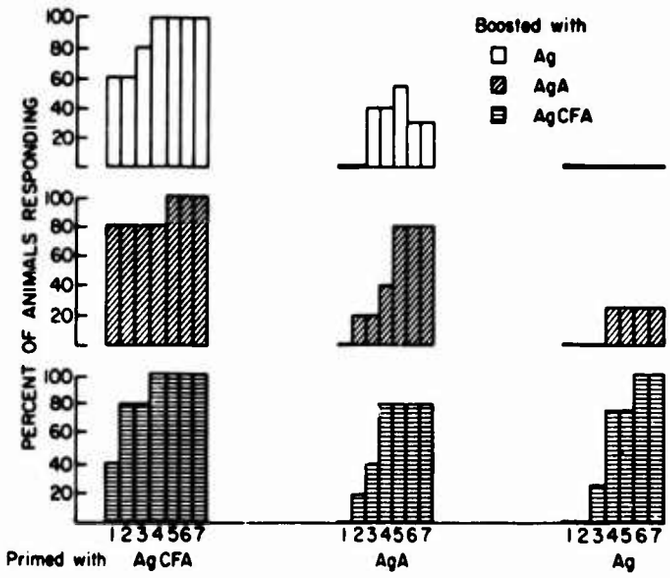

Fig. 1. Comparison of the number of responding animals to various antigen and adjuvant combinations.

of the sera are positive in Ag-CFA primed animals while only 34% have antibody activity when primed with Ag-A; none of the sera were positive with Ag priming. Further, the data demonstrates that the number of positive sera obtained from the group primed with Ag-CFA was the same whether the combination used for the booster injections were Ag-CFA, Ag-A or Ag whereas the response obtained from the guinea pigs primed with Ag or Ag-A were dependent on the antigen-adjuvant combination used for boosting. Fifty per cent of the sera obtained from guinea pigs primed with Ag (no adjuvant) and boosted with Ag-CFA were positive while only 14% and none of the sera from animals primed with Ag were positive when boosted with Ag-A or Ag, respectively.

Over 250 out of a total of 294 sera were titrated to determine the combination of priming and booster injections that gave the best titers irrespective of the group that demonstrated the largest number of antibody producing guinea pigs. To facilitate comparison among the various groups the data is expressed as the percentage of guinea pigs producing a particular titer range in Table 5. When one compares the titers obtained when using the antigen-adjuvant combination for both priming and boosting, it can be seen that priming and boosting with Ag-CFA gave higher titers than priming and boosting with Ag-A in which all of the sera had titers less than 1/500. The use of Ag alone for both priming

and boosting did not result in antibody production. The highest titers were obtained when Ag-CFA and Ag-A were used in sequence. The greatest number of titers in excess of 1/5000 were obtained with the schedule using Ag-A for priming followed by boosting with Ag-CFA.

Table 4

Number of Guinea Pigs Responding to Different Combinations of Primary and Secondary Injections

	Boost	Ag-CFA	Ag-A	Ag
Ag-CFA	1	2/5*	0/5	0/5
	2	4/5	1/5	0/5
	3	4/5	2/5	1/4
	4	5/5	4/5	3/4
	5	4/4	4/5	3/4
	6	4/4	4/5	4/4
	7	4/4	4/5	4/4
	Total 1-7	27/32	19/35	15/30
Ag-A	1	4/5	0/5	0/4
	2	4/5	1/5	0/4
	3	4/5	1/5	0/4
	4	4/5	2/5	1/4
	5	5/5	4/5	1/4
	6	5/5	4/5	1/4
	7	5/5	4/5	1/4
	Total 1-7	31/35	16/35	4/28
Ag	1	3/5	0/5	0/5
	2	3/5	0/5	0/5
	3	4/5	2/5	0/5
	4	5/5	2/5	0/5
	5	5/5	3/4	0/5
	6	5/5	2/4	0/5
	7	5/5	2/4	0/4
	Total 1-7	30/35	11/32	0/34

*Positive sera/total sera tested.

Figure 2 shows that the pattern of response of those animals primed with Ag-CFA followed by Ag-A boosts differed from animals immunized in the reverse order. Each point represents the average of 5 animals; the same pattern is obtained in the case of individual animals. When Ag-CFA was used as a priming agent followed by boosts with Ag-A peak titers were obtained within the first or second boosts and diminished with each

Table 5

Distribution of High and Low PCA Titered Sera after Various Immunization Schedules

Titer Range	Percentage of Positive Sera								
	Priming Injection	Ag-CFA			Ag-A			Ag	
	Boosting Injections	Ag-CFA	Ag-A	Ag	Ag-CFA	Ag-A	Ag	Ag-CFA	Ag-A	Ag
<500		33.3	26.0	46.7	25.0	100	88.8	53.3	100	0
501-2000		51.8	19.4	40.0	12.5	0	11.1	46.6	0	0
2001-5000		14.8	45.1	13.3	37.5	0	0	0	0	0
>5001		0	9.7	0	25.0	0	0	0	0	0

succeeding boost. In contrast, priming with Ag-A and boosting with Ag-CFA showed a progressive increase in IgG, production which did not appear to peak even after 7 boosts in spite of the high titers obtained.

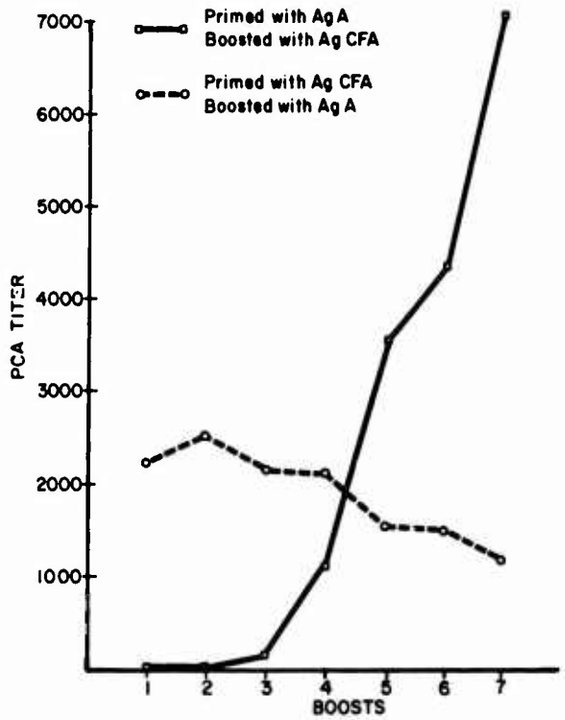

Fig. 2. IgG1 antibody production using adjuvant combinations of Alum and complete Freund's adjuvant.

Discussion and conclusion: The data obtained demonstrates the importance of first contact with antigen and the effect subsequent booster injections with a different antigen-adjuvant combination has on the production of IgG1 in guinea pigs. The group primed with antigen in Freund's complete adjuvant produced antibody earlier regardless of the antigen-adjuvant combination used for booster injection. This is in contrast to the group primed without adjuvant where the response was either delayed when booster was given with Freund's complete adjuvant or partially or totally suppressed with booster injections administered with alum or without adjuvant, respectively. The percentage of positive sera obtained from the group primed with antigen in Freund's complete adjuvant was the same regardless of what antigen-adjuvant combination was used for boosting whereas the response obtained from the group

primed with either antigen adsorbed to alum or antigen alone was dependent on the antigen-adjuvant combination used for boosting.

These results may in part be due to the particular antigen used in this investigation. The use of a hapten conjugated to homologous serum albumin may possess immunogenicity only if an extrinsic adjuvant is present. Rubin et al. (8) have demonstrated that DNP conjugated to homologous mouse serum was unable to produce antibody unless Freund's complete adjuvant was present, a result similar to that is obtained in this study. It is of interest to point out that alum as an adjuvant with this antigen is not as effective as Freund's complete adjuvant in that the number of responders was less than maximal and the titers obtained were relatively low.

The guinea pig possesses at least 3 homocytotropic antibodies; two heat stable IgG1's and a heat labile IgE (5). The two heat stable IgG immunoglobulins are antigenically similar and can be differentiated by their biological activities and electrophoretic mobilities. The question arises as to which IgG immunoglobulins are produced when different antigen-adjuvant combinations are used. Parish's (5) criteria for differentiation of the heat stable IgG antibodies is based on the latency and persistence in guinea pig skin. The 4 hour IgG confers good reactions after a latency of 4 hours which is weak or absent on the fourth day whereas the 7 day IgG gives little or no PCA activity at 4 hours, but strong sensitivity after 16 hours which persists for 7 days. The antibody assayed in this investigation appears to be primarily the type using Parish's criteria. Preliminary results indicate that IgG types are produced and investigations are in progress to determine the effect various antigen-adjuvant combinations have on the selective production of these 2 IgG antibodies as well as IgE antibody.

The most interesting finding is the synergistic effect on IgG production when either antigen adsorbed to alum or antigen in Freund's complete adjuvant are used for priming and/or boosting which produced the best titers of antibody. The enhanced effect obtained when using these two adjuvants in conjunction with one another indicates that the mode of action of the adjuvants are complementary. Regardless of the explanation for for the synergistic effect of these two adjuvants these results stress the importance that initial exposure of antigen exerts on the production of IgG antibody.

3. Induction of immunity to Plasmodium berghei in inbred mice.

Objective: This project is designed to explore the efficacy of gamma irradiated Plasmodium berghei infected erythrocytes as an immunogen in the production of immunity to the organism in strain A mice.

Description: The studies of Sadun, et al. (9) have clearly demonstrated the feasibility of immunization of mice and rats against Plasmodium berghei and of Aotus monkeys agains Plasmodium falciparum by the

use of gamma irradiated infected erythrocytes. All of these studies were performed in outbred animals, and the extension of these studies to inbred mice was required for several reasons. Passive transfer experiments have demonstrated that the serum of immune animals contains antiparasitic activity. However, the study of the specificity of this activity for parasite antigen is complicated when the possibility exists that the antigody is directed towards erythrocyte antigens. The present study was therefore designed to determine whether or not comparable levels of immunity can be induced in strain A (inbred) mice. Serum from such animals can be expected to be free of antibodies to erythrocytes.

A second motivation for these studies was to allow a better comparison of the degree of immunity obtained with irradiated erythrocytic forms of the parasite with studies by other workers (2) who have studied immunity induced by irradiated sporozoites in this same syngeneic strain of mice.

Progress: Strain A mice (The Jackson Laboratories) were infected with Plasmodium berghei and bled three or four days later for harvest of parasitized erythrocytes. The blood was centrifuged and the plasma removed, replaced by physiologic saline, and the suspensions irradiated at 25,000 rads in a cobalt 60 irradiator. This procedure was repeated 5 times at biweekly intervals, the animals receiving a total of approximately 2×10^9 parasitized erythrocytes over the 2 1/2 week period. The total packed red cell volume received was approximately 1 ml. Control animals received an equivalent number of irradiated normal erythrocytes. Additional control animals were retained without treatment. Two weeks after the last immunizing dose, all animals were challenged with 2×10^4 parasitized erythrocytes from a syngeneic donor. The results are summarized in Table 6 in which the number of animals dead by day 45 in each group is depicted. It is evident that complete protection, as measured by mortality, was observed in the immunized group whereas the controls exhibited an overall 75% mortality. There was no evidence of difference in the response of animals treated with irradiated normal erythrocytes or untreated.

Table 6

Immunization of Strain A Mice Against Plasmodium berghei

Treatment	No. Died/Total
Irradiated parasitized	0/24
Irradiated normal erythrocytes	8/10
None	7/10

Discussion and recommendations: This experiment demonstrates that irradiated erythrocytic parasites experimental immunization procedure is effective in strain A mice. The level of susceptibility of these animals is apparently lower than are the random bred mice used previously (9). These outbred animals exhibit a uniformly lethal course of disease in unimmunized animals as opposed to the 75% mortality observed in the present study. Immunized ICR mice exhibit a varying degree of mortality but almost never is the mortality rate nil as in the present experiments. These studies pave the way for future experiments involving the use of immune serum from immunized mice. They also suggest that the degree of immunization obtained through the use of erythrocytic (9) or sporozoite (10) immunogens is similar.

4. Protein synthetic capacity and infectivity of cryopreserved Plasmodium falciparum.

Objective: These studies were initiated to develop and evaluate a method for the cryopreservation of Plasmodium falciparum infected erythrocytes to provide material for the study of in vitro culture and eventual application to the study of the immunology of human malaria.

Description: Understanding of the mechanism of immunity to the malarias is derived primarily from in vivo studies and from in vitro studies with a single primate parasite Plasmodium knowlesi (11). These studies strongly suggest that antibody is an important determinant of the outcome of malaria infection. A limiting factor in further development of understanding of this phenomenon is the difficulty in approaching the study of the chemical characteristics of the antigenic determinants involved. The most promising route towards such studies is the development of in vitro methods for the assay of protective antibody, with subsequent clarification of the mechanism of antibody action and the study of the antigenic determinants by inhibition-type assays. An obstacle in the development of such systems for the study of Plasmodium falciparum has been the difficulty in obtaining viable parasites for repeated, well controlled experiments. Studies in the field with parasites of human origin have been of some value, but the impossibility of repeating experiments has made the interpretation of the data difficult (12,13). This same difficulty exists in a case of Plasmodium falciparum derived from Aotus monkeys, and in addition the occurrence of isoagglutination in mixtures of serum and erythrocytes from different individual animals greatly complicate culture experiments. It was considered that both of these difficulties could be circumvented if a method for the cryopreservation of the organism in a viable state were available. The present studies were therefore undertaken.

The approach used was to adopt methods practiced for the frozen storage of human blood for transfusion. Briefly, the methods consist of the slow addition of a cryoprotective agent containing glycerol with subsequent freezing at -70°C followed by storage at -70°C or in liquid nitrogen. After thawing, the cells are reconstituted to isotonicity by stepwise addition of solutions of decreasing solute concentration.

The parasitized erythrocytes are then used for infecting experimental animals or for short term in vitro culture experiments which are monitored by the ability of the parasitized cells to incorporate C^{14} labeled isoleucine into trichloroacetic acid precipitable material.

Progress: The initial stages of this study were reported in last years Annual Progress Report (Task 00, Work Unit 129). We reported that cells stored under conditions outlined above retained both the capacity to incorporate C^{14} isoleucine into protein and to infect animals. It was also reported that the frozen parasites remained infectious for at least 7 weeks. Animals have now been infected from materials stored for 9 months and the infectivity appeared to be quite high after this period of storage; the recipient Aotus monkey developed a parasitemia in excess of 50% of the erythrocytes parasitized on day 10 after receiving approximately 4×10^8 of the cryopreserved parasitized cells.

Initial studies of protein synthesis in vitro using short term incubation periods have now been completed. Table 7 illustrates the specificity of protein synthesis by comparing the increase in counts per minute observed with Plasmodium falciparum infected erythrocytes as opposed to control uninfected erythrocytes which were also cryopreserved. Additional evidence of the specificity of the reaction was obtained from inhibition studies with chloroquine (Table 8). In this experiment the time course of protein synthesis in the presence of various concentrations of chloroquine dihydrochloride was studied and the results are expressed in terms of the initial rate (measured over a two hour period) observed. It can be seen that minute concentrations (approximately 7×10^{-6} molar) result in 50% inhibition of the rate of protein synthesis.

Table 7

Incorporation of C-14 Isoleucine into
Protein by Cryopreserved Plasmodium falciparum In Vitro

Time of Incubation, Hours	Counts per Minute	
	P. falciparum Infected Erythrocytes	Uninfected Erythrocytes
0	230	200
1	730	180
2	1190	180
3	1390	160
5	1780	160

Table 8

Effect of Chloroquine on Protein Synthesis
by Cryopreserved Plasmodium falciparum In Vitro

Molar Chloroquine Concentration x 10^6	Initial Rate of Incorporation (CPM/hr)
0	2900
0.8	2750
2.3	2350
6.8	1350
20.3	300
61.0	0

Discussion: The studies described provide a basis for the development of methods for culture experiments of sufficiently long duration to allow experiments designed to measure the inhibitory effect of antibody on the growth and development of the parasite. Such experiments are not feasible with short term cultures since antibody acts on a mature stage of the malaria parasite and its effect can most likely be assessed after penetration of new erythrocyte by the merozoite.

Whereas quantitative comparisons are not available, it would appear that the present methodology is superior to most cryopreservation methods used in the past based on the very prompt response of recipient animals to an infectious challenge with the stored cells.

5. Inhibition of protein synthesis by antibody as an in vitro correlate of immunity to Trypanosoma rhodesiense infection

Objective: The objective of this project is to develop and characterize an assay system which quantitatively reflects functional damage to trypanosomes under the influence of antibody.

Description: Injury to trypanosomes by immune serum was described many years ago as detected by morphological evidence of damage (agglutination and/or lysis) or by neutralization tests involving injection of trypanosome-serum mixtures into recipient animals with subsequent evaluation of the infectivity of the challenge. However, only relatively few attempts have been made to devise assays for the quantitative assessment of these effects under in vitro conditions. Desowitz (14) described a respiratory inhibition test which falls into this category using Trypanosoma vivax and homologous antibody. A similar approach was used to study T. gambiense using oxygen consumption as the functional parameter measured. Assays of this type are potentially of much greater usefulness than other serologic tests since they measure actual

immune damage to the living organisms and not simply the binding of antibody to an antigen extracted from the parasite. These kinds of procedures allow the study of the specificity of protective antibodies as opposed to antibodies that react with internal antigens and are uninvolved in protection. In this way it may be possible to assess the degree to which cross reactivity between organisms occurs in nature. In addition, these in vitro systems allow approaches to the study of the mechanism of action of antibody on parasites; for example, the involvement of the complement system and the degree to which in vitro cytotoxicity correlates with neutralization tests can be studied. Finally, such in vitro tests should allow the in depth study of the antigens which are involved in the immune response to trypanosomes through estimates of ability of antigenic extracts to inhibit the cytotoxic activity of antibody.

We chose to approach the development of an assay for antibody cytotoxic to Trypanosoma rhodesiense by exploring the capacity of these cells to synthesis protein in vitro and to then examine the effect of immune serum on inhibition of this capacity. Trypanosoma rhodesiense (Wellcome strain) are isolated from the blood of rats infected four days previously by chromatography on DEAE cellulose. The organisms are centrifuged, the plasma removed, the organisms suspended in fluid containing equal volumes of normal rat serum, 5% glucose, and 0.15 M NaCl. The organisms are counted using a direct method originally developed for malaria parasites, and diluted to an appropriate concentration. The organisms are then mixed with additional serum (normal or immune) and tritiated or C-14 labeled precursor amino acids in tissue culture medium 199 supplemented with extra glucose are added. As a function of time after pulsing with the isotope, samples are removed and applied to filter paper discs. After drying these are extracted with cold trichloroacetic acid, dehydrated in alcohol and ether, redried and counted in a liquid scintillation spectrometer.

Progress: Preliminary experiments indicated that protein synthesis could be detected when a commercially supplied mixture of radioactive amino acids were added to the test system to determine which had the greatest capacity to inhibit incorporation of amino acids into protein. As a result of these studies, leucine was given a trial as a single precursor and gave satisfactory results. All further work has been done using this compound. Similarly, initial studies with a variety of buffer systems indicated that tissue culture medium 199 buffered with HEPES and with added glucose was a satisfactory medium. In addition, a high concentration of serum was apparently very beneficial to parasite survival, and this was instituted as a routine part of the reaction mixture.

Figure 3 illustrates the incorporation of C-14 leucine into the trypanosomes in the presence of normal or immune serum. Each point represents a sample containing approximately 4×10^6 organisms. The duplicate samples are taken from duplicate reaction mixtures. It is apparent from this data that essentially linear incorporation occurs

for most of the incubation period with some slight terminal fall off in rate. Immune serum completely inhibits the incorporation of leucine into protein by the organisms. In the experiment shown, serum and organisms were incubated for 20 minutes at 37°C prior to pulsing with the radioactively leucine. However, other experiments demonstrated that complete inhibition of protein synthetic capacity could be obtained with as little as 6 minutes of preincubation prior to addition of label.

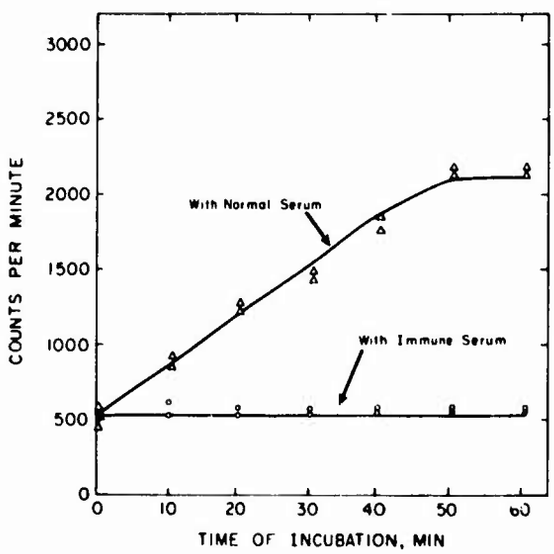

Fig. 3. Protein synthesis by Trypanosoma rhodesiense in the presence of normal or immune serum. Organisms incubated 20 minutes before addition of C-14 leucine.

Heating of the immune serum abolishes its capacity to inhibit protein synthesis (Fig. 4). This inhibition is a dose-related phenmenon with a 1:20 dilution of serum giving 50% inhibition of the protein synthesis under the conditions employed. Table 9 illustrates this dose response relationship.

When the number of cells is varied and time course experiments performed, it can be demonstrated that the initial rate of incorporation is proportional to the number of organisms used (Fig. 5). This is an important result in that it allows a quantitative interpretation of the number of cells which are active from the incorporation data.

Discussion and recommendations: These studies demonstrate that highly precise assays of immune serum mediated cytotoxicity to Trypanosoma rhodesiense can be performed in vitro. The activity of immune serum is heat labile, presumably due to a complement requirement, but with conceivably due to heat lability of the responsible antibody; studies to distinguish between these alternatives are in progress.

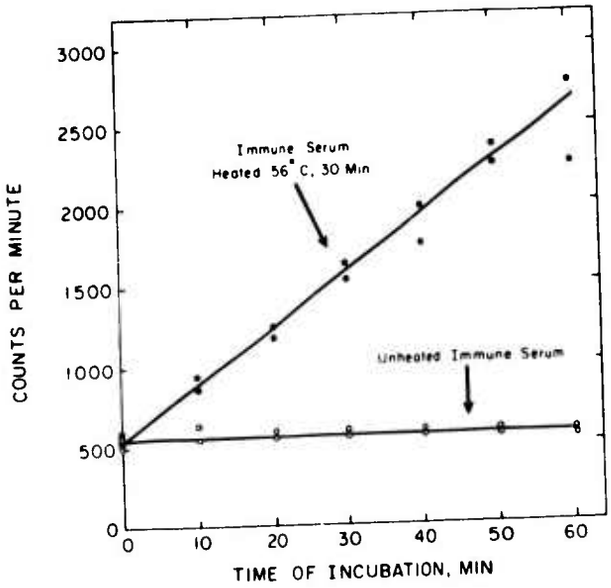

Fig. 4. Abolition of cytotoxic effect of immune rat serum on Trypanosoma rhodesiense by preheating.

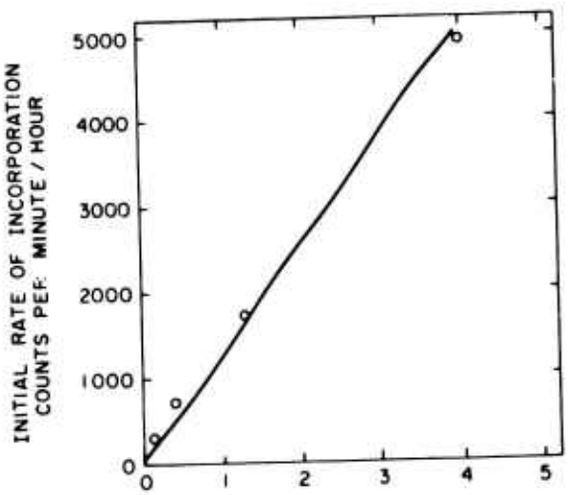

Fig. 5. Linear dependence of rate of protein synthesis on Trypanosoma rhodesiense cell concentration.

Table 9

Serum Inhibition of Protein Synthesis* by Trypanosoma rhodesiense; Dose-response Relationship

Immune Serum, ml	% Inhibition of Protein Synthesis
0.02	6
0.04	33
0.06	77
0.08	87
0.10	85
0.12	89
0.14	98

*Forty-five minutes incubation

Whether or not the in vitro cytotoxic activity will correlate with neutralizing activity has not yet been determined; these experiments are also underway. Plans are being made to study the effect of preincubation of immune serum with antigens of Trypanosoma rhodesiense to determine whether or not reversal of the inhibiting effect of antibody can be obtained. If so, the assay will provide a tool for the isolation and characterization of such materials.

6. Serological studies of experimental trypanosomiasis

Objective: The objective of these studies was to provide comparative information on various manifestations of the humoral immune response to Trypanosoma rhodesiense in Rhesus monkeys.

Description: Profound changes in total immunoglobulin levels, especially IgM, have been noted in humans and animals infected with T. rhodesiense (15). Along with specific antibodies, heterophile antibodies also appear. It is not known to what extent these various antibodies account for the immense rise in total immunoglobulin. These studies were initiated to investigate the relationship of the heterophile antibodies to antigens on the trypanosome organisms with respect to the time course of total immunoglobulin changes.

Four 7-12 pound Rhesus monkeys were injected intravenously with 1 ml of rat blood diluted in 1% dextrose-phosphate buffered saline containing 10,000 Trypanosoma rhodesiense organisms. Three monkeys were injected with saline alone. Blood was taken at various times after inoculation and sera were stored at -20°C.

Total IgG, IgA, and IgM immunoglobulin levels were determined by quantitative radial immunodiffusion analysis and IgE levels were determined by radioimmunoassay using commercially available reagents in each case.

Fluorescent antibody titers were performed using a method modified from that of Sadun, et al. (16). Specific fluorescent anti-human IgM and IgG was obtained commercially. Trypanosome organisms were isolated from 4-day infected rats by the method of Lanham and Godfrey (17), washed in 1% dextrose-phosphate buffered saline and dried onto glass slides.

The slides were stored at -20°C until use as antigen for the fluorescent antibody test.

Sheep erythrocyte (SRBC) titers were determined by microtiter hemagglutination in phosphate buffered saline, Ph 7.4 containing 1% rabbit serum. Absorptions of anti-trypanosome sera were performed using 1 ml packed sheep erythrocytes for 30 minutes at 37°C incubation. The initial absorption mixture contained 3 ml 1/40 dilution of anti-trypanosome serum. Two tenths milliliter was removed for study after each of 5 absorptions.

Anti-SRBC sera (0.2 ml) were absorbed with 0.1 ml packed glutaraldehyde fixed trypanosome organisms at 37°C for 30 minutes. Five one-hundreth ml of serum was removed and the serum was reabsorbed with 0.1 ml trypanosomes two more times.

Thirty milligrams BSA in saline was injected subcutaneously in two sites in all monkeys, control or experimental, 17 days after inoculation with T. rhodesiense. BSA titers were determined by the Farr method (18), using 0.12 μg ^{125}I-BSA.

Progress: As seen in Table 10, the rise in total IgM was marked by day 10, being threefold that of the pre-inoculation level. By day 42, the mean IgM level had risen thirty-two fold. Only one of the four monkeys had less than a thirty-fold increase in serum IgM at 42 days. The increase in IgG level was less than two-fold at its peak at 28 days. The level fell slightly after that. Individually, 3 of 4 monkeys had two two-fold IgG increases on day 28. Mean IgA levels increased from less than 10 mg/dl to 17 mg/dl on day 28 and fell slightly after that. As seen from Table 11, total serum IgE levels as measured by RIA appeared to increase slightly in one monkey (No. 3).

Specific antibody titers followed the same initial rise as did total IgM levels with a sharp rise in both IgG and IgM antibody on day 10. IgM and IgG seemed to follow parallel courses as can be seen in Table 12.

Sheep erythrocyte hemagglutination titers, however, began a sharp rise on day 17, lagging a little behind the anti-trypanosome FA titers (Table 13). Absorption of anti-SRBC activity can be accomplished with whole trypanosome organisms (Table 14). Ease of absorption seems to differ between sera taken at day 10 or day 28 after inoculation.

Table 10

Total Serum Immunoglobulin Levels of Rhesus Monkeys Inoculated with T. rhodesiense

	Days After Inoculation						
	0	6	10	17	28	35	42
Inoculated (4 monkeys)							
IgG	1463±295[a]	1308±243	1468±212	2326±716	2833±313	2620±548	2702±685
IgM	61±23	58±9	215±56	68±338	1445±386	1687±796	1970±889
IgA	<10	---[b]	4	13	17	---	14
Non-inoculated (3 monkeys)							
IgG	1437±203	---	1410±190	---	1377±267	---	1567±139
IgM	68±28	---	59±17	---	54±17	---	51±10
IgA	<10	---	---	<10	---	---	---

[a] Mean immunoglobulin level in mg/dl ± 1 standard deviation.

[b] --- not done.

Table 11

Total Serum IgE Levels in Control and T. rhodesiense Inoculated Monkeys

Monkey Number	Day After Inoculation 6	32	42
1[a]	320[b]	360	700
2	300	390	320
3	680	1210	1300
4	500	680	700
C1[c]	530	510	---
C2	580	500	---

[a]Inoculated monkeys

[b]Serum IgE in nanograms/ml

[c]Control, non-inoculated monkeys

Table 12

Log_2 Fluorescent Antibody Titers at Various Days After Inoculation of Rhesus Monkeys with T. rhodesiense

Monkey Number		Days After Inoculation 0	6	10	17	28	35	42
1	IgG	3	3	8	11	12	11	9
	IgM	2	3	9	11	12	10	9
2	IgG	1	1	6	9	10	10	10
	IgM	2	1	9	9	11	10	9
3	IgG	4	4	8	10	9	11	12
	IgM	3	6	9	10	9	11	9
4	IgG	5	4	8	8	11	9	9
	IgM	6	7	11	11	11	9	8

Table 13

Log_2 SRBC Titers After Inoculation with

T. rhodesiense on Day 0

Monkey No.	Days After Inoculation						
	0	6	10	17	28	35	42
1	1	1	3	9	8	10	8
2	1	1	2	6	8	8	7
3	1	1	4	6	11	10	9
4	2	2	10	10	10	8	7

Table 14

Absorption of Anti-SRBC Activity by
Glutaraldehyde-Fixed Trypanosomes

Day After Trypanosome Inoculation		Number of Absorptions		
		0	1	2
Day 10				
Monkey #	1	8[a]	4	3
	2	7	3	1
	3	9	7	4
	4	13	13	11
Day 28				
Monkey #	1	13	12	12
	2	12	12	11
	3	13	13	13
	4	13	13	10

[a] Log_2 anti-SRBC titer

The removal of anti-trypanosome activity can be accomplished by absorption with SRBC more easily in the IgM reaction than in the IgG reaction (Table 15). This difference appears to occur whether sera is taken from day 17 or 35.

On day 17 after inoculation of experimental animals with trypanosomes, all monkeys including controls were injected with 30 mg BSA. Antibody assay by the antigen binding capacity (ABC-33) test of Farr showed a greater response in the control animals than in the monkeys exposed to T. rhodesiense. As can be seen in Table 16, no experimental monkeys bound 33% of the radiolabeled antigen while all three control monkeys did so.

Discussion: An increase in total IgM is characteristic of trypanosome infection, but the thirty-fold increase observed in these studies is striking indeed. Slight elevations of IgG have also been previously noted. The apparent rise of IgA is noteworthy. In individual animals, the trend is easily seen. Human reagent plates were used and the values were at the low level of detection. For a more reliable estimate, low level standards and plates will be used to retest the sera. IgE levels are more difficult to interpret. Increase above control monkey IgE values seem to occur in one and possible two other monkeys. Other sera were tested using a different lot number of commercial reagents. The trend appeared in monkey No. 3, however, the total levels on duplicate serum samples between the two sets of reagents did not agree. This variability could be caused by incomplete cross reactivity of monkey IgE and the human reagents.

Specific antibody titers increase in the fluorescent antibody procedure with the rise in total globulins. IgG and IgM titers show similar patterns. This could be due to cross-reactivity of the immunological reagents or could be characteristic of the disease. Further characterization of the immunological reagents will help clarify this point. Comparison can be made with antibody titers determined by class-specific RIA in Work Unit 172 of this report.

Appearance of heterophile antibodies is also characteristic of many trypanosome infections. Absorption experiments seem to indicate that the trypanosomes contain an antigen that might induce the anti-SRBC activity. Characterization of this antibody with respect to specificity for Forssman antigen is a likely next step.

Immunodepression to injected SRBC has been reported during trypanosomiasis in rabbits and mice (19). Suppression of response to BSA in trypanosome infected monkeys also occurs in uninfected monkeys as seen in Table 16. The ability of monkeys to produce increased globulin levels with heterophile and specific antibodies yet not respond to a second antigen (BSA) stands as a contradiction. Antigenic competition can be offered as an explanation of these observations.

Table 15

Absorption of Fluorescent Anti-Trypanosome Antibody with SRBC

Monkey Number	Day After Inoculation		Number of Absorptions 0	5
1	17	IgG	1280[a]	320
		IgM	1280	160
	35	IgG	1280	1280
		IgM	640	160
3	17	IgG	1280	640
		IgM	1280	320
	35	IgG	1280	320
		IgM	1280	80

[a]Reciprocal of dilution of sera showing 1+ fluorescence as end point.

Table 16

Farr Assay Binding of ^{125}I-BSA by Sera of Monkeys Injected with BSA 17 Days After Inoculation with T. rhodesiense

Monkey Number	Days After BSA Injection			
Infected	0	11	18	25
1	0[a]	t[b]	t	t
2	0	0	t	t
3	0	0	t	t
4	0	0	0	0
Non-infected				
1	0	t	1.39[c]	1.15
2	t	6.05	5.68	3.58
3	0	0	t	1.28

[a]No binding of ^{125}I-BSA.

[b]Trace of binding equivalent to 5% binding.

[c]µg BSA bound at 33% endpoint using 0.12 µg ^{125}I-BSA.

The mechanisms of hyperimmunoglobulinemia in trypanosomiasis remain elusive, but the data provided in these studies supply a basis for the development of experimental approaches to the problem.

7. Poly (lactic acid) as an immunological adjuvant

Objective: The objective of this project is to investigate the possibility of using a biodegradable polymer of lactic acid as an immunological adjuvant.

Description: Immunological adjuvants are substances which can increase the specific antibody response to an antigen. Although the mode of action of adjuvants is not completely clear, some investigators have suggested that a substance which would release antigen slowly, thereby allowing processing of the antigen without overwhelming the lymphocyte receptor sites would probably increase antibody production. Based on this hypothesis a biodegradable matrix of polymer and protein antigen might show significant adjuvant activity.

In recent years, lactic acid polymers have been studied and used as degradable suture and surgical implants (20,21). Histological evaluations have shown that the tissue response to the polymers is minimal, similar to that elicited by stainless steel and Dacron. In vivo, the polymer is degraded by hydrolytic deesterification to lactic acid, a normal intermediate in the glycolytic cycle in carbohydrate metabolism. The bland histological response induced and the acceptability of the degradation products makes poly(lactic acid) a promising substance for controlling the release of antigens in vivo. Bovine serum albumin (BSA) was incorporated into the polymer as antigen to test the effect of slow release from the polymer on the immune response.

A polymer of lactic acid was synthesized as previously described (20, 21). The polymer-BSA matrix was formed by precipitating a pyrogen-free acetone solution of polymer and BSA with water. The resulting precipitate was ground to form particles of a size suitable for injection in saline suspension. The concentration of BSA in the polymer-BSA preparation was determined by redissolving the dried particles in acetone and assaying the protein residue by the Lowry micro method (22). The particles used in this study contained 0.37% BSA (w/w). Control polymer particles were prepared as above but without the addition of BSA. Groups of mice were injected with various preparations of BSA as indicated in Table 17. The mice used were 10-12 weeks old, ICR-WR strain outbreeds weighing 26-38 gms. Each animal was bled from the tail at two week intervals starting at day 7 post immunization. Approximately 0.5 ml of blood was collected from each mouse at every bleeding. The serum was separated and individual samples were stored at -20°C. Twenty-five hundreths ml of sera from each individual animal was pooled to form a single sample from each experimental group. Each pool was made of sera from 10 animals.

Table 17

Experimental Groups of Mice

Group	Antigen	
FBSA	Free BSA in saline	0.025 mg BSA/site
AFBSA	Acetone treated BSA in saline	0.025 mg BSA/site
FICA-BSA	BSA in Freund's incomplete adjuvant	0.025 mg BSA in 0.1 ml FICA/site
Poly + FBSA (same site)	Plain polymer particles in saline solution containing BSA	8.7 mg polymer in 0.1 ml saline containing 0.025 mg BSA/site
Poly + FBSA (different sites)	Plain polymer in saline	17.4 mg polymer/each of two sites
	Saline solution of BSA	0.05 mg BSA/each of two sites
Poly-BSA	Polymer-BSA matrix	8.7 mg polymer site with 0.025 mg BSA incorporated

10 animals per group, each animal receiving 0.1 ml of antigen subcutaneously in each of four sites as indicated above. All antigens except FICA were given in 0.9% saline.

Anti-BSA antibodies in each group were assayed by two methods. One method was a passive hemagglutination technique using glutaraldehyde-fixed BSA sensitized sheep erythrocytes (23). Antibody titers were expressed as the $\log_2$ of the reciprocal of the highest dilution of serum showing agglutination. The second method was the ammonium sulphate antigen-binding capacity (ABC-33) technique of Farr (24). The ABC values were expressed as μg BSA bound per ml of undiluted serum.

Progress: The passive hemagglutination titers of the experimental groups are shown in Figure 6. Animals that received either free BSA (FBSA) in saline or acetone treated BSA (AFBSA) in saline had no detectable primary antibody response but did show a typical secondary response when challenged with 0.1 mg BSA (Table 18). The group that received BSA in Freund's incomplete adjuvant (FICA-BSA) and the polymer-BSA (poly-BSA) group both showed a detectable antibody response, with titers increasing over a period of many weeks. The two groups that were injected with polymer and free BSA (poly+FBSA) in the same or different sites developed lower peak antibody responses which plateaued sooner than the FICA-BSA or poly-BSA groups.

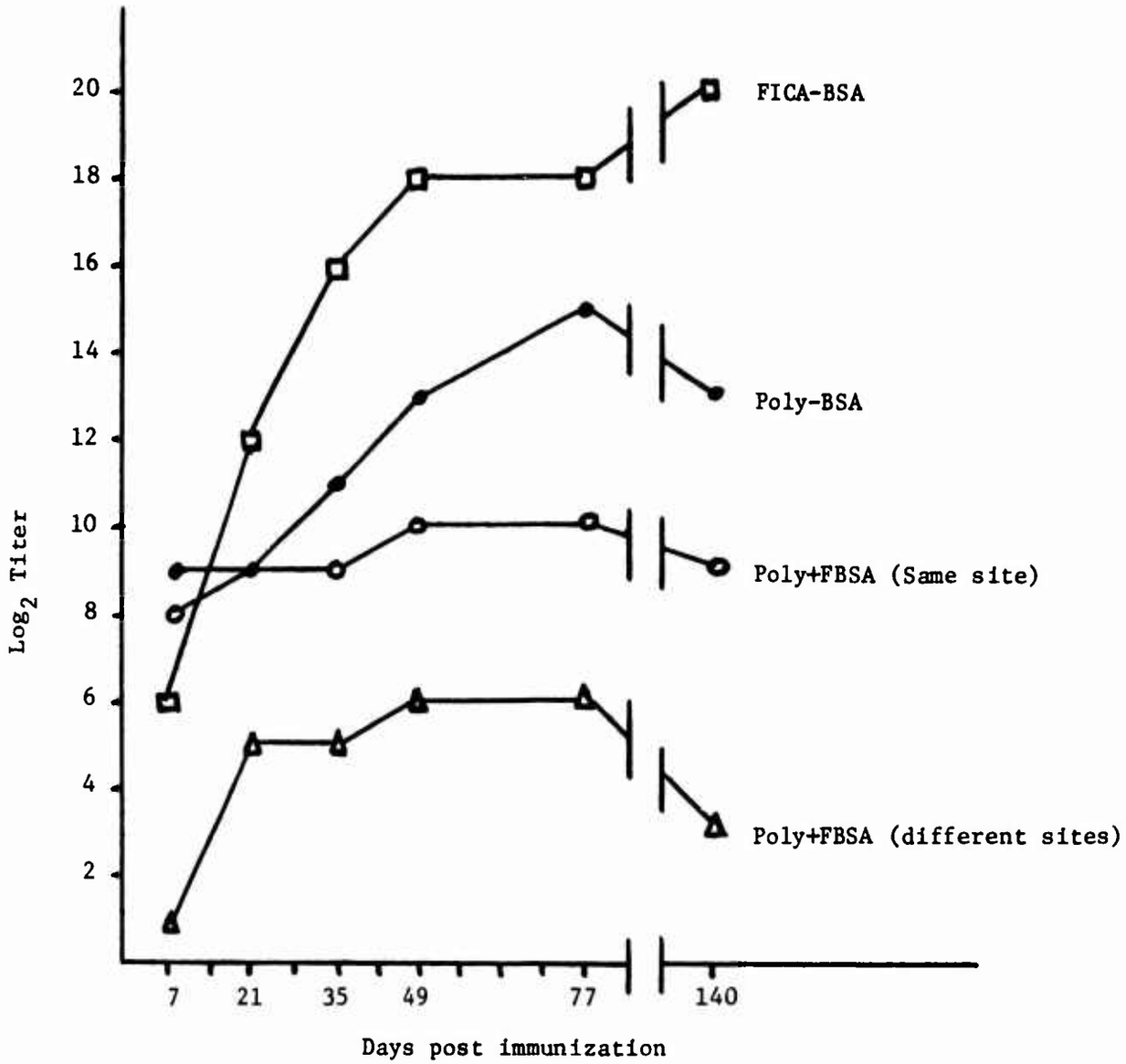

Fig. 6. Log_2 anti-BSA hemmagglutination titers of sera pooled from each group.

All animals received 0.1 mg BSA as indicated

FBSA	free BSA in saline
FICA-BSA	Freund's incomplete adjuvant with BSA
Poly+FBSA	Poly(lactic)acid and free BSA in saline
Poly-BSA	Poly(lactic)acid with incorporated BSA

Two control groups, one that received 0.1 mg FBSA in saline and one that received 0.1 mg acetone treated BSA showed no detectable HA response.

Table 18

Log_2 Anti-BSA Hemagglutination Titers of Sera Pooled From Each Group Before and After Secondary Immunization. All Animals were Given 0.1 ml BSA I.P. 21 Weeks (d147) After Initial Antigen Injection

Group	Pre-secondary Immunization Day 140	Days Post-secondary Immunization 7	14	28
FBSA	--	13	16	12
AFBSA	--	13	14	9
FICA-BSA	20	18	$\geq$22	14
Poly+FBSA (same site)	9	17	20	14
Poly+FBSA (diff. site)	3	12	15	9
Poly-BSA	13	20	$\geq$22	14

Antibody levels determined by the ABC-33 assay are shown in Table 19 for both the primary and secondary responses. The relative antibody production between groups is similar to that found with the hemagglutination assay.

Discussion: The incorporation of BSA into a poly-lactic acid polymer clearly increases the antibody response to BSA above that which occurs when the same concentration of antigen is injected in saline. This adjuvanticity may be due to multiple factors. Slow release of antigen due to bio-degradation of the lactic acid matrix, thus allowing a more prolonged and efficient stimulation of the antibody-producing cells may be a prime factor. Non-specific stimulation of the phagocytic system may also play a role. This is indicated because polymer injected along with BSA also showed some adjuvanticity. BSA in the same site could have been adsorbed by the polymer and then could have stimulated antibody production by the same means as incorporated BSA. BSA in different sites, however also stimulated antibody response greater than without polymer. The mechanism of this action may be through generatized stimulation of phagocytic activity. The response to BSA and polymer in different sites was much lower than other polymer groups in both the primary and secondary responses.

The kinetics of the Poly-BSA response is similar to that of the response to FICA-BSA. The antibody titers of the Poly-BSA group were even comparable to FICA-BSA group after a secondary injection of BSA in saline. The ability of BSA incorporated in a biodegradable matrix to elicit large antibody responses may have important applications.

Table 19

Antigen Binding Capacity (ABC-33) Expressed as μg BSA Found Per ml Serum. The ABC Values were Obtained by Determining the Dilution of Serum that Bound 33% 0.10 μg ^{125}I-BSA

	Days Post-Primary Immunization						Days Post-Secondary Immunization		
Group	7	21	35	49	77	140	7	14	28
FBSA	--	--	--	--	--	--	4.30	15.2	15.1
AFBSA	--	--	--	--	--	--	5.95	8.90	6.40
FICA-BSA	--	5.28	50.0	99.0	195	202	25.6	52.8	81.7
Poly+FBSA (same site)	--	1.22	1.52	1.14	1.32	1.15	34.7	61.0	89.6
Poly+FBSA (diff. sites)	--	--	--	--	--	--	1.47	5.78	3.52
Poly-BSA	0.97	1.90	2.48	2.48	6.75	3.14	64.4	71.0	59.3

By chemically varying the amount of polymerization, the rate of degradation of the polymer can be controlled. Additionally, the ratio of antigen to polymer can be varied resulting in further capabilities to control the rate of antigen reaching the immunoglobulin producing cells. Another variable which can be adjusted is the particle size of the polymer. Antigen deposits range from phygocytizable particles to large, slowly degrading pieces of polymer. The variety of parameters of antigen release that can be controlled allows a means to study the effect of presentation of antigen on the immune response.

8. Forssman-containing liposomes: Complement-dependent damage due to interaction with a monoclonal IgM

Since 1968 liposomes have been employed as a model for studying the molecular basis of immune lysis. The liposomes consist of closed, concentric shells of lipid bilayers alternating with aqueous interspaces. The aqueous portions within the liposomes generally contain marker molecules (e.g., glucose) and the membrane damage is measured by release of the marker compound. By using liposomes with various amphipathic lipids as antigens, a wide variety of specificities have been described. Forssman substance was the first antigen used in these studies and its properties in liposomes have now been thoroughly investigated. The initial work demonstrated that liposomes containing Forssman hapten could bind anti-Forssman antibodies (hemolysin) and this led to membrane damage and glucose release due to complement fixation. A subsequent report showed that this was a highly specific phenomenon, and anti-Forssman antibodies did not cross-react with globoside I, which is closely related glycolipid.

Recently a Waldenstrom macroglobulin (McG) has been discovered which agglutinates sheep erythrocytes and hemolyzes them in the presence of complement. In this report we are presenting preliminary observations which demonstrate that this protein also interacts with liposomes containing highly purified Forssman hapten. The binding of the macroglobulin leads to complement fixation and liposomal membrane damage in a manner apparently analogous to that caused by hemolysin (Fig. 7).

9. Comparative properties of four galactosyl lipids as antigens in liposomes

Two sphingosyl galactolipids, galactocerebroside and mixed gangliosides and two glyceryl galactolipids, mono and digalactosyl diglycerides were compared for immunological activity in liposomal model membranes. The liposomes were prepared with one of several synthetic lecithins or with beef sphingomyelin. Specific complement-dependent glucose release was found in all cases, although high titer antiserum was not obtained against monogalactosyl diglyceride. With galactocerebroside the degree of glucose release was inversely related to the phospholipid fatty acid chain length. The relative efficiencies of these substances in terms of antigenic expression and or immunogenicity was in the order: ganglioside > digalactosyl diglyceride > galactocerebroside > monogalactosyl diglyceride. Reciprocal serological cross-

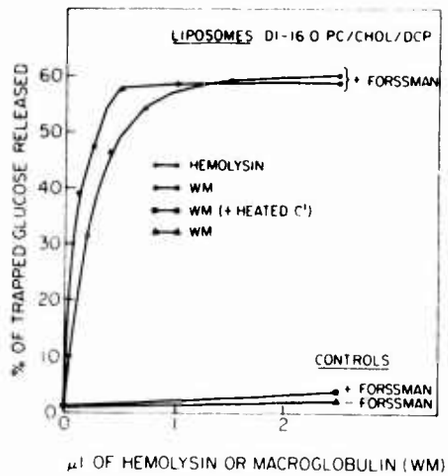

Fig. 7. Each point corresponds to the glucose measured in a cuvette which contained 5 μl of liposomes, 500 μl of glucose assay reagent (5), 120 μl of either fresh guinea pig serum as a complement source or, as a control, guinea pig serum which had been inactivated by heating at 56°C for 60 min (heated C). Hemolysin (rabbit antiserum in 50% glycerol, Difco Laboratories, Detroit, Mich., diluted 1:5 with 0.15 M NaCl and heated at 56°C for 60 min) or macroglobulin (4.9 μg protein/μl) were added in the amounts shown on the abscissa, and the volume was brought to 1.0 ml with 0.15 M NaCl. Glucose release was measured at room temperature (*ca.* 22°C) 30 min after starting the reaction.

reactivity was observed between ganglioside and galactocerebroside on the one hand and between mono and digalactosyl diglycerides on the other. Cross-reactivity oetween these two groups occurred in four out of six possible combinations studied. Anti-digalactosyl diglyceride was the only antiserum with an apparent high degree of specificity and it did not cross-react with either cerebroside or ganglioside (Table 20). Cross-reactivity was influenced by the phospholipid fatty acid chain length, liposomal antigen concentration and the antibody titer against the immunizing antigen.

10. The influence of retinal on complement-dependent immune damage to liposomes

Retinal was incorporated into liposomes containing dipalmitoyl-lecithin cholesterol, dicetyl phosphate and galactocerebroside; the latter substance served as antigen. They were compared to control liposomes, lacking retinal, with regard to glucose release due to complement-dependent immune damage in the presence of anticerebroside serum. The liposomes were indistinguishable from each other in the amount of total glucose trapped, light scattering characteristics and

Table 20

Serological Cross-reactivity of Galactosyl Lipids

Antiserum[+]	% of Trapped Glucose Released in 30 Minutes from Liposomes Containing*				
	No Antigen (Control)	Cerebroside	Ganglioside	Monogalactosyl Diglyceride	Digalactosyl Diglyceride
Anti-cerebroside (9)	1.6	64.3	19.7	35.1	70.0
Anti-ganglioside (2)	7.6	21.2	64.9	20.4	59.2
Anti-digalactosyl diglyceride (2)	5.5	4.1[++]	8.2[++]	39.4	67.4

*Each liposome preparation contained di-16:0 PC chol DCP plus, except where indicated, 150 μg of antigen per μmole of di-16:0 PC. Each liposome was assayed for glucose release in the presence of 128 μl of complement and 37 μl of each of the three antisera. Each value is an average of between two and six experiments. Variation of immunological lucose release was never greater than 10 per cent of the mean.

[+]The values shown were taken from a single representative rabbit antiserum. The numbers in brackets indicate the number of different rabbits tested. Only antisera which gave at least 50% glucose release against the specific immunizing antigen were used. All the rabbits in each antiserum category gave results which were qualitatively, but not necessarily quantitatively identical with those shown. Occasionally, with cerebroside and monogalactosyl diglyceride, higher antigen concentrations (two-fold higher) were required in order to demonstrate unequivocal cross-reactivity.

[++]Cross-reactivity could not be demonstrated with either of these antigens, even when twice the antigen concentrations were employed.

phosphate content. The rate and extent of glucose release in 30 minutes was inhibited by the incorporation of retinal (Fig. 8). In addition, inhibition was directly related to retinal concentration (Fig. 9) and was also observed in the presence of a wide range of concentrations of antigen and complement. Damage to liposomes in the presence of either guinea pig or human complement was inhibited by retinal; this was in contrast to the erythrocyte system in which the hemolytic activity of guinea pig complement was inhibited while that of human complement was enhanced by retinal. Addition of retinal to preformed liposomes did not influence complement-dependent damage. Inhibition occurred only when retinal was present during the initial formation of the model membranes. Inhibition persisted even after washing the liposomes free of any unincorporated retinal. The data indicate that liposomes may be an excellent model for studying the influence of retinal on complement mechanism in membranes.

11. Immune damage to liposomes containing lipids from Schistosoma mansoni worms

A protein-free lipid extract ("F2") consisting mainly of glycolipids, a small amount of phospholipids and an unidentified brown pigment, was obtained from Schistosoma mansoni adult worms. The FA was tested for the presence of haptenic molecules by incorporation into liposomal model membranes containing trapped glucose. Sera from five infected monkeys were assayed for serological activity against the F2 in liposomes at different times following infection. Complement-dependent damage leading to liposomal glucose release was observed with sera from four out of five monkeys (Fig. 10). Glucose release did not occur when the F2 was omitted from the liposomes nor when heat-inactivated complement was used (Fig. 11). All of the activity was removed by absorption of monkey serum with adult schistosomes (Fig. 11). The IgM-containing fraction of serum accounted for all of the antibody activity. It was concluded that a complement-dependent immune response against lipids may be observed during the course of schistosomiasis in monkeys. The antibody activity can be detected by utilizing liposomal model membranes which contain schistosomal lipids.

12. Immunochemistry of A_1 and A_2 human blood group substances; differences in the reactivities of homologous and heterologous antisera in quantitative hemagglutination inhibition reactions with hog and human saliva A_1 and A_2 substances

The chemical basis for the differences in the serological reactivities of the subgroup A1 and A_2 antigens of human erythrocytes has been widely disputed. One view holds that the differences are merely quantitative in that A_1 cells possess a greater number of A sites. The opposing view contends that the determinants on A_2 cells differ structurally from those on A_1 cells. The latter view is strongly supported by recent findings of Moreno et al. (25) with A_1 and A_2 substances purified from human ovarian cyst fluids. This report describes the differences in reactivities of antisera prepared in humans to hog A

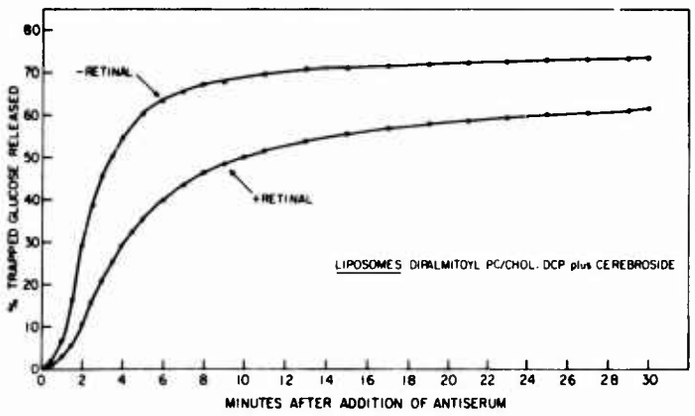

Fig. 8 Effect of retinal on the time course of immune damage to liposomes. The liposomes contained sufficient retinal to make a final concentration of 1.0 mM in the swollen suspension. 4.8 μl of liposomes were preincubated for 10 min with 128 μl of fresh guinea pig serum (complement), 500 μl of glucose assay reagent and sufficient 0.154 M NaCl to make a volume of 976 μl in a semi-micro cuvette. After reading the absorbance at 340 nm the immunological reaction was started by adding 24 μl of rabbit anticerebroside serum. The final absorbance readings were measured at the time intervals shown and the % of trapped glucose released was determined from the difference compared to the initial reading. Less than 4% of the available trapped glucose was released in 30 min in control cuvettes in which complement had either been omitted or inactivated by heating at 56 °C for 60 min.

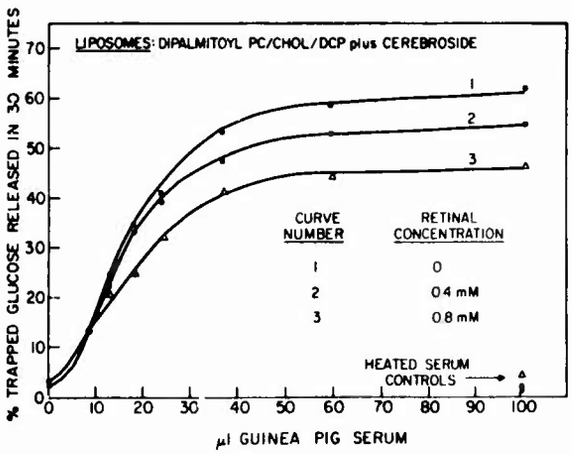

Fig. 9 Effect of retinal concentration on immune damage to liposomes. The procedure was the same as described in the legend of Fig. 8. Retinal was incorporated directly into the liposomes as described in Materials and Methods, and was present in the concentrations, with respect to the swollen dispersions, indicated in the figure.

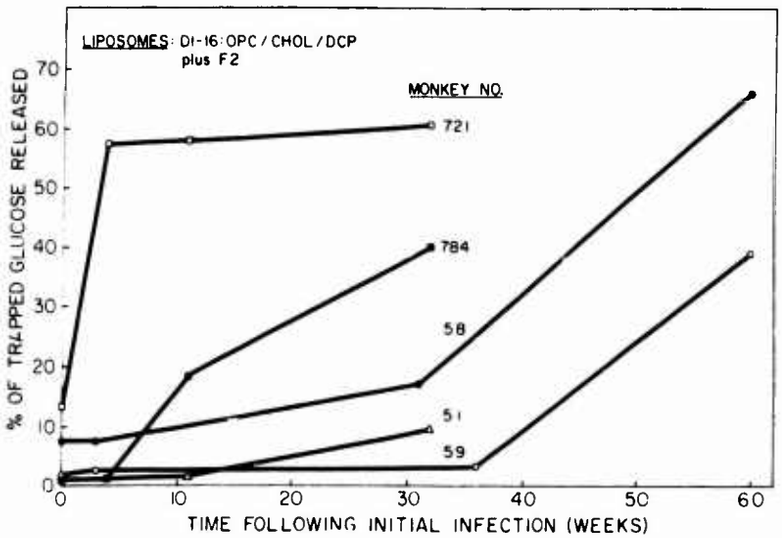

Fig. 10. Time course of serological activity of 5 infected monkeys against schistosomal lipids. Each point corresponds to a separate reaction cuvette which contained 4.7 μl of liposomes, 50 μl of appropriate monkey serum 120 μl of fresh guinea pig serum as a complement source, 325 μl of 0.15 M NaCl and 500 μl of glucose assay reagent (10, 12). Glucose release was measured at room temperature (ca. 22°) 30 min. after starting the reaction. See the text for further details.

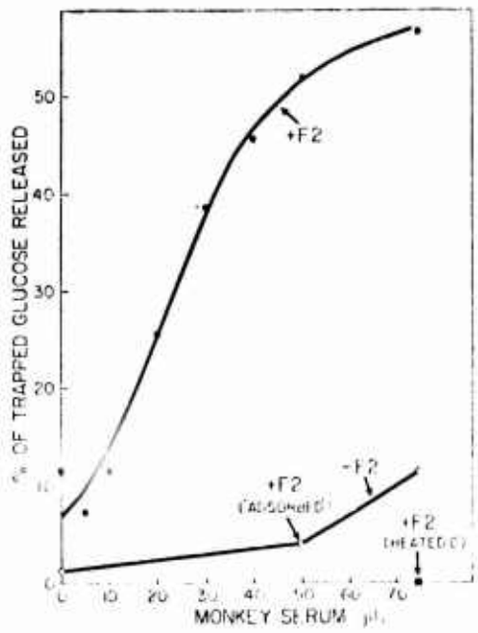

Fig. 11. The interactions of infected-monkey serum with liposomes. The assay procedure was the same as described in the legend of Fig. 10, except that the quantity of monkey serum was varied as shown on the abscissa. The reaction volume was kept constant (1.0 ml) by adjusting the amount of 0.15 M NaCl. Two liposome preparations were used which either contained F2 (●--●) or, as a control, lacked it (O--O). "Heated C" refers to guinea pig serum which had previously been heated at 56° for 60 min. (■). In one case, monkey serum was used which had been "absorbed" twice at 20° with 4 mg of a homogenized suspension of adult worms (▭). The worms were removed following each adsorption by centrifuging at 27,000 g for 10 min.

and human saliva A_1 and A_2 substances observed in quantitative hemagglutination inhibition (HI) assays of homologous and heterologous substances.

Materials and Methods

The source and preparation of the purified hog A substance and the four human antisera to this substance have been described. Human A substances were isolated and purified from the individual salivas of donors having A_1, A_2, A_1B and A_2B blood types by the method recommended by Kabat (26). Antisera to the A_1 and A_2 substances were prepared in selected male volunteers by subcutaneous injection of 0.5 ml of the appropriate solution containing 1 mg substance/ml sterile saline on two successive days. Fifteen days after the second inoculation, blood was drawn from each donor for serum.

The hemagglutinating $dose_{50}$ (HD_{50}) units of anti-A activity of one each of the anti-hog A, the anti-human A_1 and anti-human A_2 sera were determined simultaneously by the probit-log assay method of Wilkie and Becker (27) using A1 test cells. Dilutions of each of the three antisera containing 6 HD_{50} antibody units were then used in simultaneous quantitative HI assays of the inhibitory strength of one of the blood group A substances; i.e., either hog A, human A_1, A_1B, A_2 or A_2B substance. The remaining substances were assayed on subsequent days with the same set of three sera of different specificities. The inhibition assay procedure was the same as published (28). The hemagglutination inhibitory $dose_{50}$ (HID_{50}) was interpolated from the assay curve obtained by plotting percentages of agglutination against the 2-fold concentrations of substance on probability-log paper. HID_{50} is defined as the micrograms of blood group substance required to inhibit 6 HD_{50} units of antibody activity from 97% to 50% agglutination.

Results and Discussion

The 50% endpoint anti-A titers ($1/HD_{50}$) of the pre- and post-immune sera in Table 20 accord with the findings of Kabat et al. (29) that A_2 substance is less immunogenic in humans than are hog A and human saliva A_1 substances. The results in Figure 13 and Table 21 obtained with an anti-hog A, anti-human A_1 and an anti-human A_2 serum summarize the observations made in this investigation. The inhibition assay curves produced by this set of antisera in assays of human saliva A_1B and A_2B substances were similar to those depicted in the figure for A_1 and A_2 substances, respectively. With each of the Group A substances, assay curves were linear and parallel. The differences in position of the assay curves in Figure 13 were highly significant ($p = >0.01$).

The HID_{50}'s listed in Table 21 show that hog A substance was no more effective in inhibiting its homologous than the heterologous anti-human A sera. The respective amounts of human saliva A_1 and A_1B substances inhibiting anti-human A and anti-human A sera were identical,

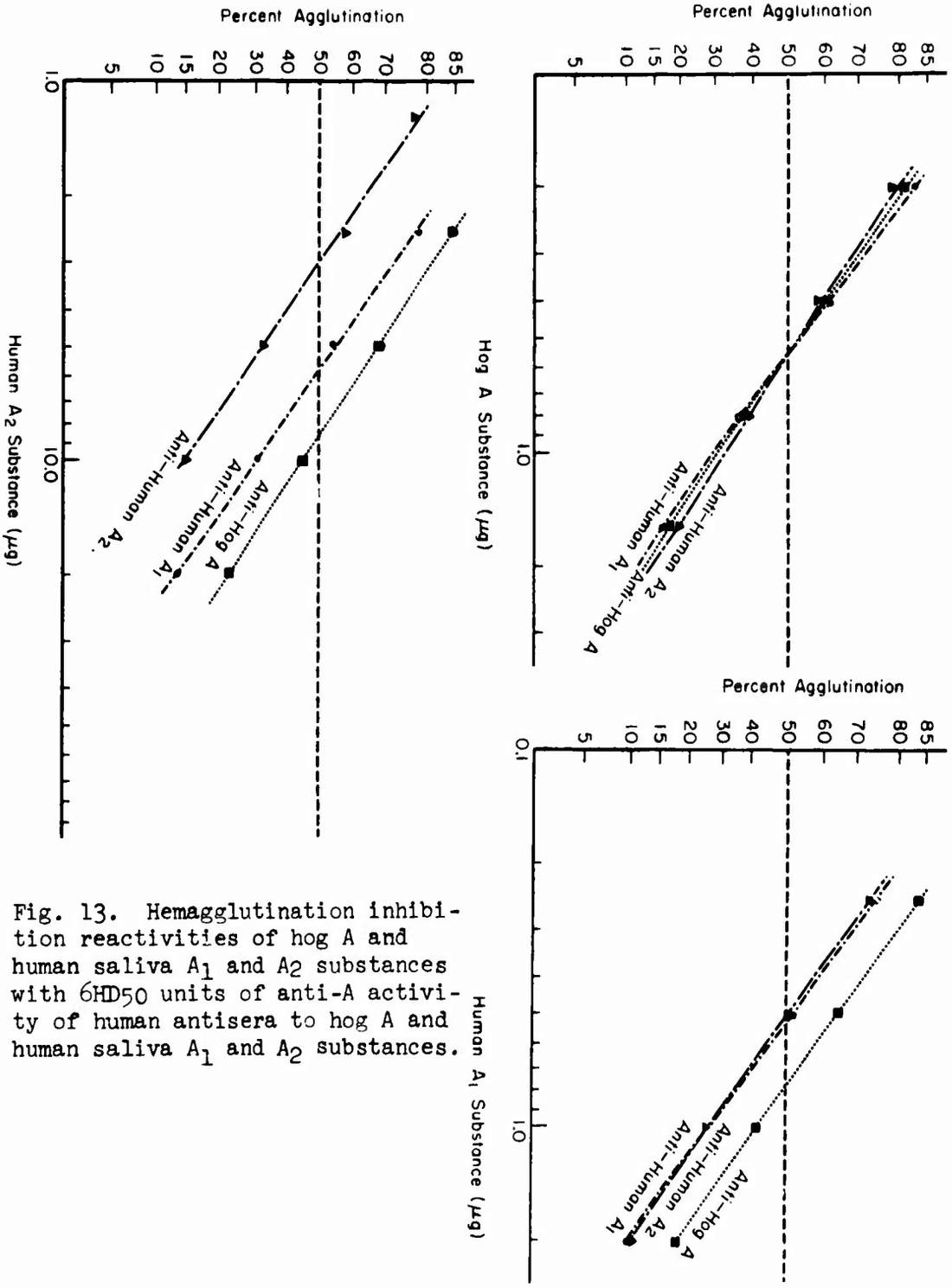

Fig. 13. Hemagglutination inhibition reactivities of hog A and human saliva A_1 and A_2 substances with 6HD50 units of anti-A activity of human antisera to hog A and human saliva A_1 and A_2 substances.

Table 21

HID_{50}'s Obtained in Hemagglutination Inhibition Assays of Blood Group A Substances with Immune Antisera and A_1 Test Cells

Blood Group Substance	Micrograms of Blood Group Substances Inhibiting Hemagglutinating Activity of Antisera		
		Human sera	
	Anti-hog A	Anti-human A_1	Anti-human A_2
A_1 cells and 6 HD_{50} antibody units	μg	μg	μg
Hog A	0.52	0.52	0.52
Human A_1	0.77	0.52	0.52
Human A_1B	0.24	0.15	0.15
Human A_2	8.60	5.60	2.90
Human A_2B	590.00	476.00	223.00

however, approximately 1 1/2 times more of these materials were required to inhibit the activity of the anti-hog A serum. The HID_{50}'s for human saliva A_2 and A_2B substances indicate that twice as much of these preparations were involved in the inhibition of anti-human A_1, and three times more in the inhibition of anti-hog A serum, than was concerned in the inhibition of homologous serum activity. The much weaker capacity of A_2B saliva substance per unit weight to inhibit hemagglutination of A_1 cells is apparent from the inordinately large quantity needed for inhibition. The fact that A_2 and A_2B substances were much more effective in inhibiting hemagglutination of four homologous anti-A_2 sera than of four anti-A_1 sera shows that antibodies which are elicited in humans by immunization with human saliva A_1 and A_2 substances do not possess identical specifications for the inhibitor A_2 substances. This interpretation is made in accordance with the hapten inhibiting findings of Landsteiner and van der Scheer (30) that the closer the complementary configuration of the inhibitor hapetn to the homologous antibody the less hapten required for inhibition of antibody activity. Additional inferential evidence on a structural difference in the saliva A_1 and A_2 substances is to be obtained from kinetic studies of the rates of dissociation of homologous and heterologous antibodies from the A_1 and A_2 substances.

13. Filtration of microaggregates formed in vivo

The extent of microembolization which occurs during autotransfusion is unknown, however, small microemboli formed during shock (31) and extracorporeal circulation (32,33) cause alteration in structure and function of the lungs and other organs. Although these findings suggest that the microemboli are primarily platelet aggregates, reliable quantitation and characterization of the particulate matter removed during in vivo filtration has been difficult with existing microscopic, filtration (34) and ultrasonic (35) technics. Our previous studies utilizing the Model T particle size analyzer have shown that platelets contribute to the formation of two types of aggregates; those composed of neutrophils and platelets which develop in stored blood (36) and those consisting of platelets which form in vivo by hemostasis (37) or by injection of ADP (38). In the present study, the filtration characteristics of these two types of microaggregates are compared.

Matierals and Methods

In all studies, samples of blood were collected in plastic syringes and 3 ml were passed through Dacron wool and small 120 μ and 40 μ pore mesh filters constructed in the barrels of plastic syringes. Induction of platelet aggregates (PA) by extravasation of blood was accomplished by severing the right iliac artery of ether-anesthetized Wistar rats after injection of heparin and allowing the blood to collect in the peritoneal cavity. Size distribution analyses were made 60 seconds after arteriotomy (30 seconds for collection in the peritoneum and 30 seconds for filtration). These measurements were compared to those of unfiltered blood collected in the same manner for 30 seconds and held in a plastic syringe for an additional 30 seconds. PA were also induced in vivo by injection of ADP as previously described (38). Thirty seconds after injection, 4 ml of inferior vena caval blood was withdrawn and size distribution analyses performed before and after filtration through the various filters. PA were induced in vitro by drawing blood from the inferior vena cava of rats into a syringe containing heparin and ADP. Twenty one day old human blood donors were the source of the microaggregates (MA) of stored blood.

Results

Filtration of particles of known size: The effective pore size of the filter was determined by measuring the size distribution of a saline suspension a mixture of corn (75-80 μ), pecan (45-50μ), lycopodium (27-28 μ) and mulberry (12-14 μ) pollens before and after passage through each of the filters (Figure 14). The size distribution of the mixed pollens after passage through the 210 μ pore filter in Figure 1 was the same as that of the unfiltered suspension. The 40 μ pore mesh removed all the pecan (45-50 μ) and corn (75-80 μ) pollen, reduced the lycopodium (27-28 μ) by over one half, but did not remove the smaller particles. The Dacron wool completely removed all particles larger than 16 μ and

reduced the number of 13 μ mulberry pollen. The correlation of the known size of these particles with that detected by the instrument demonstrates the validity of the size distribution measurement.

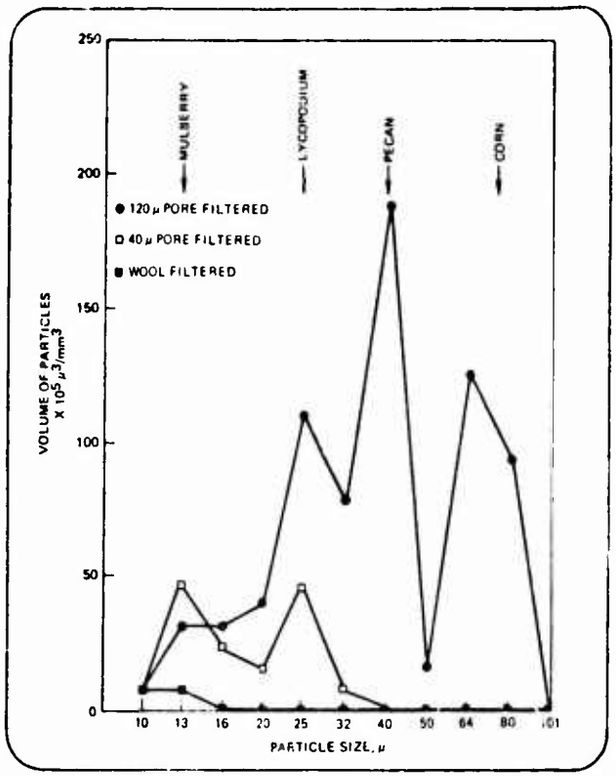

Fig. 14. Effects of filtration of particles of known size. Size distribution of volume of particles in μ^3/mm^3 (ordinate) plotted against particle size in μ (abscissa) after filtration with 120μ and 40μ pore mesh and Dacron wool filters.

Filtration of microaggregates in stored blood: The volume size distribution of MA of stored human blood before and after passage through use of the filters is plotted in Figure 15. Although the 120 μ pore mesh was relatively ineffective in reducing the volume of particles in the blood, there was a significant removal of particles 40 μ and larger in size which is in contrast to the inability of this mesh to filter pecan (45-50 μ) and corn 75-80 μ) pollens (Fig. 14). The 40 μ pore filter completely removed particles 50 μ and larger and reduced the volume of particles 40 μ and smaller. Dacron wool removed all particles larger than 13 μ.

Filtration of platelet aggregates induced by extravasation of blood by ADP: The size distribution of the PA in unfiltered extravasated rat blood was identical to that after filtration with the 120 μ pore mesh

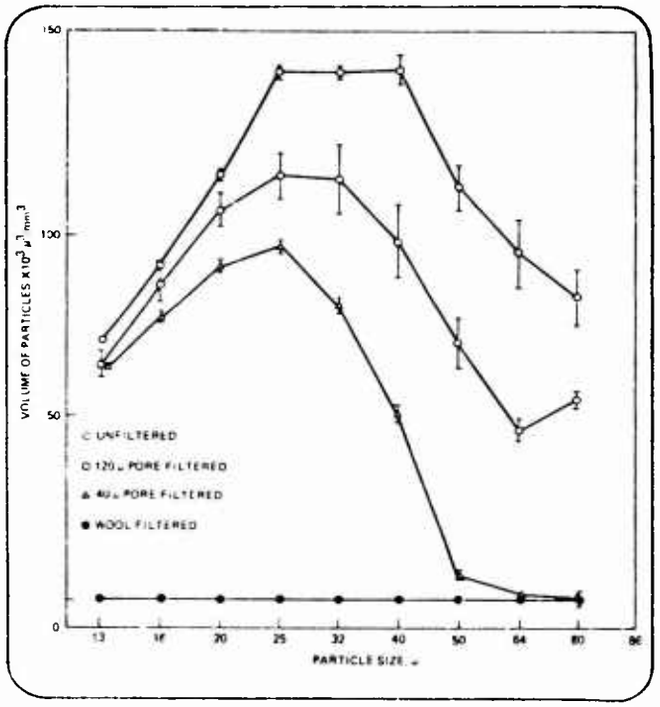

Fig.15. Effects of filtration of microaggregates in stored blood. Size distribution of mean volume of particles (±S.E.) plotted against particle size in μ before and after filtration of aliquots of a unit of stored blood through 120μ and 40μ pore mesh and Dacron wool filters (n=8).

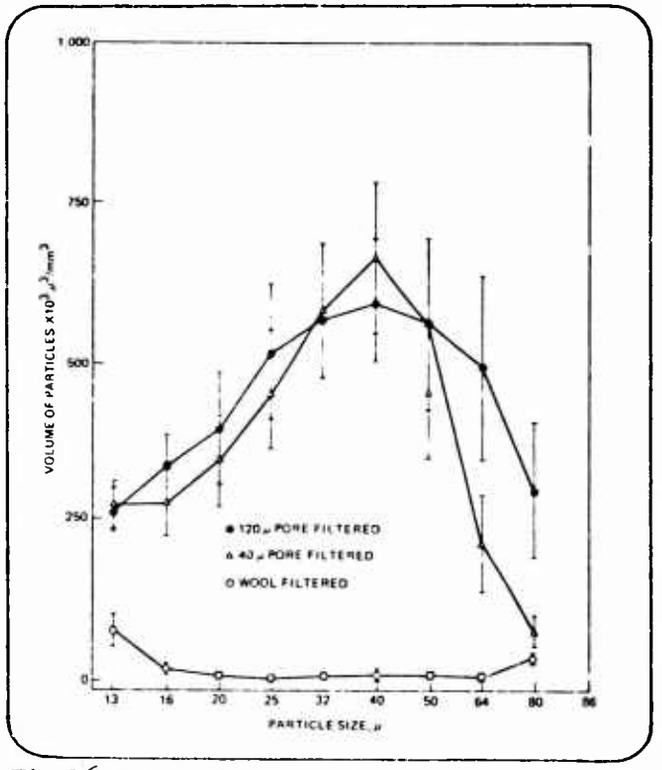

Fig.16. Effects of filtration of platelet aggregates formed during hemostasis. Size distribution of mean volume of particles (±S.E.) plotted against particle size in μ after filtration of extravasated blood with 120μ and 40μ pore mesh and Dacron wool filters (n=10).

shown in Figure 16. In striking contrast to the ability of the 40 μ mesh to remove 45-50 μ pecan pollen in Figure 14 and the same size MA of stored blood in Figure 15, a considerable number of PA 50-65 μ in size remained in the blood after passage through this filter (Fig. 16). However, the Dacron wool filtered virtually all particle 13 μ and larger.

Although PA formed in vivo after ADP injection are more stable in vitro (38) and hence are presumed more tightly bound than those formed during extravasation (37), both types of in vivo aggregate larger than 40 μ were found to be present after passage through the 40 μ pore mesh filter. In fact, there was no significant difference in the volume of these two types of PA before and after filtration through the two pore mesh filters, whereas a significant volume of the MA of stored blood of the same size was retained by these filters. The filtration characteristics of PA induced in vitro by ADP were similar to those of the in vivo aggregates.

Filtration of platelets and leukocytes: Platelet and leukocyte counts of extravasated rat blood before and after filtration in Table 22 show that there was no significant change in leukocyte counts after passage through the three filters, nor in platelet counts after the two mesh filtrations. However, Dacron wool markedly reduced the platelet count. Similar results were obtained with blood drawn from ADP-treated rats. Platelet concentrations of heparinized rat blood containing no PA were markedly reduced after Dacron wool filtration, however, there was no significant difference in platelet concentrations of rat blood drawn into ACD after similar filtration.

Discussion

The results of the present study suggest that the two major sources of microemboli during heart-lung bypass are the MA infused with stored blood and the PA infused with extravasated blood collected in the cardiotomy return line. The effectiveness of Dacron wool in removing both types of aggregates is determined by the adhesiveness of these particles. Thus, platelets in blood drawn into heparin and known to be more adhesive (39) are trapped on Dacron wool while those drawn into ACD are not. The fact that MA of stored blood larger than 40 μ were completely removed by the 40 μ pore mesh surface filter (Fig. 15) while the PA of equal size apparently passed through this filter (Fig. 16) shows that the effectiveness of mesh filters is not only dependent on pore size but also on the stability of the particles being filtered. Because platelets are more weakly bound in aggregation, the large PA break up at the surface of and reform after passage through the 440 μ pore mesh, whereas the more strongly bound MA of stored blood are trapped by this filter. Although Dacron wool is most effective in removing PA from blood, it also removes aggregated platelets. Thus, in considering this filter in autotransfusion, the possible harmful effect of PA embolization must be weighed against the thrombocytopenia which may result.

Table 22

Platelet and Leukocyte Counts in Extravasated Rat Blood Before and After Filtration*

Filter	Platelets/mm^3		Leukocytes/mm^3	
	Before Filter	After Filter	Before Filter	After Filter
120 μ mesh	1,074,000 ± 103,000	1,053,000 ±89,000	10,210 ± 700	9,020 ± 630
40 μ mesh	676,000 ±75,000	643,000 ±36,000	9,440 ± 780	10,160 ±1,040
Dacron wool	784,000 ±46,000	175,000 ± 103,000	10,680 ± 870	9,350 ± 780

*Each value represents the mean ± standard error of eight experiments.

14. Immune mechanisms in Schistosoma masnoni infection of the inbred rat

Objective: These studies were designed to gain insight into the mechanisms of immunity to Schistosoma mansoni in the rat.

Description: Despite the public health and military importance of schistosomiasis, information on host defense mechanisms in the disease is limited. Controversy exists regarding the role of the immune system in both the primary and chronic or reinfection aspects of the disease. In many ways the inbred rat represents an ideal animal for experimentation as it is highly resistant to schistosomes and allows for critical transfer experiments. This report expands and defines previous findings as to conditions for reproducible exposure and the kinetics of development of host immune mechanisms during the initial exposure to Schistosoma.

Fischer rats were exposed to Schistosoma mansoni cercariae and the intensity of infection assessed by perfusion and direct counting of the worm burden. The effect of animal age at exposure upon the subsequent worm burden is summarized in Figure 17. Increasing resistance with age was found. The effect of exposure magnitude upon subsequent burden is summarized in Figure 18. Maximum resistance was established by the exposure of animals to a minimum of 250 cercariae. All animals in subsequent experiments were 4 1/2 weeks of age at exposure, and were exposed to 1000 cercariae and sacrificed 3 1/2 weeks after exposure.

EFFECT OF ANIMAL AGE AT EXPOSURE UPON
SUBSEQUENT SCHISTOSOME WORM BURDEN

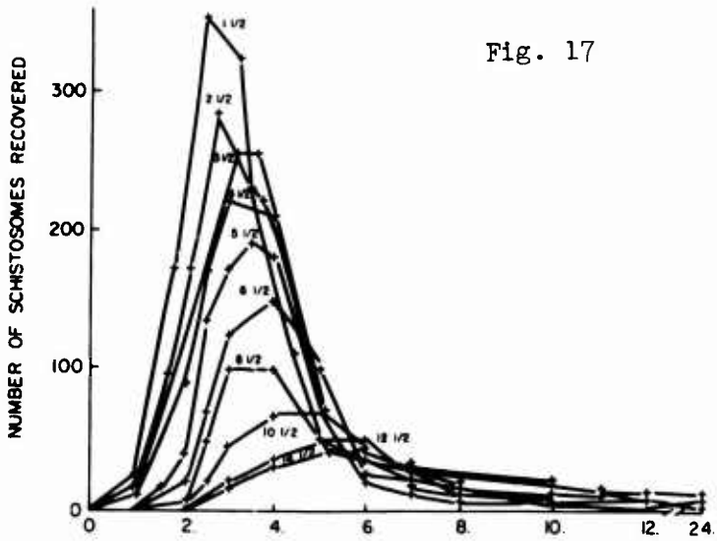

Fig. 17

EFFECT OF EXPOSURE MAGNITUDE UPON
SUBSEQUENT SCHISTOSOME WORM BURDEN

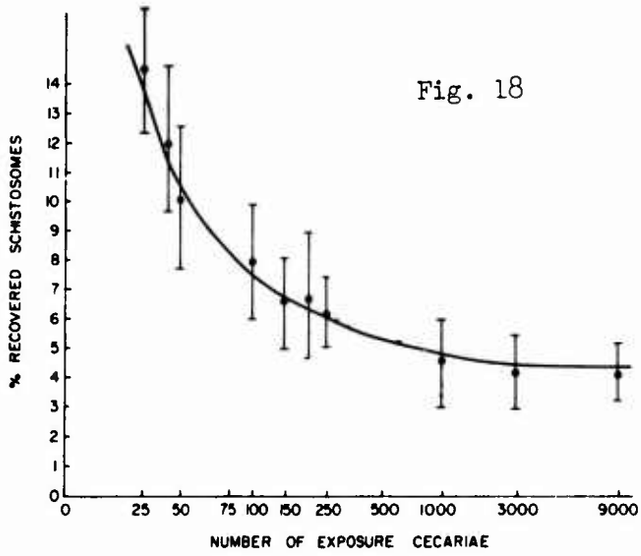

Fig. 18

Studies on the mechanisms of host defense were next performed. Cells from animals previously exposed to schistosomes were injected into recipient animals which were subsequently exposed to schistosome cercariae. Figure 19 demonstrates that resistance could be specifically transferred with the use of cells from previously infected animals. Maximum effect was seen using cells from animals exposed to cercariae 3 weeks earlier. Additional studies on subpopulations of cells showed that the cells responsible for resistance were probably lymphoid in morphology, and thymic derived in origin. These conclusions are based on studies using cells of various origins (bone marrow, thymus, lymph node, spleen and peritoneal exudate) which were characterized by their adherence properties and susceptibility to antibody and complement using antibodies with defined activity against specific cell types. The results of many experiments are briefly summarized in Table 23.

Table 23

Cell Source and Treatment	Mean Decrease in No. of Worms Recovered (%)
0	66*
Removal of adherent cells	57*
Removal of non-adherent cells	12
Removal of thymic lymphocytes	17
Removal of bone marrow lymphocytes	48*

*Significant reductions at 0.05 confidence level.

These results indicate that the cells responsible for protection are thymus derived lymphocytes.

Similar studies on protection were accomplished using serum from exposed animals. Figure 20 shows that serum could successfully protect animals when it was taken 7 weeks after exposure. Of interest was the observation that earlier serum could actually enhance the worm survival. In addition this early serum would block the expected protection afforded by cells when transferred simultansouly with those cells.

Preliminary studies using multiple exposures indicate that upon re-exposure the serum mediated responses are most important. Work continues in this area and is augmented by antigen fraction and in vitro cell culture work.

15. Immune mechanisms of endogenous virus release in mice

There is a growing realization that endogenous (possibly leukemogenic) viruses are affected by and affect immunogenic factors. We have been studying some of these reactions in collaboration with the Harvard Medical School and the Tufts University School of Medicine.

The results may be summarized as follows: When mice are challenged with an ongoing immunogenic disease, i.e. graft vs host reaction induced by the injection of parental cells into F, recipients - several interesting

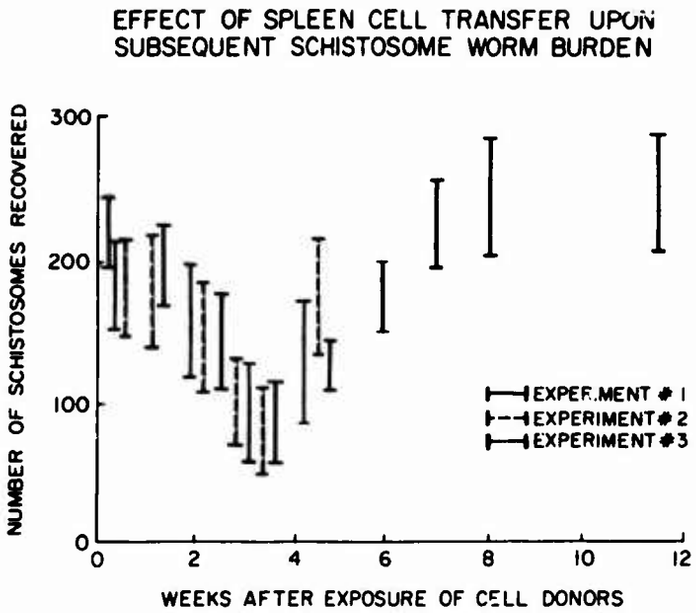
EFFECT OF SPLEEN CELL TRANSFER UPON
SUBSEQUENT SCHISTOSOME WORM BURDEN
NUMBER OF SCHISTOSOMES RECOVERED
300
200
100
0
0
2
4
6
8
10
12
WEEKS AFTER EXPOSURE OF CELL DONORS
EXPERIMENT # 1
EXPERIMENT # 2
EXPERIMENT # 3

Fig. 19

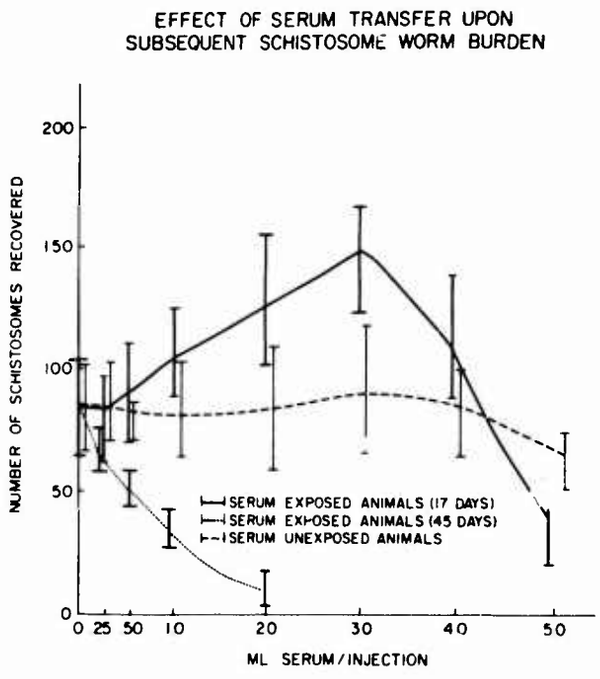
EFFECT OF SERUM TRANSFER UPON
SUBSEQUENT SCHISTOSOME WORM BURDEN
NUMBER OF SCHISTOSOMES RECOVERED
200
150
100
50
0
0 25 50 10
20
30
40
50
ML SERUM / INJECTION
SERUM EXPOSED ANIMALS (17 DAYS)
SERUM EXPOSED ANIMALS (45 DAYS)
SERUM UNEXPOSED ANIMALS

Fig. 20

phenomena are observed. First one finds the liberation of virus to occur as early as one week after challenge. Table 24 demonstrates this phenomenon. It also shows the second major point of this study. Concomitant with the release of virus, the lymphocytes display altered in vitro response to thymic lymphocyte mitogens such as phytohemagglutination (PHA) and allogeneic cells in mixed lymphocyte culture (MLC). This alteration of cellular reactivity is further defined in Table 25. Here one sees that the cellular response is complex, increasing early and decreasing later in the course of disease. Finally a variety of stimuli have been used to stimulate virus release. These studies are summarized in Table 26. Briefly, in vivo and in vitro allogeneic stimulation as well as the drug Iododeoxyuridine release virus. Other nonspecific agents do not.

These studies are potentially of great importance since the release of virus may play a role in altering immunity to infection and neoplasia. In addition, it is known that infectious virus can suppress cellular reactivity; for example measles vaccination leads to a transient tuberculin anergy.

Table 24

Relationship of In Vitro Proliferation and Activation of Leukemia Virus

	Stimulation Source					
	0		PHA		MLC[a]	
Cell	CPM[b]	Virus titre[c]	CPM	Virus titre	CPM	Virus titre
GUHR Animal	1040	1.2	9,700	1.2	3,800	-
Normal	980	0	29,500	0	10,600	2.2

[a]Mixed lymphocyte culture utilizing addition of irradiated normal C57BL/6 spleen cells.

[b]Mean counts per minute, incorporated tritiated thymidine.

[c]Mean virus titre--TCID50/ml (50% tissue culture infectious doses/ml).

16. Effects of thymic products on the immune response in vitro

There is an increasing awareness of the importance of the thymus in modulating cellular immunity and potentiating humoral immunity through the production of soluble mediators. We have studied two such mediators, the immunosuppressive alpha globulins. They are derived from the serum or thymus of animals and possess potent immunosuppressive capabilities in vivo. Our studies were conducted under in vitro conditions.

Table 25

In Vitro Cellular Reactivity During Course of GvH Reaction

Stimulant	Time in weeks 1[a]	8[b]
PHA	+44[c]	-64
CON-A	+24	-57
SRBC	+97	-70
C57BL/6mito[d]	+133	-87

[a] 1 week following single injection of parental cells.

[b] 8 weeks following first injection of parental cells.

[c] Results expressed as percent change of response of 6VHR animal compared to normal F_1 animal.

[d] Mitomycin treated allogeneic cells ($N \geq 9$).

Table 26

Activation of Leukemia Virus from $CIAF_1$ Spleen Cells Cultured in vitro

Stimulus	Number tested/ positive	% positive
GvH	26/31	84
MLC	9/16	56
IUDR	5/12	42
0	4/34	12
PHA	1/24	4
CON-A	1/12	8
SRBC	0/19	0

Studies of the effect on afferent arc of the immune response were first conducted. When these alpha globulins were added to proliferating cells, they possessed the ability to both augment and suppress that proliferative response (Fig. 21). The analysis of this in vitro effect showed it to be nonspecific since response to nonspecific mitogens such as PHA or specific alloantigens in mixed culture or sheep red blood cells (following in vivo exposure) were similarly effected. That the most profound effects were on DNA synthesis was demonstrated when various synthetic pathways were analyzed (Fig. 22). Furthermore, the stimulatory and suppressive effects were functionally separable on the basis of in vitro reactivity when added early and in low concentrations to culture. Dominant stimulatory effects were observed (Table 27). Later addition and higher dosage showed suppressive effects.

Because of the intimate relationship between cell proliferation and the subsequent development of effector cells the cells resulting from stimulation were next assayed for their ability to destroy target cells which were identical to those used to stimulate the MLC. Figure 23 demonstrates an excellent correlation between suppression of MLC proliferation and subsequent effector capability.

After sensitization, lymphocytes are known to develop specific response characteristics defined by in vitro assays. One such ability is the destruction of targets (see above) and another is the ability to release mediators of known biologic activity; for example, the ability to inhibit the migration of macrophages (MIF). The effect of immunosuppressive alpha-globulin on pre-existing effector capability was next tested. Table 28 demonstrates that a wide range of concentrations of the immunosuppressive alpha-globulins had no demonstrable effect on those effector capabilities.

These studies indicate that antigenic recognition and effector capability are not influenced whereas the proliferative lymphocyte response is either augmented or suppressed, depending upon the concentration of alpha globulin. The significance of the metabolic control on DNA synthesis is of potential importance in augmenting an immune response. Since similar thymic factors are found in man, the possibility of their application in specific immunization schema are becoming increasingly manifest.

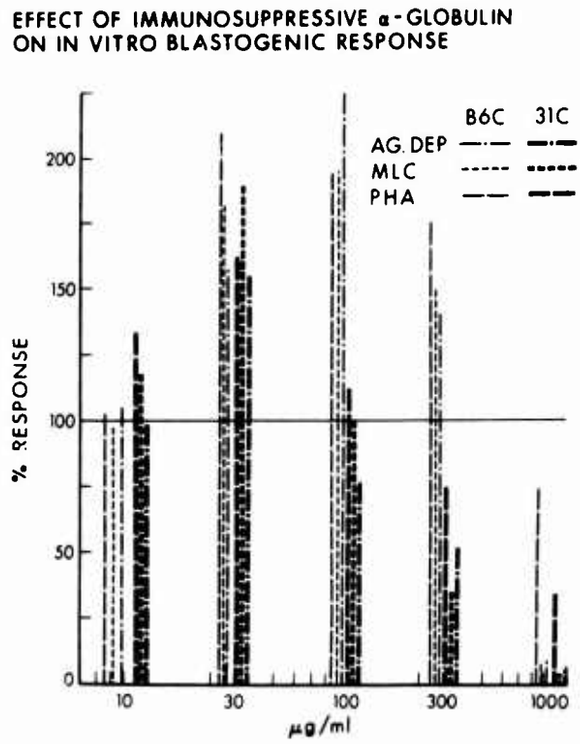

Fig. 21. Effect of immunosuppressive alpha globulins upon in vitro blastogenic responses. Various concentrations of thymic (T31C) and bovine (B6C) derived alpha globulins were added to cultures of C57Bl/6 spleen cells containing either medium 15µg/ml PPD (Ag. Dep.), 1.2µg/ml PHA, or BALB/c spleen cells (MLC) the concentration of each fraction is plotted against the resulting degree of isotope incorporation (determined by ^{3}H-thymidine) as a percentage of the untreated control. Note a dose-dependent stimulatory and suppressive effect by both alpha globulins on all responses. Mean cpm of unstimulated cultures: 1480 ± 315 at 48 hours (PHA), 1690 ± 427 at 72 hours (MLC and Ag. Dep.). No significant direct effect was observed upon spontaneous isotope incorporation of unstimulated cultures by the addition of the fractions.

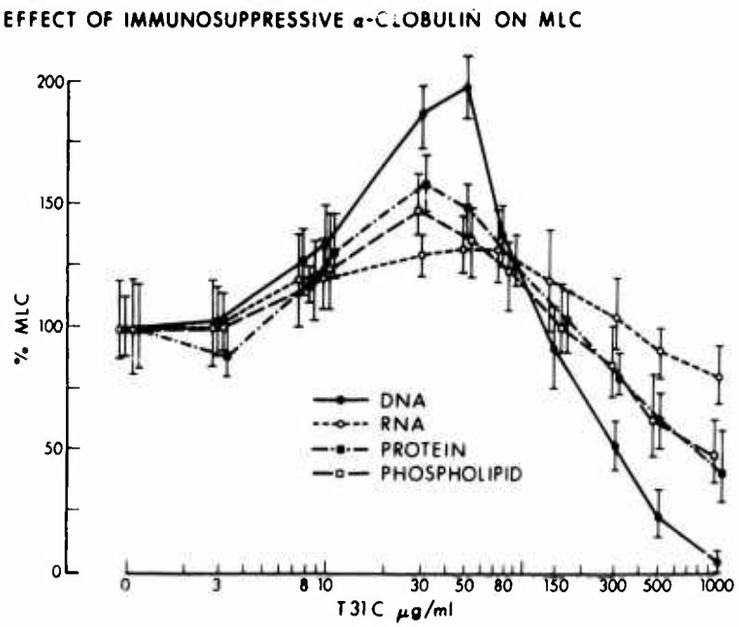

Fig. 22. Effect of immunosuppressive alpha globulin on MLC. 75×10^6 C57Bl/6 + 75×10^6 BALB/c spleen cells were incubated in vitro in the presence of various concentrations of immunosuppressive alpha globulin. After 68 hours of incubation ^{3}H-thymidine, ^{3}H-5-uridine ^{14}C-DL-Leucine, or ^{3}H-myoinositol were added to replicate cultures which were terminated at 72 hours. The concentration of fraction is plotted against the resulting degree of isotope incorporation shown as a percentage of the untreated control. Each point represents the mean and 95% confidence interval of replicates. Note the dose-dependent stimulation and suppression of all four synthetic rates; however, the most profound effects are upon DNA synthesis.

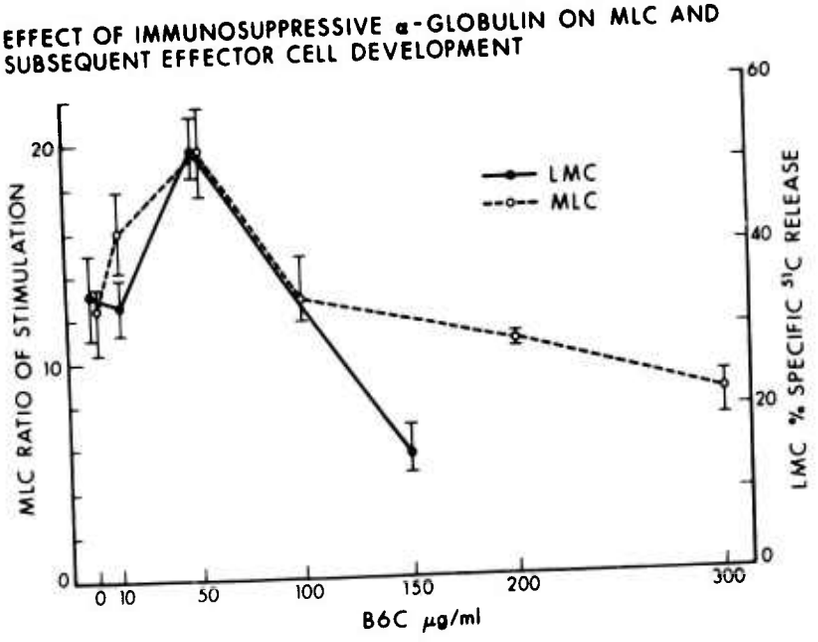

Fig. 23. Effect of immunosuppressive alpha globulin upon MLC and subsequent effector cell development. Various concentrations of immunosuppressive alpha globulin were added to MLC upon initiation (BALB/c + C57Bl/6 irradiated spleen cells) and the resultant cell population tested for MLC activity by ^{3}H-thymidine incorporation and LMC by ^{51}Cr releast. The ratio of stimulation and percent specific chromium release is plotted against concentrations of immunosuppressive (B6C) alpha globulins added to culture. Note the dose-dependent correlation of stimulation and suppressive effects upon both phenomena. MLC cpm mixture, 34,300 ± 4500; C57Bl/6 irrad, 220 ± 150; BALB/c, 2650 ± 310. LMC cpm freeze-thaw, 3920 ± 36 cpm, spontaneous release, 1283 ± 71 cpm.

Table 27

In Vitro Effectiveness of Immunosuppressive Alpha Globulin Varies with Time of Addition to Cultures

Fraction		Time of Addition to Mixed Lymphocyte Cultures[a]				
		0[b]	12	24	48	72
B6C	30μg	214 ± 24[c]	149 ± 11	109 ± 12	102 ± 6	98 ± 7
B6C	1000μg	4 ± 2	4 ± 1	16 ± 4	69 ± 8	90 ± 6
T31C	30μG	163 ± 25	128 ± 14	93 ± 12	106 ± 12	112 ± 14
T31C	1000μg	3 ± 1	7 ± 3	28 ± 11	79 ± 10	81 ± 9

[a] C57B1/6 + BALB/c

[b] Hours after culture initiation.

[c] % of control MLC ± S.D.

Control MLC = 32,400 ± 4800, unmixed cells = 2010 ± 730 cpm ^{3}H-thymidine incorporation at 72 - 76 hours.

Table 28

Effect of Immunosuppressive Alpha Globulin on Pre-Existing Effector Capability

Assay	Fraction	μg/ml Fraction Added to Assay: 0	30	100	300	1000	3000
LMC[a]	B6C	2709 ± 115[b]	2592 ± 201	2609 ± 106	2463 ± 8	2590 ± 175	2391 ± 69
	T31C		2801 ± 6	2558 ± 63	2668 ± 156	2815 ± 46	2493 ± 106
MIF[c]	B6C	60,66[d]	60,68	59,63	65,69	64,68	56,62
	T31C		57,61	58,66	62,68	58,64	56,60

[a] Lymphocyte Mediated Cytotoxicity

[b] cpm ^{51}Cr-release at 4 hours. (Freeze-Thaw cpm = 4651 ± 35; spontaneous cpm = 1265 ± 25).

[c] Macrophage Migration Inhibition Factor

[d] % inhibition of migration (2 experiments): cells from guinea pigs sensitized to complete Freund's adjuvant. 15μg/ml Weybridge PPD in assay, read at 30 hours. Control migration inhibition: 2.6 ± 0.7%, without PPD; 1.9 ± 1.2%, unsensitized cells + PPD.

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 070 Antigen-antibody reactions in vivo and in vitro

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6453	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71P	01	074
b. CONTRIBUTING				
c. CONTRIBUTING XXXXXX	CARDS 114F			

11. TITLE (Precede with security classification code)

(U) Molecular Basis of Biological Regulation and Chemotherapeutic Drug Pharmacology

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	6	128
	CURRENT 75	6	128

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with security classification code)

(U) R-factors; (U) Bacterial Drug Resistance; (U) Narcotics; (U) Antimalarials

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security classification code.)

23. (U) The scientific objective is to study the molecular pharmacology of elimination of R-factor-mediated bacterial multiresistance to chemotherapeutic drugs as well as of antimalarials, antibiotics and narcotics. The ultimate objective is the development of clinical anti-R-factor drugs **for use in treating drug-resistant diarrheas, an important military problem.**

24. (U) The approaches are fundamental laboratory experimentation using advanced microbiological, biochemical and biophysical methods.

25. (U) 73 07 - 74 06 Potencies of DNA-complexing compounds to eliminate resistance determinants from a bacterial R-factor correlated with a relative measure of stoichiometry of binding to DNA (methyl green displacement). One nitroacridine, at 2 micrograms/ml, sterilized ampicillin-resistant (R-factor determined) S. typhimurium in combination with ampicillin. Biochemical observations indicate that it is possible to study R-factor replicative DNA synthesis in cell-free systems. The DNA-binding antibiotic, distamycin A, which interferes with the transmission of R-factors among bacteria binds preferentially to poly dA and single-stranded DNA of phage ϕX174. The dissimilation of bacterial ribosomes and ribosomal RNAs under the influence of primaquine has been investigated in vivo and in vitro. Kinetics and endpoints of association and dissociation of E. coli ribosomes have been measured in vitro by light-scattering techniques. The role of the three "initiation factors" as well as the effects of streptomycin and one experimental narcotic on ribosomal kinetics have been studied. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 074 Molecular basis of biological regulation and chemotherapeutic drug pharmacology

Investigators.

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Description.

Molecular biological and pharmacological as well as microbiological research studies with three objectives. (1) To provide fundamental information on the replication and elimination of bacterial plasmids (R-factors) as a basis for developing anti-R-factor compounds for clinical use in man; (2) To elucidate modes of action of major antimalarials and antibiotics, and (3) To gain a better understanding of the molecular pharmacology of narcotic drug action. Objective (2) is being deemphasized and Objective (3) has been phased out. Progress reported here has been curtailed by an extensive shut-down of laboratories owing to rebuilding.

Progress and Results.

Elimination of resistance determinants from R-factors. Experiments have been continued on the elimination of resistance determinants from an R-factor in Salmonella typhimurium. This organism has been selected for both its medical importance and the fact that elimination of resistance determinants works well in Salmonellae, as contrasted, for example, to Escherichia coli. Tables I and II summarize results of elimination experiments which were carried out by growing cultures of the test organism overnight in the presence of the test compounds, followed by differential plating and colony counting. Three facts emerge from an examination of the Tables. (1) Most compounds (with the exception of nalidixic acid and coumadine) are known to form complexes with DNA; nalidixic acid inhibits DNA biosynthesis by a different and still unknown mechanism. (2) Elimination of resistance determinants was selective, i.e. the test compounds produced genetic segregation of individual resistance markers. "Curing" en bloc was rarely seen. (3) The eliminating potencies of the studied compounds (Table II) fell into a systematic hierarchic sequence which showed only a few inversions which may not all be statistically significant. Conclusive statistics are difficult to obtain owing to the tendency of S. typhimurium to produce some spontaneous segregation of genetic markers from plasmids. The activity sequence evident in Table II

TABLE I

Per Cents Elimination of Resistance Determinants in S. typhimurium

Compounds	Kana	Chloramph	Strepto	Ampicill
6.25×10^{-6} M				
Nalidixic Acid	70	56	60	64
2.5×10^{-5} M				
Nitroacridine II	99	96	96	97
Daunomycin	86	80	77	81
Proflavine	86	79	64	68
5×10^{-5} M				
Nitroakridin 3582	82	69	70	69
8×10^{-4} M				
Coumadine	70	56	60	64

TABLE II

Per Cents Elimination of Resistance Determinants in S. typhimurium

Compounds at 10^{-4} M	Kana	Chloramph	Strepto	Ampicill
Ethidium	96	82	84	82
Miracil D	91	82	81	82
Quinacrine	92	79	81	71
Propidium	89	78	71	71
Tilorone	85	80	74	78*
p-Rosaniline	87	71	73	66
Acridine Orange	85	71	69	68
Berberine	83	68	70	62
Quinine	81	60	66	57
Spermine	81	56*	71	58
Chlorpromazine	78	63	67	72*
Hoechst 33258	72	64	64	67*
Chloroquine	80*	58	52	53
Methylene Blue	68	50	48	41

*Significant inversions in activity sequence

is a biological consequence of the DNA-binding capacity of the tested compounds. A diagram in which the elimination frequencies were plotted as a function of the endpoints of displacement of methyl green from DNA (a relative measure of stoichiometry of drug binding to DNA) revealed a direct correlation between the two parameters, i.e. the elimination potencies of the studied compounds are a function of their abilities to form complexes with (R-factor) DNA. Most of the tested compounds bind to DNA by intercalation (except nalidixic acid, coumadine, p-rosaniline and spermine). Intercalation into closed circular supercoiled DNA (R-factor DNAs have this conformation) causes progressive unwinding of the constrained molecule and, at higher drug concentrations, upwinding into supercoils of opposite handedness. When the stoichiometries of binding (methyl green displacement) were plotted as a function of the unwinding stoichiometries for a series of compounds for which both sets of numbers are available, again a linear correlation was seen. It is assumed, therefore, that the elimination of resistance determinants from R-factors by DNA-intercalating drugs is a consequence of the binding of these compounds to R-factor DNA and of the resulting conformational changes in this DNA which incapacitate it from acting as a template for its own replication. Our results and their interpretations converge, therefore, on the conclusion that compounds which share the group property of the intercalation binding into DNA possesses, for this very reason, the group property of eliminating chemotherapeutic drug resistance determinants from bacterial R-factors. This was originally proposed by us as a working hypothesis in 1970 and appears by now to be an established theory which can guide the search for superior compounds which might restore drug sensitivity to multi-resistant Gram-negative pathogens, clinically. We have also continued combination experiments in which cultures of S. typhimurium, resistant to 2,500 µg/ml of ampicillin are sterilized by combinations of 60 µg/ml of this antibiotic plus concentrations of anti-R-factor compounds which, by themselves, do not inhibit bacterial growth. We reported such results last year for combinations of ampicillin with ethidium or quinacrine. This work has been successfully extended to include two nitroacridines and tilorone. The most active compound was Nitroakridin 3582 ("Entozon") which in combination with ampicillin sterilized test cultures at the low concentration of 5×10^{-6} M, i.e. 2 µg/ml.

In vitro biosynthesis of R-factor DNA. In very preliminary experiments, Escherichia coli RS-2 (from which the R-factor in the S. typhimurium experiments originates) was selectively extracted by a procedure which has been shown by others to yield a plasmid DNA as well as enzymes for its biosynthesis. The bacterial extracts incorporated radioactive deoxyribonucleoside triphosphates into a polymer presumed to be de novo synthesized R-factor DNA. This incorporation was inhibited by ethidium and was (in freshly prepared extracts) stimulated by added ribonucleoside triphosphates. These observations suggest that in vitro plasmid DNA synthesis has been accomplished. Next logical steps will be the identification of the template as well as of the biosynthetic product

obtained.

Distamycin A. This antibiotic has received wide scientific attention because of its unique binding to DNA; it also inhibits the transfer of R-factors between Gram-negative bacteria. Studies on the Distamycin-DNA complex have been carried out in this laboratory since 1970.

Spectrophotometric titrations of distamycin A with double-helical calf-thymus DNA progressively shifted the absorption maximum of the antibiotic to longer wavelengths as well as decreased and subsequently increased its absorption intensity. No red shift was observed upon dilution of free distamycin in solution. These spectral changes suggest decreasing intramolecular interactions between the antibiotic's chromophores rather than decreasing intermolecular interactions between distamycin molecules. No clear isosbestic point was observed. This indicates the existence of more than one spectrally distinct form of bound distamycin. Resolution by computer revealed two Gaussian contributions to the composite absorption spectrum of distamycin bound to low concentrations of DNA and a third component in the presence of higher DNA concentrations. Existence of three absorption components for bound distamycin may represent a manifestation of a slight heterogeneity of the three antibiotic chromophores resulting from different substitutions at the three N-methylpyrrole ring systems. This heterogeneity became progressively apparent with increasing concentrations of DNA as distamycin became more and more bound in the manner which reduced the intramolecular interactions between the antibiotic's chromophores. No stoichiometries and association constants can be derived from these observations; the results, nevertheless, demonstrate a progressive change in relative occupancy by distamycin of two classes of binding sites in DNA.

Studies of the interaction of distamycin with different DNAs have shown preferential binding of the antibiotic to A-T rich regions of DNA or to synthetic double-helical polymers devoid of guanine. Distamycin also binds selectively to single-stranded DNAs. The antibiotic's absorption was reduced and shifted to longer wavelengths by ϕX174 DNA, poly dA, poly dT, poly dG and poly dC; the shift was small with poly dC but large with poly dA and ϕX174 DNA. Poly dA induced a Cotton effect of large molecular amplitude in the optical rotatory dispersion spectrum of distamycin; a smaller effect was induced by poly dG, while poly dT and poly dC produced no such effect. ϕX174 DNA melted cooperatively with the antibiotic as did denatured DNA, while the complex of poly dG with distamycin partly dissociated with heat, whereas the complexes with poly dA, poly dT and poly dC remained intact. We infer that the selective interaction of distamycin with single-stranded DNAs may inhibit their template activity. Effects of the antibiotic on the RNA polymerase reaction primed by single-stranded DNAs are under investigation; any template specificity might correlate with our biophysical results above.

Influence of R-factor eliminating compounds on the conformation of closed circular DNA. The rationale of this study and preliminary results were reported last year. The closed-circular supercoiled DNA was isolated from bacteriophage PM-2; this is currently the most widely studied supercoiled DNA species. This work has been completed for publication. A new, interesting result is the fact that nalidixic acid has no influence on the conformation of supercoiled DNA. This adds to the sum of different lines of biophysical study which have led to the conclusion that nalidixic acid does not bind to DNA and produces its well-known inhibition of bacterial (but not mammalian) DNA biosynthesis by a mechanism which is not related to template toxicity. The work has now entered into its next phase in which the influence of R-factor eliminating compounds will be studied biophysically with an isolated R-factor DNA. Such DNA is being obtained from E. coli harboring the R-factor R6K whose replication is under relaxed control and, therefore, produces numerous copies per cell. This is important for yield as well as from the viewpoint of studying the conditions underlying relaxed replication. R-factors so replicated might be difficult to eliminate.

Continuation of studies on the mode of action of primaquine. We reported last year that primaquine, like chloroquine, elicits a disassembly of ribosomes and concomitantly induces a degradation of ribosomal RNA in B. megaterium. We now report that, after global RNA of B. megaterium was radioactively labeled with ^{14}C-uracil, exposure to primaquine resulted in a slow linear (with time) release of ^{14}C-uracil-labeled material from the cells into the medium: after 60 minutes at a primaquine concentration of 6×10^{-4} M, 20 per cent of the radioactive label was lost from cells; at 8×10^{-4} M, the loss was 30 per cent. Evidently ribosomes are disaggregated to structures of low sedimentation rate, but the RNA is not degraded to small (oligonucleotides and/or mononucleotides) molecules. This corroborates our previously reported zone centrifugation studies of extracts from primaquine-treated B. megaterium demonstrating that the majority of the breakdown products of ribosomes and of ribosomal RNA accumulated in the "lighter" sRNA region of sucrose gradients. Zone centrifugation analysis following in vitro addition of primaquine prior to or after preparation of extracts (by passage through a French pressure cell) of B. megaterium, failed to show any detectable ribosome destruction. Other investigators have shown that high Mg^{2+} (10 mM) protects isolated ribosomes from disaggregation and degradation but that at concentrations of the order of 10^{-5} M Mg^{2+}, dissociation of the protein and RNA moieties of isolated ribosomes is accompanied by the liberation of a latent ribonuclease and subsequent hydrolysis of ribosomal RNA. Crude extracts prepared from cells washed and suspended in peptone phosphate growth medium, in which the Mg^{2+} concentration is approximately 0.5 mM, and incubated with primaquine, produced extensive breakdown of ribosomal particles. However, ribosomes in primaquine-free control extracts were also degraded. A comparison of the breakdown profiles as revealed by sucrose

gradient centrifugal analysis suggests a slightly greater destruction of ribosomes in primaquine-containing extracts. A technique other than zone centrifugation, namely light-scattering, is being employed as an alternate and, perhaps, more sensitive method to study primaquine-induced disaggregation and degradation of isolated B. megaterium ribosomes.

Studies on ribosomal kinetics. Reactions providing for the initiation of protein synthesis in bacteria are of interest not only to molecular biology in general but also as concerns the action of aminoglycoside antibiotics, e.g. streptomycin which inhibits bacterial growth through prevention or distortion of the initiation process. Initiation of protein synthesis is, in part, catalyzed by three protein "factors" which possess multiple functions, among which are dissociation and reassociation of 70S ribosomes, in order to position correctly the initiation codons contained in messenger RNA (mRNA). Individual subunits (50S and 30S) of the 70S ribosomes alone are incapable of synthesizing protein. Past techniques for direct measurement of reactions between ribosomes and their subunits have been deficient; zonal centrifugation for determination of reaction endpoints in terms of the distribution of equilibrium components influences these distributions through exertion of hydrodynamic pressure which induces ribosomal dissociation. Therefore, light scattering was used to study ribosomes and their reactions. Efforts were directed toward answering five questions: (1) What is the influence of the major cellular cations on the distribution of ribosomal species? (2) What are the time courses and kinetic characteristics of association and dissociation? (3) What is the influence of the initiation factors upon the distribution of ribosomal species? (4) What are the changes in the ribosomal distribution during initiation of actual protein synthesis? (5) What is the influence of streptomycin upon the action of the initiation factors? In addition, the influence of one narcotic on the initiation reaction was investigated. (1) The concentration of Mg^{2+} controls the proportion of ribosomes and their subunits in ribosomal suspensions. When the concentration of Mg^{2+} was varied systematically between 1 and 20 mM, the distribution of 70S ribosomes and their 50S and 30S subunits, depended upon this concentration between 1 and 8 mM; at 10 mM Mg^{2+} the population consisted almost entirely of 70S ribosomes with an average molecular weight of 2.66×10^6 daltons, while at 1 mM Mg^{2+} the population consisted entirely of subunits having an average molecular weight of 1.35×10^6 daltons. However, when ribosomes were obtained from sucrose gradients and their dissociation and reassociation followed as a function of time and endpoint, a much sharper Mg^{2+} dependency was observed, as well as a markedly different time course. The conclusion was that ribosomes undergo changes in conformation which may affect their ability to carry out synthesis of protein. While this research was in progress, another laboratory, also using light scattering, reported that ribosomes and their subunits obey the laws of mass action and that a true chemical equilibrium, controlled by the concentration

of Mg^{2+}, exists between these various species. (2) The time course of ribosome dissociation and association and the nature of their kinetics remained to be elucidated. We found that Mg^{2+} induced alterations in the state of association of ribosomes despite the high velocity of these reactions. The equipment had a dead time of 10 milliseconds (msec); reactions occurring more rapidly than 10 msec could not be measured. Ribosomal subunits associated at two rates which maximally possessed half times of 250 msec and 15 sec, respectively; both reactions were controlled by the concentration of Mg^{2+}. Similar kinetics were observed for the dissociation of ribosomes. The slower rate of ribosome dissociation was subsequently found to be related to the effect of the initiation factors. (3) The influence of the initiation factors (IF-1, IF-2, IF-3) upon the state of ribosome association was measured by normal light scattering. IF-3 induced a slow dissociation of ribosomes, the rate being similar to the slower of the two dissociation rates following reduction in the concentration of Mg^{2+}. Both the spontaneous as well as the factor-induced dissociation exhibited first order kinetics, suggesting an excess of IF-3 to act indirectly through attachment to the 30S ribosome rather than directly through attachment to the 70S ribosome. Dissociation, therefore, results from a shift in the equilibrium between ribosomes and subunits. Initiation factor 2 (IF-2), conversely, induced formation of 70S ribosomes from subunits: a light scattering increase always occurred when ribosomes were mixed with IF-2. Addition of IF-1 to ribosomal suspensions did not alter their light scattering characteristics, suggesting that IF-1 on its own exerted no effect. However, when IF-1 was added to ribosomes in the presence of IF-3, decrease in light scattering occurred more rapidly and to a greater extent than with ribosomes and IF-3 alone. Thus, IF-1 could increase the IF-3-induced rate of dissociation as well as the number of ribosomes dissociated by IF-3. IF-1 could also increase light scattering when ribosomes were mixed with IF-2. We further found that the ribosome dependent GTPase activity of IF-2 was increased by IF-1 and that this increase could be eliminated by addition of IF-3, a pattern consistent with our other observations. (4) Since it is important to know whether the reactions studied do occur during the actual initiation of protein synthesis, the components necessary stably to attach the initiator tRNA (formyl methionyl-tRNA) were included in reaction mixtures. The synthetic messenger p(A,U,G) was utilized since it contains a large number of initiation codons (ApUpG). When this RNA was added to ribosomes alone, there occurred an unexpected increase in light scattering consistent with the formation of a ribosome-messenger RNA complex. Ribosomes could be saturated with p(A,U,G), the formation of this complex was dependent upon Mg^{2+} and could be eliminated either by RNAase, or IF-3 with or without IF-1. Even IF-1 alone reduced the concentration of the complex. The composite system containing p(A,U,G) as well as factors exhibited far greater decreases in light scattering at more rapid rates than systems lacking mRNA, although first order kinetics again were measured. It was concluded that factor-induced

dissociation occurred more rapidly and to a greater extent in the presence of mRNA than in its absence. This was tested through use of sucrose gradient centrifugations which yield the end-product of such reactions. The greatest dissociation occurred in the presence of IF-3, IF-1 and mRNA, hence, corroborating results obtained by light scattering. When the initiation system was completed by inclusion of (^{3}H)-fMet-tRNA and GTP and analyzed by tracer analysis as well as by light scattering, we observed each reaction that had been previously found in the separate systems, and also a stable attachment of (^{3}H)-fMet-tRNA to the ribosome. (5) Streptomycin reduced the rate of, or inhibited, IF-3-induced dissociation of ribosomes, regardless of the presence of other initiation components. The antibiotic appeared to exert two related effects: (1) reduction in the rate of factor-induced dissociation, and (2) restriction of light scattering decreases to the initial level which existed prior to addition of p(A,U,G), suggesting that light scattering increases due to the presence of p(A,U,G) could be eliminated by factors despite their inability to dissociate 70S ribosomes in the presence of streptomycin. Ribosomes from either streptomycin-resistant, or -dependent mutant strains were less active than were ribosomes from the parent strain, not only with respect to factor activity, but also with respect to complex formation with p(A,U,G). We reported last year that several potent narcotics, including the meperidine derivative NIH 7591, inhibit growth of E. coli. Protein synthesis was less sensitive to 7591 than was RNA and DNA synthesis. When tested with respect to the effect of 7591 upon the stable attachment of fMet-tRNA to ribosomes, a stimulation of ∿30% was observed when the concentration of Mg^{2+} was suboptimal. A similar stimulation was seen with spermine, suggesting that the narcotic's effect was a function of its polyamine character rather than its specific drug structure.

Conclusions.

Potencies of DNA-complexing compounds to eliminate resistance determinants from a bacterial R-factor correlated with a relative measure of stoichiometry of binding to DNA (methyl green displacement). Several such chemicals, foremost one nitroacridine at 5×10^{-6} M, sterilized ampicillin-resistant (R-factor determined) S. typhimurium in combination with ampicillin. Preliminary biochemical observations indicate that it is possible to study R-factor replicative DNA synthesis in cell-free systems in vitro. The DNA-binding antibiotic, distamycin A, which interferes with the transmission of R-factors among bacteria, binds preferentially to poly dA and single-stranded DNA of phage ϕX174; it binds much less strongly to poly dG, poly dC and poly dT. The dissimilation of bacterial ribosomes and ribosomal RNAs, under the influence of primaquine, has been investigated in detail in vivo and in vitro. Kinetics and endpoints of association and dissociation of E. coli ribosomes have been measured in vitro by light scattering

techniques. The role of the three "initiation factors" in these processes as well as the effects of streptomycin and one experimental narcotic on ribosomal kinetics have been studied.

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 074 Molecular basis of biological regulation and chemotherapeutic drug pharmacology

Literature Cited.

Publications:

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2. Hahn, F.E.: Distamycin A and Netropsin. In Antibiotics III. J. Corcoran and F.E. Hahn edts. Springer, Berlin-Heidelberg-New York 1974, pp 79-100.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6434	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71P	01	075
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Metabolic Problems Associated with Disease and Injury

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 003500 Clinical Medicine 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 06	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	1	54
FISCAL YEAR CURRENT 75	6	108

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Genetic Interrelationships; (U) Gene Isolation; (U) Protein Synthesis; (U) Receptor Sites; (U) Enzymes

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The objective of this work unit is to define the responses of the host to infecting agents causing diseases of military medical importance.

24. (U) Purification, characterization and structural elucidation of macromolecules by physico-chemical methods, and the interaction and alteration of macromolecules in biological systems are being investigated. Both in vivo and in vitro systems are employed to study genetic and phenotypic alterations and interaction between host and infecting agents. Studies on the characterization of the receptor sites of metabolic antagonists and drugs are being pursued. The biochemical bases of lymphocyte transformation will be studied.

25. (U) 73 07 - 74 06 A chromatographic method for determination of size of DNA obtained by controlled shearing techniques was developed. Using genetically defined animals and MLC systems it was determined that there is a correlation between cAMP levels and occurrence and absence of blast transformation. In vitro binding of 7,8-dihydromorphine in hypothalamic synaptosomes and microsomes of tolerant rat brain is higher than in control rat brains. DNA relatedness studies in the tribe protease was shown to be very diverse. With the advent of multiple drug resistance and gross atypical biochemical reactions, differentiation of these organisms by DNA relatedness has become more important. Trials for the identification of unidentifiable clinical isolates by rapid modified assay based on DNA relatedness is very accurate. Using in vivo and in vitro techniques it was observed that the 4,5-diphenylhydantoin does not associate either with brain or other nucleic acids or the brain subfractions which contain nucleic acids. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 075 Metabolic problems associated with disease and injury

Investigators.

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The objective of this work unit is to apply the knowledge gained through basic and molecular research to military medical problems. Studies are carried out on disease-induced variations in cellular processes to characterize biochemical, genetic and molecular alterations using the physico-chemical and structural aspects of macromolecules in bacteria, viruses and mammalian cells. These investigations are:

1. Enrichment and characterization of antibodies to morphine.

2. 4-methylpyrazole-induced changes in alcohol intake and blood alcohol levels of alcohol-preferring rats.

3. Enrichment of tRNA cistrons from E. coli using antibody affinity chromatography.

4. Fractionation and molecular weight determination of DNA fragments using agarose column chromatography.

5. The role of cAMP modulation in major histocompatablity complex-induced activation of subpopulations of human splenic lymphocytes.

6. DNA relatedness among enteric bacteria.

7. Studies on the binding of 5,5-diphenylhydantoin (DPH) to nucleic acids in rat brain.

8. Uptake of catecholamines by brain synaptosomes from opiate-treated rats.

9. Stimulation of in vivo incorporation of ^{14}C-2-glucose into free amino acids of rat brain by 5,5-diphenylhydantoin treatment.

1. Enrichment and characterization of antibodies to morphine.

Since the commercially available antibodies to morphine used in FRAT analysis were not suitable for systematic characterization studies, attempts were made to further purify and characterize these antibodies Residual albumin from a commercially available antibody sample was removed by ammonium sulfate precipitation. The globulin fraction was subjected to Sephadex 200 gel chromatography. The fractions thus obtained were examined for their morphine binding capacity by using spin labeled morphine (3-SLM) and ESR spectrometry. The binding activity was contained in IgA and IgG fractions. The separation of IgA and IgG was accomplished by pooling the active fractions from the Sephadex G-200 column eluates and chromatographing them on a DEAE-cellulose column. Only the IgA fraction contained the morphine binding activity. Further efforts are being made to purify and characterize the antibody to morphine using immunological as well as physico-chemical parameters.

Preliminary results obtained with the opiate binding antibody show the following: (1) maximal affinity for 3-SLM was at pH 6-7, (2) there were between 3.7 and 4.9 nanomoles of binding sites for morphine per mg protein. Syva (commercial) morphine antibody preparation for FRAT on the other hand, had a rather broad pH optimum for 3-SLM binding (pH 6.5-7.6). There were between 1.1 and 1.4 nanomoles of binding sites for morphine/mg Syva antibody protein; therefore the enrichment of morphine binding sites was some 350%. The enriched antibody preparation appeared to react instantaneously with 3-SLM. The complete Syva antibody preparation bound 3-SLM for about 10 min, released it in the next 15 min and finally reassociated with 3-SLM maximally after a total of 45 min. Furthermore, about 45 min were required for equilibrium to be reached for the displacement of 3-SLM from the Syva antibody by morphine. Further efforts are underway to fully characterize this reaction and to extend these studies to other immunoassays for drugs of abuse, i.e., RIA, EMIT.

2. 4-methylpyrazole-induced changes in alcohol intake and blood alcohol levels of alcohol-preferring rats.

This project was conducted in collaboration with Drs. L. Lumeng and T.-K. Li of the University of Indiana School of Medicine, Indianapolis, and D. Hawkins, Division of Neuropsychiatry, WRAIR. The following is the summary of the results.

Alcohol-preferring rats were placed on three different feeding schedules: 1) solid food ad lib and an unsweetened alcohol solution, 2) food ad lib and a sweetened alcohol solution, and 3) food restriction and sweetened alcohol. Water was present in all instances as an alternative fluid source. A single intraperitoneal injection of 4-methylpyrazole (4MP - 90 mg/kg body weight) inhibited alcohol intakes in all three

models for as long as 4 days. Blood alcohol levels in the food-restricted rats remained unchanged or decreased slightly after the administration of 4MP whereas the animals given food ad lib showed substantial increases in blood alcohol levels with either sweetened or unsweetened alcohol. The administration of 4MP produced transient neurologic dysfunction.

3. Enrichment of tRNA cistrons from E. coli using antibody affinity chromatography.

This project has come to a stage where it can be employed for enrichment of cistrons from mammalian systems. The progress to date is summarized here.

Antibody affinity chromatography was used to enrich E. coli DNA fragments that contain tRNA cistrons. The method uses solid phase antibody to 2,4-dinitrophenol (DNP) to bind specifically DNA which was covalently attached to tRNA in a DNA/tRNA hybrid. DNA was covalently linked to the periodate oxidized 3' terminal ribose of tRNA by reaction with the DNA hydrazine (DNPH). The DNPH of tRNA (tRNA-DNPH) thus formed was bound specifically and quantitatively by the anti-DNP antibody which was attached to agarose gel (A-150M) via cyanogen bromide activation. The tRNA-DNPH was quantitatively eluted from the gel with 1 mM DNP. Specific hybridization of tRNA-DNPH to E. coli DNA sheared under pressure (4000 psi; approx. 400,000 + 40,000 daltons) was accomplished by incubation at 37° for 10 min in 40% formamide containing 1.3 x SSC. The DNA/tRNA-DNPH hybrid was bound and eluted from anti-DNP agarose gel column just as with tRNA-DNPH but with 35 to 40% of the efficiency. Unhybridized DNA passed through the column with less than 0.1% non-specific binding. The procedure is rapid and uses low temperature hybrid isolation. DNA fragments containing tRNA cistrons are enriched approx. 200 to 400-fold using one purification cycle. This method is being applied to several studies, such as isolation of cistrons that code for stable messenger RNAs.

4. Fractionation and molecular weight determination of DNA fragments using agarose gel column chromatography.

DNA fragments of several different sizes were produced by shearing E. coli DNA under different pressures. These fragments were used to demonstrate that column chromatography on agarose bio-gel (A-15M) can provide a rapid and inexpensive fractionation and sizing method for single-stranded nucleic acids having masses between 10^5 and 10^6 daltons. Both chromatographic and electrophoretic analysis of the sheared DNA indicated that discrete fragment populations were produced at each shearing pressure and that these fragments were distributed essentially symmetrically around a mean piece size. The average molecular weight of the several DNA fragment size distributions was determined electrophoretically by comparison with standard DNA fragments obtained from

restriction endonuclease cleavage of SV40 viral DNA. The molecular weights of the denatured, sheared fragments (single-stranded) ranged from 1.25×10^5 to 7.4×10^5. The single-stranded DNA fragments were chromatographed over agarose bio-gel (A-15M) and a linear relationship was found between the mobilities and logarithms of the molecular weights. Readily available tRNA, 5s RNA and ØX174 single-stranded, circular DNA chromatographed at the extremes of this linear relationship and could be used to calibrate the column chromatography.

5. The role of cAMP modulation in major histocompatibility complex induced activation of subpopulations of human splenic lymphocytes.

The human spleen contains distinct subpopulations of lymphocytes which can be separated by discontinuous gradient centrifugation. The fractions thus obtained were shown to be functionally different as judged by modulation of the adenylate cyclase-cAMP system in mixed lymphocyte cultures (MLC). The results presented here show that in splenic lymphocytes from a given donor, the total effect of a particular drug on DNA synthesis was controlled by three factors: (1) drug concentration, (2) spleen cell subpopulation used, and (3) time of interaction between responding and stimulating cells in the MLC prior to addition of the drug. The experimental design used in this investigation provides a model by which the timing and/or duration of signal transfer between histoincompatible cells may be more fully explored. The results further show that agents known to elevate intralymphocytic cAMP levels modulate the human transplantation antigen complex-stimulation of lymphocytes. This suggests a role for cAMP in allogenic recognition. Finally, the use of highly purified preparations of lymphocytes may clarify some of the controversy about cAMP metabolism in lymphocytes.

6. Deoxyribonucleic acid relatedness among enteric bacteria.

The family Enterobacteriaceae contains the bacterial causative agents of a variety of diseases including enteric fever, diarrhea, urinary tract infection, food poisoning and bacteremia. DNA relatedness in representatives of all groups of enteric bacteria is being determined. The data obtained are used for the following purposes:

a. To develop a method for rapid identification of biochemically atypical bacteria in the clinical laboratory.

b. To correctly identify cultures that cannot be routinely identified due to grossly atypical biochemical reactions, unusual serological characters or the presence of plasmids.

c. To accurately classify newly described organisms.

d. To develop a molecular (genetic) definition of a bacterial species.

e. To develop a classification based on genotypic relatedness, instead of relying solely on a few phenotypic characteristics. The average bacterium contains sufficient DNA to specify some 3,000 average size genes, yet most organisms are classified on the basis of 10-25 characters.

f. To isolate and characterize specific portions of the bacterial genome such as the genes that specify transfer ribonucleic acids, 5s ribonucleic acid, and the lactose operon.

g. To characterize relatedness among bacterial viruses and between these viruses and their host bacteria.

Data obtained previously indicate that enteric bacteria are all significantly related. In the main there is a core of some 25% relatedness among these organisms. The main exceptions are found in the genera Proteus and Providencia. Most of the proteei and providencia exhibit about 10-15% relatedness to members of all other genera.

During the past year work was concentrated on assessing relatedness within specific genera of medical importance, and on developing a clinical method for identification of atypical cultures.

Polynucleotide sequence relatedness among species of Klebsiella and Enterobacter. It is difficult to distinguish between Klebsiella and Enterobacter aerogenes solely on the basis of biochemical differences. We have shown that these organisms are easily separated on the basis of DNA relatedness. The three species of Enterobacter: E. aerogenes, E. cloacae and E. hafniae are also significantly different. E. aerogenes is about 40% related to E. cloacae and E. hafniae is about 25% related both of these species. It was unexpectedly found that E. cloacae strains form two relatedness groups; one containing all unpigmented strains and the other containing all strains with a yellow pigment. There is no biochemical basis for these two groups which are 40% related to one another.

Polynucleotide sequence relatedness to Enterobacter agglomerans. E. agglomerans strains until recently have been classified with the genus Erwinia - as plant pathogens. These organisms have been isolated with increasing frequency from human infection. In fact they were one of the two causative organisms in the Abbott serum bottle epidemic of bacteremial infection some three years ago. Ewing and Fife have proposed that these organisms be placed in Enterobacter as E. agglomerans. They have identified 11 different biogroups. We have tested some 50 strains and find that there are at least five different hybridization

groups; some of which do not correlate with the biogroups. We have now prepared some 100 additional strains including representatives of all of the biogroups. These strains are currently being tested.

Polynucleotide sequence relatedness in the tribe Proteeae. Bergey's Manual of Determinative Bacteriology describes one genus, Proteus containing five species: P. mirabilis, P. morganii, P. rettgeri, P. vulgaris, and P. inconstans. Ewing has placed E. inconstans into a second genus, Providencia with two species: Prov. alcalifaciens and Prov. stuartii. The former species has been divided into four biogroups and the latter into two biogroups. These organisms are prime causative agents in urinary tract infection and are often implicated in nosacomial outbreaks. In addition, several of these species are highly resistant to a wide variety of antibiotics. Correct identification of these organisms is therefore extremely important for epidemiological purposes, as well as for treatment purposes. Extensive DNA relatedness studies indicate the following:

1. Proteus and Providencia species exhibit 8-15% relatedness to other genera of enteric bacteria. The sole exception is P. morganii which is 20-25% related to other enterics. The percentage of guanine + cytosine in P. morganii is 50%; that of other proteii is 38-42% and that of other enteric bacteria is 50-58%. Therefore it is not surprising that P. morganii exhibits higher relatedness to other genera of enterics.

2. All P. morganii strains form one relatedness group. They are distantly related to other proteii.

3. P. mirabilis strains form one relatedness group. They are about 50% related to P. vulgaris and 25% or less related to other proteii

4. P. vulgaris strains form two relatedness groups. They are both about 50% related to P. mirabilis and 10-25% related to other proteii.

5. P. rettgeri strains form three relatedness groups. Two of them are 30-50% related to Providencia species and 15-20% related to Proteus species. The third group is inseparable from Providencia biogroups 4, 5, and 6 (see below).

6. Providencia alcalifaciens biogroups 1 and 2 form one relatedness group which is 15-40% related to other proteii.

7. Providencia alcalifaciens biogroup 3 strains form one relatedness group which is not highly related to other proteii.

8. Providencia alcalifaciens biogroup 4, Providencia stuartii biogroups 5 and 6, and one of the relatedness groups of Proteus rettgeri

are all part of one relatedness group. The Providencia alcalifaciens biogroup 4 strains will probably become part of Prov. stuartii. Further study is necessary to determine the proper status of the Proteus rettgeri strains.

Reference system for identification of clinical isolates. These data are being used to develop a system of clinical identification of biochemically atypical bacteria. Thus far we have identified the following relatedness (or hybridization) groups in enteric bacteria (a relatedness group may be defined as a group of bacteria that are distinguishable from all other organisms based on DNA relatedness group is usually 80% or higher):

1. E. coli, Shigella species
2. Edwardsiella tarda
3. Salmonella species
4. Arizona hinshawii
5. Citrobacter freundii
6. Citrobacter freundii H_2S^-, ind^+, KCN^-
7. Citrobacter diversus
8. Klebsiella species
9. Enterobacter aerogenes
10. Enterobacter cloacae, unpigmented
11. Enterobacter cloacae, yellow pigment
12. Enterobacter hafniae
13. Enterobacter agglomerans - 4-8 groups
14. Serratia marcescens
15. Serratia liquifaciens
16. Serratia rubidaea
17. lys^+, H_2S^-, "Citrobacter-like" organisms
18. Red mouth bacteria
19. Erwinia dissolvens and Erwinia nimipressuralis
20. P. mirabilis
21. P. vulgaris - 2 subgroups
22. P. morganii
23. P. rettgeri - 2 subgroups
24. P. rettgeri, P. alcalifaciens biogroup, 4, P. stuartii biogroups 5 and 6

25. P. alcalifaciens biogroups 1 and 2

26-30. Erwinia species

31-36. Pectobacterium species.

Those who use DNA reassociation are often asked whether the system can be used routinely for clinical purposes. The answer was always "no" because of the time involved and due to technical considerations. Among the Enterobacteriaceae we can identify any organism thus far tested as to genus, and almost every organism as to species, solely on the basis of DNA relatedness. It now takes us 7-10 days to carefully characterize an organism. We have modified our system to a point where most identifications can be completed within 48-72 hrs. The major modifications are: a) rapid and simplified isolation and purification of DNA; 2) separation of reassociated from unreacted DNA using a single-strand DNA specific nuclease; and c) preparation of labeled reference DNAs from representative Enterobacteriaceae.

In the absence of biochemical and serological data virtually all organisms can be identified as to genus (see list of relatedness groups above). In many cases species or even biotype designations are obtainable. In conjunction with biochemical data almost every organism is identifiable at the species level. The inclusion of biochemical data also allows many genera to be ruled out and therefore diminishes the number of DNA relatedness reactions necessary to identify an organism. Ideally this method allows the larger clinical laboratories to identify organisms not readily identifiable by biochemical tests. It also enables reference laboratories to readily identify most atypical strains. Furthermore, DNA relatedness provides a rapid and sensitive method for determining the taxonomic status of newly isolated strains.

7. Studies on the binding of 5,5-diphenylhydantoin to nucleic acids.

The binding of 5,5-diphenylhydantoin (DPH) to nucleic acids (rat brain, rat liver, Torula RNA, E. coli tRNA; calf thymus and E. coli DNA) was studied using UV spectroscopy, gel chromatography, and thermal transition procedures. Utilizing a variety of experimental conditions, it was found that DPH did not bind to nucleic acids in vitro as previously reported. No evidence of DPH intercalation with DNA was noted during thermal transition studies. DPH did not interfere with DNA reassociation. Further studies into the nature of the in vivo subcellular distribution of ^{14}C-DPH in rat brain revealed accumulation of the drug primarily in the soluble fractions of brain, with the nuclear fraction and microsomes demonstrating the greatest particular association at 2 and 12 hrs, respectively. This association with particulate fractions was not observed after Sephadex G-25 column chromatography. An hypothesis relating DPH binding to nucleic acids or brain subcellular fractions as a possible biochemical mechanism of action of the drug appears unlikely on the basis of the results obtained from this investigation.

8. Uptake of catecholamines by brain synaptosomes from opiate-treated rats.

The acute administration of morphine has been reported to result in changes in brain catecholamine metabolism, producing a decrease in the level of noradrenaline in whole brain and in hypothalamus. Morphine was reported to increase the in vivo biosynthesis of catecholamines from [^{14}C] tyrosine. Also, a morphine-induced increase in the in vivo biosynthesis of dopamine and norepinephrine (NE) from [^{14}C] tyrosine which was antagonized by naloxone has been reported.

Uptake of [^{3}H] dihydromorphine by rat brain was demonstrated in vitro by synaptosomes. This uptake was inhibited by narcotic antagonists. When adult rat brain homogenates were incubated with [^{3}H] noradrenaline, most of the radioactivty was localized in the synaptosomal fraction.

In a recent study the inhibition of the in vitro synaptosomal uptake of NE by morphine, methadone, thebaine, and cocaine was demonstrated. The present study shows the in vivo effects of acute narcotic drug administration on the in vitro uptake of NE by synaptosomes.

After 10 mg kg^{-1} of heroin, the mean uptake of ^{3}H-NE was 75-84% of the control values in the hypothalamic synaptosomes. Morphine at 50 mg kg^{-1} did not change ^{3}H-NE uptake by hypothalamic synaptosomes. Both heroin and morphine inhibited in vitro ^{3}H-NE uptake by whole brain synaptosomes. At 10 mg kg^{-1} of heroin, the uptake was 65-77% of the control values. At 50 mg kg^{-1} of morphine, the uptake was 75-87% of the control values.

These experiments indicate a differential inhibitory in vivo effect of heroin compared to morphine on the in vitro synaptosomal ^{3}H-NE uptake when the opiates were administered in comparable pharmacological dosages.

9. Stimulation of in vivo incorporation of ^{14}C-2-glucose into free amino acids of rat brain during 5,5-diphenylhydantoin treatment.

In vivo administration of 5,5-diphenylhydantoin (DPH) at dosages of 80 mg/kg and 30 mg/kg for 14 days increased the in vivo incorporation of ^{14}C-2-glucose into free amino acids of rat brain. DPH-treated and control animals were sacrificed 3 hrs after the I.P. administration of 30 µCi of ^{14}C-2-glucose and the free amino acids of serum, liver, and brain were determined by cation exchange chromatography (µM/g wet tissue; µM/100 ml serum; cpm/µM). DPH stimulated glucose incorporation into aspartic acid, glutamine, glutamic acid, and GABA of brain. These results were not reflected by similar changes in serum or liver free amino acids. When the DPH groups are compared to each other, a dose-response effect was

noted. DPH at 80 mg/kg produced a 10% (aspartic acid), 23% (glutamine), 28% (GABA), and 43% (glutamic acid) increase in incorporation over that noted in animals receiving 30 mg/kg. This effect of DPH on glucose incorporation into free amino acids of brain may be related to the known and postulated effects of DPH on amino acid transport/metabolism, membrane transport phenomena, and ion fluxes.

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 075 Metabolic problems associated with disease and injury

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6448	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71P	01	076
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			CH ER 036

11. TITLE (Precede with Security Classification Code)

(U) Basic Pharmacological Studies

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	1.0	337
FISCAL YEAR CURRENT 75	1.0	337

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Pharmacology; (U) Medicinals; (U) Drugs; (U) Secretion; (U) Toxicity

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Research is directed toward investigating special areas of the pharmacology of potential drugs of military importance, their interactions, their mechanisms of action, and the development, characterization and improvement of animal models for defining specific applicable parameters.

24. (U) Drugs are tested in animal models specifically designed to pinpoint mechanisms of pharmacological effects, effects on both physiological and drug secretion, and effects on protozoan systems. In vitro models are being used to correlate bioavailability with pharmacological efficacy.

25. (U) 73 07 - 74 06 The cardiovascular effects of WR 181,023 were found to be consistent with beta-adrenergic antagonism; isolated uterine segments are being used to elucidate this blocking action. Wide variations in disaggregation and dispersion of clinical dosage forms of experimental drugs have been correlated with clinical response. The choroid plexus preparation of the cat lateral ventricle is being used to study drug effects on plasma:cerebrospinal fluid fluxes of sodium and potassium. WR 33,063 effects on chloroquine-induced pigment clumping in P. berghei were modified by microsomal enzyme inhibitors. The change in clumping has been used to demonstrate bioavailability of experimental drugs in mice. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

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Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 076 Basic pharmacological studies

Investigators.
Principal: Melvin H. Heiffer, Ph.D.
Associate: LTC L. Miner, Dr. R. Rozman, CPT H. Ellis, III, Dr. A. Einheber, CPT D. Foley, D. Palmer

1. Description.

The basic research efforts of the department are directed to investigating: the pharmacology of promising medicinal agents; biological responses to toxic substances; drug interactions with and the nature of adrenergic receptors; and the development of new or modification of existing techniques to characterize drug effects.

Appropriate pharmacological, physiological, biochemical and electrophysiological studies are conducted in vivo and in vitro. These studies encompass the acute and subacute responses to potential drugs and their interaction with standard pharmacological and physiological agents. An important feature is ready access to the vast inventory of serially related and diverse chemicals which can be used in detailed studies of the nature of drug interactions with biological systems.

2. Autonomic nervous system pharmacology.

Studies on elucidating autonomic nervous system action are in progress on one potential IND drug, WR 181,023.

While delineating the cardiovascular effects of 8-aminoquinolines, it was observed that WR 181,023 abolished the blood pressure response to isoproterenol. This suggested the possibility that WR 181,023 may possess beta-adrenergic blocking properties. Studies have therefore been initiated using the in vitro isolated tissue technique reported by Levy and Tozzi (1963). This method consists of suspending rat uterine segments in Locke's solution within a constant temperature water bath at 37°C, with spontaneous motility recorded by means of a force displacement transducer. Epinephrine, isoproterenol and carbachol are the principal agonists used and are allowed to act for 3 min prior to flushing. Results indicate that this procedure will be successful in further elucidating the nature and mechanism of the beta-adrenergic antagonist actions of WR 181,023.

3. Disaggregation and dispersion of experimental drugs.

Although capsules, in possible contrast to tablets, are often assumed to be free of disaggregation problems, hydrophobic materials may interfere with the process. This can lead to decreased bioavailability of orally administered drugs. Since many of our drugs are hydrophobic, the distinct possibility exists that the physical properties of the formulated drug product may be part of the observed problem of drug failure. Thus studies of the disaggregation and dispersion of various dosage forms begun in FY 73 have been continued, with attention directed primarily toward newly developed dosage forms and compounds. Except when specified otherwise, the method used remains the USP tablet disintegration test, without discs, in simulated gastric fluid at 37°C.

a. Tablets:

New sulfadiazine tablets (placebo, 50, 250 and 500 mg sizes) were tested with discs. The 250 mg tablets required 12 min to disintegrate, but the others disintegrated within 5 min. These results comply with USP requirements.

b. Capsules:

The effect of drug-excipient ratios was studied in two compounds. Placebo capsules of WR 158,122 disaggregated in 8 min. Two lots of 25 mg capsules (7.6% drug) were 90% disaggregated in 90 min; the remaining small chunks disaggregated very slowly. Fifty mg capsules (14.8% drug) were 90% disaggregated only after 5 hr of agitation. Similarly, placebo capsules of WR 159,412 disaggregated within 3.5 min and 5 mg capsules (1.4% drug) in 4 min. The 25 mg capsules (6.8% drug) required 7 min and the 50 mg capsules (11.4% drug) were only 80% disaggregated after 4 hr. One interpretation of these data is that the drug acts as a binder of the excipients.

When one clinical contractor reported that 250 mg capsules of WR 142,490 appeared much less effective than comparable doses of a 50 mg capsule, various preparations of this drug were examined. Both the placebo and 50 mg capsules (13.3% drug) disaggregated within 5 to 10 min. When the 250 mg capsules (44.2% drug) were tested, the gelatin readily dissolved, but the capsule masses remained for at least 6 hr. By that time, a few very small pieces had abraded off, but at least 95% of each capsule mass remained in its original size and shape. When these were removed and air dried, the mass hardened, becoming brittle. When snapped open, the outer 2-3 mm were seen to be stained pink (by the dye in the gelatin) and cemented together; the interior remained an easily poured powder. In response to these observations, tablets containing 250 mg of WR 142,490 (44.5% drug) and 2.8 mg (0.5%) of the nonionic surfactant Pluronic L101 were developed. These

disintegrated within 10 min (with discs), and are reported to be clinically very effective. These results imply that a surfactant can be valuable excipient with refractory compounds such as many of our experimental antimalarials.

The effect of excipients was investigated further with WR 171,669 capsules. In the original formulations the main diluent was microcrystalline cellulose (Avicel ®). Both the placebos (68.9% cellulose) and the 60 mg capsules (17.1% drug, 57.1% cellulose) disintegrated slowly: about 80% in 60 min, the remainder over several more hr. In tablet formulations microcrystalline cellulose is very effective as a disintegrant. It is evident that it is less effective in these non-compressed capsule fills. When a larger capsule dosage was requested, the formulation contractc developed a 250 mg capsule (71.9% drug) containing only the surfactant Pluronic L101 (2.2%) and corn starch (an excellent tablet d'sintegrant) as excipients. These capsules disaggregated within 8 min.

From the above data, and that produced earlier, one can conclude the following: there is a significant possibility of producing dosage forms of WRAIR investigational antimalarials with poor bioavailability properties; and proper formulation, particularly using surfactants, may be able to alleviate the difficulties.

4. Choroid plexus preparation for cerebrospinal fluid studies in the cat.

a. Background:

It is well known that the electrolyte composition of the cerebrospinal fluid is of paramount importance to normal neuronal activity within the brain. There is evidence that the electrolyte composition of the cerebrospinal fluid is determined primarily at the time of formation and that most of the fluid is formed by secretion at the choroid plexus, the membranes of which separate the plasma from the fluid in the four ventricles of the brain. In addition to its secretory role, the choroid plexus forms the blood-cerebrospinal barrier which is part of the blood-brain barrier. The choroid plexus thus plays a vital role in regulating the composition of the cerebrospinal fluid, thereby maintaining the proper milieu interieur for the brain by means of specialized transport processes across the choroidal epithelium. In studying the neuropharmacology of experimental drugs it is appropriate to investigate the formation and composition of the cerebrospinal fluid by the choroid plexus.

Inasmuch as Na^+ and K^+ are the electrolytes primarily responsible for the generation and propagation of nerve action potentials, the objective of the present study is to investigate the transport mechanisms responsible for the movement of Na^+ and K^+ across the epithelial

cells of the intact choroid plexus. It has been suggested (Bradbury and Davson, 1965) that the poisoning of the Na^+ - K^+ pumps of brain cells allows a leak of intracellular K^+ across the ependymal cells lining the ventricles. It has also been postulated that K^+ transport is linked to the influx of Na^+ and that it might be located at the choroid plexus and/or the blood-brain barrier (Bradbury and Stulcova, 1970). Based on previous studies (Miner and Reed, 1971) one would expect a decrease of 15 to 30% in the rate of movement of Na^+ and/or K^+ across the choroid plexus as a result of addition of a known transport inhibitor (oubain) to the cerebrospinal fluid. This study was designed to help clarify the primary anatomical site of Na^+ - K^+ exchange between plasma and the cerebrospinal fluid as well as further elucidating the underlying mechanisms involved.

As a model for conducting transport studies in the central nervous system, a method was employed which involves the in situ isolation of the choroid plexus in the lateral ventricle of the cat. This technique makes it possible not only to study the movement of a drug between the plasma and the cerebrospinal fluid across the cuboidal cells of the choroid plexus without the modifying influence of surrounding neural tissues, but also to control experimentally the concentrations of electrolytes and pharmacologic agents on the cerebrospinal fluid side of the choroid plexus. The technique thus makes it possible to conduct definitive studies of the transport systems in the choroid plexus responsible for cerebrospinal formation and for removal of substances from the cerebrospinal fluid as well as modifications of their responses to various pharmacological agents.

b. Methods:

This method involves the surgical implantation of a plexiglass chamber consisting of two parts, a concave base which forms the bottom of the chamber and a top which rests on the base and forms the wall. The base has a handle to permit clamping it to the micromanipulator during implantation. Cats anesthetized with sodium pentobarbital were used as experimental animals. The primary operative procedures consisted of an extensive bilateral craniotomy performed by rongeur, removal of the cerebral cortex overlying the left ventricle, and exposure of the choroid plexus within the lateral ventricle. The choroid plexus was then reflected posteromedially over the lateral aspect of the thalamus and an appropriate segment separated from its underlying connective tissue attachments. The chamber base was lowered into position adjacent to the segment of free choroidal tissue which was gently teased into the grooves on the upper edge of the chamber base and allowed to settle to the bottom. The isolation was completed by placing the chamber wall on the base and attaching it with tissue adhesive. The chamber was stabilized in position by filling the entire space surrounding it with white petrolatum which had been warmed to a semi-solid consistency. The tissue in the bottom of the chamber was

covered with mineral oil to avoid contact with air and then cerebrospinal fluid was allowed to accumulate in the chamber. Chamber fluid was sampled with a glass micropipette positioned inside the chamber with the aid of a micromanipulator and the operating microscope.

The rate of formation of fluid by the isolated choroidal tissue was determined by measuring the rate of fluid accumulation in the chamber and dividing this value by the weight of the choroid plexus tissue in the chamber at the end of the experiment.

c. Results and discussion:

Experiments were performed to determined the feasibility of studying the fluxes of Na^+ and K^+ across the choroidal cells between plasma and cerebrospinal fluid by experimentally altering the K^+ concentrations in the plasma. Hyperkalemia was induced by an intravenous infusion of 0.15 M KCl at 0.16 mg/kg/min. Hypokalemia was subsequently induced in the same animal by a combination of a 10% glucose: 1 unit/ml insulin intravenous infusion and introduction into the rectum of K^+ cation exchange resin (Kayexolate (R)). Control samples of plasma taken prior to treatment contained 3.4 mequiv/l of K^+ and 146.5 mequiv/l of Na^+. These values agree well with normal controls in other similar experiments. After treatment to induce hyperkalemia, the plasma contained 4.8 mequiv/l of K^+ and after treatment to induce systemic hypokalemia, the plasma was found to contain 1.7 mequiv/l of K^+. Hence the results thus far are encouraging and suggest that it is feasible to use these methods and procedures to study the fluxes of Na^+ and K^+ between the plasma and cerebrospinal fluid across the choroidal epithelium.

5. Morphologic changes in Plasmodium berghei after oral administration of antimalarials to malarious mice.

a. Inhibition of chloroquine-induced pigment clumping:

Inhibition of chloroquine-induced pigment clumping (CIPC) after oral administration of certain antimalarials (quinolinemethanols and phenanthrenemethanols) has been further explored by phase-contrast microscopy of unstained thin blood smears from P. berghei-infected mice. The following agents were reported last year and were all found to have a direct effect on the parasite morphology within 60 min after oral administration: WR 142,490 AE, 20 mg/kg (partial inhibition of CIPC 30 min after chloroquine [CQ]);WR 171,669 AC, 20 mg/kg (complete inhibition of CIPC 30 min after CQ); and quinine $HCl \cdot 2H_2O$, 200 mg/kg (partial inhibition of CIPC 30 min after CQ). The inhibitory patterns of these, as well as of WR 30,090 AJ, 80 mg/kg or 10 mg/kg and WR 33,063 AN, 80, 160 or 640 mg/kg, were followed for 6 hr after CQ.

b. Retarding influence of SKF 525-A pretreatment on the CIPC-inhibitory effect of WR 33,063:

Several variations on WR 33,063 effects in this CIPC-inhibitory system were examined. For example, SKF 525-A pretreatment (50 mg/kg i.p. in saline) 60 min before WR 33,063 AN, 80 mg/kg, p.o., in our CIPC-inhibitory system produced a retardation of the inhibitory effect of WR 33,063. Blood films examined at intervals after CQ revealed that SKF 525-A pretreatment retarded development of the pattern produced by WR 33,063 alone except at 6 hr after CQ. By that time, the proportion of pigment types had equalized for both groups. When the course of infection in these groups was followed, there was a trend toward prolonged survival and greater parasitemia suppression in the WR 33,063 group pretreated with SKF 525-A.

c. Comparative CIPC-inhibitory properties (bioavailability comparison) of two preparations of WR 33,063:

The Roche formulation of WR 33,063 (Ro 21-2427/001) was tested against WR 33,063 AN, both given p.o., at a dose of 80 mg/kg, for CIPC-inhibitory properties. There appeared to be a greater inhibition of type 2 pigment formation (granulation) 30 min after CQ by the Roche formulation in comparison with the AN lot in MCT (methylcellulose 0.2% and Tween 80 0.4% in 0.9% saline), but no outstanding difference was detected. No difference in extension of survival time was seen, but there was a trend toward greater parasitemia suppression by the Roche formulation on Day 10 of infection.

d. Reversal of CIPC:

In following the patterns of these agents to 6 hr after CQ, those of WR 33,063 yielded a surprising apparent breakdown of already formed pigment clumps (reversal of CIPC) with time. This led us to give a variety of CIPC-inhibitory agents by gavage 80 min after CQ. At this time, pigment clumps usually comprise 100% of the pigment types seen. With the following agents tested in this system we were able to demonstrate a direct effect on parasite morphology (viz., by the onset of reversal) within 30 min after oral administration of the agent: WR 142,490 AE, 20 mg/kg; WR 171,669 AC, 20 mg/kg; WR 30,090 AJ, 80 mg/kg; WR 33,063 AN, 640 mg/kg; and quinine $HCl \cdot 2H_2O$, 200 mg/kg. Quinine was unique in its patterns, both of CIPC inhibition and reversal. Both the inhibiting and the reversing effects of quinine diminished by 6 hr after administration of quinine such that CIPC reasserted itself. This may reflect the rapid elimination of the form active in CIPC inhibition and reversal, and is consonant with quinine's known extensive metabolism and rapid excretion. None of the other agents demonstrated this diminution.

e. WR 30,090-induced reversal of CIPC in previously uninfected mice hypertransfused with CIPC donor blood:

CIPC blood (1.5 ml) from donor mice (exsanguinated 80 min post-CQ, 40 mg/kg, i.p.) was hypertransfused into the tail vein of uninfected mice. Upon challenge with WR 30,090 AJ, 80 mg/kg, p.o., CIPC reversal was noted at a time (60 min after WR 30,090) when no reversal was seen in control CIPC-hypertransfused recipients not receiving WR 30,090. Interestingly, these latter controls did demonstrate a smaller, but nonetheless distinct, reversal of CIPC by 6 hr after vehicle alone. This "spontaneous" reversal may indicate that a certain CQ concentration in blood is needed for maintenance of pigment clumps, a concentration affected by the experimental procedure. However, if the situation in CIPC hypertransfused, previously uninfected receipient mice reflects the situation in infected CIPC mice, our results strongly suggest that CIPC reversal represents an actual breakdown of pigment clumps in infected CIPC mice. This would be in contrast to a drug-induced entry into the circulation of pigmented but non-CIPC parasites which may have previously been sequestered in infected mouse tissues.

f. Course of chloroquine-induced depigmentation:

The loss of pigment with time after CQ (40 mg/kg, i.p.) was also investigated by examining Giemsa-stained parasites in which pigment was also clearly discernible. Such depigmentation after CQ had been previously reported, but not quantitated. At the beginning of the experiment, about half of the parasites possessed pigment. Throughout the sampling intervals to 6 hr after CQ a progressive decline in the percentage of parasites with pigment was noted, until at 6 hr after CQ this percentage had been approximately halved. In a control group receiving CQ's vehicle (water) only, the percentage of pigmented parasites showed a slight increase.

g. Lack of temporal association between onset of WR 30,090-induced CIPC reversal and course of depigmentation:

In view of the possibility that reversal might reflect an acceleration by the reversing agent of depigmentation due to CQ, (i.e. that the loss of pigment clumps after CQ might be preceded by their breakdown)we quantitated depigmentation at the various sampling intervals during a CIPC reversal regimen. WR 30,090 AJ, 80 mg/kg p.o., when given after CQ's vehicle (water), ultimately resulted in about 10% reduction in the proportion of pigmented parasites by 6 hr after WR 30,090. When WR 30,090 was given after CQ, we found by 6 hr after CQ a depigmentation intermediate between that of CQ "alone" and WR 30,090 "alone." At the intervals when CIPC reversal was clearly proceeding, we did not see an increased depigmentation in groups receiving CQ followed by WR 30,090 when compared with groups receiving

CQ followed by control vehicle. Thus, we were able to detect no enhancement of depigmentation by WR 30,090 when given after CQ, at times when reversal was in process.

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01 Biomedical Sciences

Work Unit 076 Basic pharmacological studies

Literature Cited.

References:

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Publications:

1. Caldwell, R. W. and Heiffer, M. H.: The genesis of the initial blood pressure peaks following I.V. administration of certain catecholamines. Proc. Soc. Exp. Biol. Med. 144:89-95, 1973.

PROJECT 3A161102B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03
Human Ecology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6431	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D,CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71P	01	025
b. CONTRIBUTING		CH FR	03	035
c. CONTRIBUTING	CARDS 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Ecology and Control of Disease Vectors and Reservoirs

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 005900 Environmental Biology 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 74	7	55
FISCAL YEAR CURRENT 75	3	55

19. RESPONSIBLE DOD ORGANIZATION

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RESPONSIBLE INDIVIDUAL

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Arboviruses; (U) Ecology; (U) Mosquitoes; (U) Disease Vectors; (U) Control; (U) Taxonomy

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Studies emphasize control of vectors of arbovirus and parasitic diseases of military significance. Objectives are incrimination of vectors and understanding of host-parasite relationships initially, understanding of vector biology and disease transmission mechanisms ultimately in order to develop more effective control procedures

24. (U) Invertebrate vectors and vertebrate reservoirs and hosts are collected in areas of known disease activity. Infection rates are determined, as are flight ranges, blood meal sources, breeding habits, and other biological characteristics. Other biological processes, such as pathogen transmission, flight physiology, and diapause are studied in the laboratory.

25. (U) 73 07 - 74 06. California encephalitis virus (Keystone subtype) was isolated from field-collected larvae of Aedes atlanticus as well as males and females reared from field-collected larvae. This virtually proves the occurrence of transovarial transmission of the virus in nature as a means of virus survival over winter. Also, 63 isolates of California encephalitis were obtained from 106,000 mosquitoes collected. Keystone and Jamestown Canyon subtypes were isolated, all from Aedes atlanticus and Aedes canadensis. Environmental conditions instrumental in breaking embryonic diapause in these two species were studied. In eggs collected March-May, temperature appears to be the only significant factor involved. Culex restuans was found to be able to undergo various functions (blood digestion, metamorphosis, etc.) at considerable lower temperatures than its sympatric counterpart, Culex salinarius. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03 Human Ecology

Work Unit 035 Ecology and control of disease vectors and reservoirs

Investigators
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Description

This task involves field and laboratory studies of the relationship between selected arthropods and various aspects of their natural environment, especially those aspects relating to certain pathogenic organisms, their hosts, and their reservoirs. Included are ecological and physiological studies on arthropods, studies of transmission mechanisms and the development of improved methods of control of arthropod of medical importance.

Progress

1. Ecology of arboviruses in the eastern United States

a. Introduction. A program to investigate the occurrence and ecology of California encephalitis group viruses (CE) was continued. Emphasis during the year was placed on elucidating details of the infection and transmission process in Aedes atlanticus, the principle vector of Keystone subtype of CE, the principle subtype present in the area and on studying the bionomics of forest pool aedine mosquitoes likely to be involved in the ecology of CE. At the beginning of the reporting period, several CE isolates had been made. Two of these isolates had been identified as the Jamestown Canyon and Keystone subtypes. The remaining isolates from mosquitoes collected in 1971 had not been identified. Mosquitoes collected during 1972 had been pooled and identified, but processing for virus isolation was not yet done. Circumstantial evidence from the population curve of Aedes atlanticus collected during 1972 and the CE isolation rate suggested the occurrence of transovarial transmission in nature. The obtaining of direct evidence of this was a major aim during this reporting period. Other studies dealt with the ecology of Aedes atlanticus, A. canadensis, and Psorophora ferox, the 3 most abundant floodwater mosquito species in the area. This report covers field and laboratory data accumulated since last year's annual progress report. Virological aspects of this program are reported under Project 3A061102B71Q,

Communicable Disease and Immunology, Work Unit 166, Viral Infections of Man. For background of the overall study and descriptions of the study areas, see WRAIR annual progress reports for previous years and published reports.

b. Light trap collections of mosquitoes. Identification, pooling, and processing for virus isolation for all mosquitoes collected during 1972 was completed. The number of 1972 mosquitoes collected, pooled and processed for virus isolation is shown in Table 1. Of the mosquitoes collected during that year, only Aedes and Psorophora mosquitoes were identified and pooled. Mosquitoes in other genera were not sorted but were stored for possible future studies.

During 1973, light trapping was conducted from May through September, Light traps were operated weekly at night at 6 sites in and around the Pocomoke Cypress Swamp. The sites trapped and the number of traps operated were as follows (site numbers refer to sites described in Saugstad, et al. (1972)): Site 2 (1), Site 3 (1), Site 7 (3), Site 10 (4), and Site 11 (2). Sites 2, 3, and 7 are within the swamp. Sites 10 and 11 are in upland forest. Site 10 is adjacent to woodland pools which form the typical habitat for Aedes atlanticus. Supplemental trapping was done during the period when A. atlanticus mosquitoes were emerging following heavy rains in August 1973 and continuing until mid-September. Light traps were operated during both a day cycle (0700-1800) and a night cycle (1800-0700) during the supplemental trapping period, and were placed in a triangular grid pattern to determine (1) diurnal vs nocturnal abundance of Aedes atlanticus and (2) dispersion of this species from breeding sites.

Although identification of 1973 trap catches is not yet complete, preliminary data from the traps for 2 days following emergence of A. atlanticus show that: (1) Light traps from all sites operated during the day cycle have about 30% more A. atlanticus per trap than those operated during the night cycle. (2) Abundance of A. atlanticus females decreases rapidly away from the area immediately adjacent to known larval breeding sites. Traps placed within 25 m. of breeding sites caught from 2 to 4 times as many A. atlanticus per trap as did traps greater than 100 m from such sites, suggesting that most A. atlanticus females do not travel far from their breeding sites in search of blood meals.

c. Virus isolations from mosquitoes

(1) Characterization of 1971 virus strains

The final identification of the 1971 virus strains isolated from mosquitoes collected from the swamp is presented in Table 2. Results of chloroform sensitivity tests and filtration experiments are also presented in the table. Virus strains were identified by plaque reduction neutralization testing (PRNT). A detailed discussion of testing techniques, antigens and antisera used and the relationship of the

subtypes isolated here when compared to the CE group as a whole is presented under Work Unit 166.

These data on filterability and chloroform sensitivity of the 1971 field strains establish these viruses as arboviruses. The specific determinations of group and subtype complete the characterization of the 1971 strains recovered. We considered it advisable to characterize these initial recoveries fully to assure the accuracy of our identifications. However, the 1972 strains and all future strains recovered from these mosquito species will be directly identified by PRNT without filtration or chloroform sensitivity experiments. As noted in the table, with the exception of a single Jamestown Canyon subtype, all strains isolated in 1971 were Keystone subtype CE. The suspected third subtype referred to in last year's Annual Report was concluded to be variation within the Keystone subtypes recovered.

(2) 1972 Virus recoveries and identifications

The objective of the 1972 field collections was to test several ecologically distinct habitats in an effort to define an area of active CE transmission for future long term surveillance. This program was begun before the 1971 material had been processed, consequently at that time we had no knowledge of the existence of both Keystone and Jamestown Canyon subtypes of CE being actively transmitted in the swamp.

In 1972, in addition to the swamp, studies were conducted in swamp and upland forest habitats adjacent to the Pocomoke River near Snow Hill, Md., in Chincoteague National Wildlife Refuge, Assateague Island, Va. and at the Wallops Island NASA Station, Va. Both Assateague Island and Wallops Island NASA Station consist of salt marsh vegetation and mixed hardwood-coniferous forests. Freshwater swamps similar to the Pocomoke Cypress Swamp are not present at either of these sites.

Adult mosquito collections were made intensively for a brief period during each of the warmer months at each of the sites sampled. Special day and nighttime collections were made in the swamp in 1972 during the emergence of A. atlanticus adults. Adult mosquitoes were identified and pooled as described by earlier reports. Based on our previous failure to detect CE virus from numerous pools of Culiseta melanura and Culex salinarius mosquitoes tested, and the demonstrated association of CE virus with floodwater mosquitoes by others, only floodwater mosquitoes were processed for virus.

Mosquito pools were divided equally, one-half being processed in suckling mice, and the other half inoculated into tube cultures of continuous BHK-21, Clone 13 cells. Mosquitoes selected for cell culture were pooled in groups of 100 females or less, ground in Wasserman tubes by adding several 6 mm glass beads and vibrated on Vortex mixer. One ml of BME (Earle's, 20% FBS, antibiotics) medium was added to each pool, triturated a second time, 1.0 ml of BME Earle's added again,

triturated a third time, then centrifuged for 20 minutes at 1500 rpm. An aliquot of supermate was removed, diluted and mixed 1:2 with the same media, and 0.2 ml of this added to duplicate tube monolayers of BHK-21 cells. Remaining original suspensions were saved for reisolation. Monolayers were observed for cytopathic effect (CPE) on days 2-7 post inoculation. Suspect positive pools were frozen, then later passed to a second set of cell cultures. Passage material was clarified at 8000 rpm for 45 minutes to eliminate virus aggregation and stored in aliquots at -70°C pending identification. Contaminated and toxic pools were retested either in cell culture or suckling mice. Pools processed in suckling mice followed procedures described in previous reports.

Viruses, isolated were identified by PRNT on LLC-MK 2 cells. Eight selected isolates were tested against mouse hyperimmune ascitic fluids for Keystone, Jamestown Canyon, LaCrosse, Snowshoe Hare, and Trivittatus viruses. These preliminary tests indicated that six of the eight were strains of Keystone and two were strains of Jamestown Canyon; subse quently isolates were tested only against those two MHAFs. Additional discussion of PRNT results is presented under work unit 166 (see above).

In 1972 over 106,000 mosquitoes were collected and processed from the combined Del Mar Va Peninsula collection sites (Table 1), from which 63 viruses were recovered. Of the 63 isolations made, all but 3 were from A. atlanticus, the remaining being from A. canadensis. Isolations were made from material collected both from the swamp (56) and Snow Hill (7) sites. No virus was recovered from mosquitoes collected at either the Wallops NASA Station or Assateague Island collection sites. Virus was reisolated from 54 of 63 pools collected. Based on these observations, efforts to select a new study site were abandoned and all subsequent efforts were concentrated around the swamp sites.

Many more A. atlanticus were tested in 1972 than in 1971. Again a high rate of virus isolation was detected, even among newly emerged adults (Table 3, Fig. 2). The overall isolation rate for 1972 approximated that for 1971.

All isolations made from the 1971 and 1972 A. atlanticus pools (total of 92 strains) were neutralized by MHAF made against reference Keystone strain CE and were not neutralized by Jamestown Canyon MHAF. The neutralization test results indicate that all 92 strains from A. atlanticus are similar or identical to the Keystone strain.

A total of four virus isolations were made from the A. canadensis mosquitoes tested. The single 1971 strain and one strain recovered in 1972 were neutralized by reference Jamestown Canyon MHAF and were not neutralized by reference Keystone MHAF. Two strains from 1972 were similar to the strains from A. atlanticus being neutralized only by Keystone MHAF.

Aedes atlanticus is a floodwater mosquito that overwinters in the egg stage and whose larvae occur in deeply shaded pools in upland

forests. Eggs apparently hatch in response to flooding after heavy summer rains. In this study area a substantial rain (minimum of 3 inches in 24-48 hours) is needed to flood these pools. Such rains may occur only once a summer, so that most years only a single brood emerges. However, newly hatched larvae have been found after a second heavy rain. Since these larvae may be either from eggs laid earlier in the year, or from eggs laid during the previous year, it is impossible to state at this time whether or not A. atlanticus is univoltine. Adult females are abundant for a relatively few weeks, during which they readily attack a variety of mammalian and cold blooded hosts during daytime hours.

Isolations of Keystone strain CE made in 1971 and 1972 show a clear association of this virus with A. atlanticus mosquitoes. The remarkably high field infection rate remained roughly constant during the two years studied, as well as throughout each season. In both years, virus was recovered from the very first emerging females. Also, each year, as the number of adults present rapidly increased, there was no dilution detected in the rate of virus recovered. (Fig. 1 and Fig. 2). Two possibilities exist to explain these observations. First, these mosquitoes may have taken an infectious blood meal almost immediately after emergence, rapidly digested it and had virtually no extrinsic incubation period. Second, they may have emerged infected through transovarial virus transmission. Two factors support the latter mechanism. First, engorged mosquitoes were removed before testing, thus eliminating the possibility of these isolations representing viremic blood meals. Second, the likelihood that an entire brood of mosquitoes in two successive years could rapidly acquire infections of the described magnitude is minute. We thus believe, based on the temporal relationship between A. atlanticus populations and isolations of Keystone strain CE made from wild caught females, that this virus is maintained through transovarial transmission of the agent from infected females to their progeny in this area.

The isolations of Jamestown Canyon from A. canadensis do little more than to establish its presence in the mid-Atlantic states.[14,15] This finding is not surprising, since A. canadensis feeds avidly on deer in this area, and deer apparently are significantly involved in the maintenance of this virus in other parts of the United States. The failure to isolate Jamestown Canyon virus from A. atlanticus and its very low infection rates detected in A. canadensis imply a different mechanism of virus amplification and persistence in nature from that described for Keystone virus and A. atlanticus.

d. Demonstration of transovarial transmission

The temporal relationship of isolations of the Keystone strain of CE virus to populations of adult Aedes atlanticus mosquitoes collected on the Del Mar Va. Peninsula in 1971 and 1972 led us to suspect that this strain of CE virus is also maintained at least in part through transovarial transmission. This was based on the observations that (1) A. atlanticus showed very high minimum field infection rates, (2) this

rate remained constant even in the face of significant numbers of emerging adults, and (3) isolations were made from the very first adult A. atlanticus collected and tested.

Aedes atlanticus is a floodwater mosquito which has but one generation of adults on the Del Mar Va. Peninsula most years. Its eggs are deposited in shaded forest depressions and will hatch only after substantial rains have flooded these depressions. Such restrictive requirements allow an accurate estimate of date of hatching and result in a rather uniform development of mosquitoes. In 1973 such conditions allowed us to sample at discrete intervals during the development of the populations of A. atlanticus and be assured a lack of exposure of collected mosquitoes to viremic hosts.

Developmental stages of A. atlanticus mosquitoes were collected from their breeding pools in the Pocomoke Cypress Swamp, 5 km southeast of Pocomoke City, Worcester County, Maryland, and in pools outside Snow Hill, Worcester County, Maryland. Our collections of A. atlanticus were limited to the uplands hardwood-coniferous forest adjacent to the swamp proper. Collections at Snow Hill were in Hardwood-coniferous forests very similar to those at the swamp. The Snow Hill and swamp collecting sites were approximately 15 km apart. Collections were made between 26-31 August 1973 after substantial rains had flooded these sites. Flooding was so great that it eliminated the possibility of sampling from discrete ponds. Larvae had emerged at a time when all breeding areas were inundated, and subsequent draining left larvae in most depressions holding water.

Third and fourth larval instars and pupae were collected by dipping and were transported to a field laboratory alive. Only A. atlanticus larvae were removed for testing. Larvae were pooled into groups of 25 or fewer individuals in a Wasserman tube, and excess water removed by filtration through cloth netting. After removal of water, 0.5 ml of 4% bovine plasma albumin in phosphate buffered saline containing antibiotics was added to each pool. Larvae pools were then frozen at -70°C until processed for virus isolation.

Pupae were placed in enamel pans and allowed to emerge in small holding cages. To avoid possible virus cross contamination, no source of nourishment was provided newly emerged adults. At intervals of not greater than 24 hrs, pans still containing pupae were removed and all emerged adults were killed by chilling at -70°C for 15 minutes. All A. atlanticus were removed, segregated by sex and pooled in groups of 50 or less. Pools were held at -70°C pending processing for virus isolation. The laboratory in which these mosquitoes were reared had never been used for virus work.

Larvae and reared adult mosquito pools were assayed for virus in suckling mice following techniques described earlier. Identification of isolates was done at the first suckling mouse passage level using

plaque reduction neutralization tests on LLC-MK_2 cells. Isolates were tested against mouse hyperimmune ascitic fluids made from CE virus seeds supplied by the American Type Culture Collection and Dr. Wayne Thompson, University of Wisconsin, and against local isolates of CE virus. All reisolation attempts were made by directly plaquing the original mosquito suspension in confluent monolayers of LLC-MK_2 cells.

Mosquito pools were processed at a time when no other viral agents were being handled in the laboratory. Inoculated suckling mice were housed in two separate animal rooms, neither of which had ever been used for virus isolation or passage.

A total of 518 larval A. atlanticus collected from the swamp comprising 21 pools were tested for virus. Reared females were processed in 35 pools representing 1,688 mosquitoes, while 2,040 reared males were tested in 42 pools. From these pooled specimens, 1 virus isolate was obtained from larvae, 3 from reared adult females and 5 from reared adult males (Table 4). Virus was recovered from reared adults taken from both the swamp and the Snow Hill larval collection sites.

All isolates were identified as the Keystone strain of CEV by PRNT. Eight of the nine isolates were reisolated on LLC-MK_2 cells with titers varying from 25 to 2500 pfu per 1.0 ml original mosquito suspension (Table 5).

The fact that virus was isolated from both larvae and reared adult A. atlanticus processed under conditions designed to minimize the possibility of introducing contaminant virus, and the subsequent reisolation of relatively high titered virus from these pools strongly support the validity of these isolations. The epidemiological association of A. atlanticus with the Keystone strain of CE virus presented earlier add further credence to the conclusion that this agent is maintained at least in part through transovarial transmission of the virus by A. atlanticus in this area.

In retrospect, transovarial maintenance of CE virus is not surprising. Most CE viruses occur in temperate zones where year-round transmission of virus by mosquitoes is improbable. The principal vectors of CEV do not overwinter as adults (rather as eggs) which precludes the possibility of virus maintenance by overwintering adults. Further, primary vertebrate hosts of CE are non-migratory mammals, so that reintroduction of virus through migrating viremic hosts is unlikely. However, the possibility of CE maintenance by recurrently viremic vertebrate hosts, similar to that demonstrated with WEE and coldblooded vertebrates cannot be eliminated without further study.

In early October a number of larval A. atlanticus were again collected from their breeding pools. All larvae were pooled for virus isolation attempts in suckling mice as described earlier. None were reared to adults. Weather conditions prevented the larvae in the field

from maturing to adults. A total of 108 larval pools were tested representing 2695 larvae, and a total of 10 virus strains were recovered. All of these strains recovered were identified as the Keystone subtype of CE by PRNT. (Details reported under work unit 166.) Reisolation attempts are still in progress, but to date only 2 of 7 pools tested have been reisolated in tissue culture, and both of these pools were very low titered.

These additional isolations from larval A. atlanticus adds further support to the observation made earlier that this species is capable of transovarial transmission of the Keystone subtype of CE. The overall isolation rate of those adult A. atlanticus females tested in suckling mice in 1972 was 1:279, and for the 1973 larval A. atlanticus was 1:286. Since collections of A. atlanticus in 1972 were centered primarily in the area of woodland pools supporting breeding of this species (Site 10), it was questionable whether the isolation rate from A. atlanticus would reflect the distribution pattern of virus in the entire swamp. Altogether, 37 virus isolates have been obtained from 106 pools of A. atlanticus collected from various collection sites in the swamp. The isolation rate from A. atlanticus collected at Site 10 is 1:283, and this compares favorably with the overall isolation rate of 1:279.

e. Mosquito bloodmeal identifications

(1) Eastern U.S. mosquitoes. As done in previous years, mosquito bloodmeals were identified by the capillary tube precipitin test method. The exact techniques used have been presented in detail in previous Annual Reports. A total of 514 engorged mosquitoes collected from the Pocomoke Cypress Swamp, Md. and representing 10 species were identified. All specimens were collected in the routine CDC miniature light trap collections, and from the special light trap collections made to monitor the A. atlanticus population. These later collections explain the increased number of A. atlanticus tested this year.

Results of screening tests and totals tested are presented in Table 6. Specific bloodmeal determinations are presented in Table 7 and 8. The discovery that A. atlanticus mosquitoes are capable of transmitting the Keystone subtype of CE transovarially adds greater importance to the feeding patterns of this species if auxillary virus maintenance routes are to be discovered. With this in mind, the increase in poikilothermic host feeding by A. atlanticus is of special interest. While it had previously been recorded as feeding on cold-blooded hosts, such hosts had not been considered a major source of nourishment. However, with this observation of approximately 20% of those bloodmeals identified being from cold-blooded vertebrates, this clearly establishes the importance of such hosts as an important blood source for this species. The possibility of cold-blooded vertebrates being involved in the maintenance of CE in the swamp is discussed under work unit 166.

As has been suggested in the past, A. atlanticus remains primarily a mammal feeder. Among the mammalian bloodmeals identified, the squirrel

feedings are especially interesting since squirrels from the swamp environs have frequently been found to have CE antibody. Further, information reported under work unit 166 suggests that squirrels may circulate CE virus at high enough titers to infect feeding mosquitoes.

The catholic feeding patterns of A. canadensis is again confirmed. As in the past, mammals appear to be the primary blood sources, but again significant feeding on poikilothermic hosts was detected. Too few engorged mosquitoes of the other species were collected to draw meaningful conclusions regarding their feeding patterns. However, through the continued routine collection and identification of these engorged specimens we are accumulating good host feeding data on even the less common mosquito species found in the swamp. A comparison of feeding patterns of A. atlanticus and A. canadensis based on bloodmeals identified from 1969 to 1972 is presented in Figure 3.

(2) Other bloodmeal determinations. A number of bloodfed insects were collected by the WRAIR field team while visiting Zaire. All bloodmeals identified proved to be of mammalian origin; specific determinations are presented in Table 9.

f. Bionomics of floodwater mosquitoes

(1) Collection of eggs. Knowledge of the biology of the major floodwater mosquito species in the upland forest study area is scanty. Work was begun, in early February, 1974, to study the location, collection, and handling of eggs of the 3 principle species, Aedes atlanticus, Aedes canadensis, and Psorophora ferox. To avoid the necessity of obtaining material only during a few weeks of the year, when adults are active and easily collected, it was necessary to locate a source of eggs and to develop techniques for collecting and separating large quantities of eggs from soil. Approximately 15,000 eggs were collected from February to May, 1974.

Sites best suited for egg collections proved to be either natural depressions with moderate to steep profiles or very old drainage ditches with steep sides situated in heavily shaded areas. Greatest numbers of eggs were found along the steep ditch profiles in or at the edge of mature forest. Fewer eggs were found in shallow depression with shallow profiles in clearings or in recently cut forest.

Eggs were collected by gently scraping the recently accumulated leaf and pine needle litter away, leaving older litter and duff intact. The top 2-3 cm. of litter and soil was carefully lifted with a bricklayer's trowel and placed in plastic bags.

(2) Separating Eggs from Soil. Initially, soil samples were washed in a Horsfall Egg Separator, but failure to obtain eggs forced abandonment of this device. Subsequently, soil samples were placed in

the top sieve of a vertical 4-sieve series, with the finest mesh at the bottom (60 meshes/sq. cm.) Fine debris and eggs were separated from coarse organic and inorganic material in the top sieve, by directing a jet of water on the debris, the finer material washing through the coarser sieves to the 60 mesh screen. Most floodwater mosquito eggs and fine debris were caught in the 60 mesh screen, with a few eggs washing through to the 45 mesh screen. All material, including eggs on the 60 and 45 mesh sieves were then washed into a saturated salt solution and stirred. Mosquito eggs and some organic debris floated in the salt solution; silt and other inorganic debris sank. Eggs were then decanted through a filter paper cone.

(3) Egg sorting and storage. Eggs were collected from the wet filter paper under a stereoscopic microscope with a fine brush, identified to species, sorted and placed on wet filter paper in plastic petri dishes. The petri dishes were sealed with masking tape and labelled. Eggs were stored in a controlled climate chamber with a temperature cycle of 68°F mm., 88°F max. and RH approximately 85%. The temperature regime selected was taken from a thermograph record of 13 August 1973 at the Pasture Point weather station.

(4) Effect of photoperiod on breaking of diapause of floodwater mosquitoes. To determine if floodwater mosquito eggs are in a diapause state in the spring which is broken by a photoperiod trigger, soil samples were collected from the field on 8 March 1974 and 3 May 1974, brought to the laboratory for processing. One-seventh of the 8 March sample was flooded immediately after its arrival at the laboratory with a mixture of distilled and hay infusion water in a 10:1 ratio. The remaining soil was divided into 2 parts, one half placed in enamel pans in a climate chamber at constant temperature (24°C) and RH (85%) and LD 8:16, the other half in another climate chamber with LD 16:8. One third of each soil sample was flooded on the 7th, 14th and 21st day following collection respectively. All larvae were removed 3 days after flooding and the samples sieved for eggs. Results are present in Table 10.

Eggs collected in the field on 2 May 1974 were processed in the same way as discussed above. Since no Aedes canadensis eggs were collected in the samples, this species is not included in the tabular data. Results of flooding the 2 May sample are presented in Table 11.

For both the 8 March and 2 May samples, there is no difference in the hatching rate of eggs of any of the species exposed to long day conditions or short day conditions. The relatively low hatching rates occasionally observed, such as the 68.75% for Ps. ferox on Day 28, LD 8:16 may be due to the small sample size (17 eggs). It was concluded that either A. atlanticus and P. ferox are not in diapause on 8 March or else photoperiod conditions do not determine termination of diapause in these species. Additionally, photoperiod differences do not affect the rate at which eggs of the various species are conditioned

to habit. Eggs at given stages in conditioning habit at approximately the same rate and in the same time interval when exposed to either long day or short day conditions (see discussion of conditioning below). Future experiments will be conducted to determine when the onset of diapause occurs in floodwater mosquito eggs, how long diapause lasts and when it is terminated. A. canadensis eggs are obviously not diapausing in March.

(5) Egg storage. Following sieving of mosquito eggs from soil samples, all eggs were placed on wet filter paper in plastic petri dishes sealed with masking tape. To insure maximum hatch rate when subsequently flooded, eggs were kept in a climate chamber with a temperature range of 68°-80°F and RH of 85%.

To determine the hatch rate of eggs held in storage for 40-90 days, 3,714 eggs were flooded on 6 May 1974. The hatching rate varied from 0 for A. atlanticus held 60-75 days to 52.43% for Psorophora ferox eggs held 45 days. The hatch rate for all samples is given in Table 12. The hatch rate for all species and all samples was 14.97%. Psorophora ferox eggs had the greatest hatch rate in most samples, followed by Aedes canadensis and Aedes atlanticus.

The poor hatch rate for stored eggs is probably due to desication of eggs in the climate chamber or an unfavorable combination of temperature and humidity th[illegible]onditions the eggs, making them refractory to hatching.

In an attempt t[illegible]ve the hatch rate of stored eggs, 2,447 eggs were moistened and returned to the climate chamber for 14 days. All eggs were then flooded. Results are not yet complete, but initial hatch rates are given in Table 13. Hatch rate for all eggs was 23%. Compared with unmoistened eggs in Table 5, Aedes atlanticus showed the greatest rate of increase in hatching. When all hatching data are complete, hatch rate is estimated to be about 30%, about 50% greater than for unmoistened eggs, still far below adequate per colonization purposes.

(6) Spatial distribution of floodwater mosquito eggs. From the approximately 15,000 floodwater mosquito eggs collected from February-May 1974, preliminary data allow a summary of spatial distribution of eggs of the 3 major species studied (Aedes atlanticus, Aedes canadensis and Psorophora ferox). Data are most complete for a small drainage ditch (A-3) that has subsequently been destroyed by a logging operation. This ditch had a steep profile and eggs were overlain by a leaf litter layer. Table 14 summarizes the distribution of eggs in the 3 horizons (elevations) sampled. Horizon A was the highest point of flooding in the ditch, horizon C was the slope just above the bottom of the ditch. Each horizon was about 8 cm. high.

Based on rather crude sampling methods. Aedes canadensis eggs were most abundant in the top horizon, A. atlanticus most abundant in the lower horizon (based on percent of eggs recovered), and P. ferox most abundant in the middle horizon. The relatively low number of A. canadensis eggs in C horizon may be due to prior hatching of many eggs flooded in January and February.

Number of eggs collected in a given sample was extremely variable, from 0 to over 500, indicating a strongly clumped distribution. Possibly, the female mosquitoes lay many eggs in particular spots where moisture, microclimate and substrate are most attractive, avoiding other superficially attractive sites.

Virtually all eggs occurred in the top 1-2 cm. in the soil-litter, on heavily shaded sites, obviating the necessity of taking very deep samples of mud and sand. Few eggs have been collected in exposed ditches.

Preliminary sampling in another drainage ditch (AS-1) indicates that the egg distribution pattern given in Table 7 is similar in other ditches in the upland forest, with eggs also highly localized at particular sites along the ditch.

(7) Conditioning of floodwater mosquito eggs. Following termination of diapause in floodwater mosquito eggs, they must be "conditioned" for a certain period of time before they will hatch. From numerous conditioning experiments conducted from February to May 1974, it appears that Aedes canadensis requires only a very short conditioning period before all eggs flooded will hatch. Eggs collected in February all hatched immediately when flooded in the laboratory. Larvae of A. canadensis were seen in the field in January. Eggs of Aedes atlanticus and Psorophora ferox are not conditioned to hatch immediately until probably early to late June, although a few eggs will hatch as early as May. "Conditioning" of A. atlanticus and P. ferox eggs in the laboratory require a variable number of days at temperatures above 70°F. A. atlanticus eggs require a longer conditioning time than those of P. ferox. Table 15 summarizes conditioning information on 3 species to date.

As environmental temperature increases, the conditioning time of A. atlanticus and P. ferox decreases. For samples collected in March, conditioning in P. ferox is complete in 7 days at 78°F, in 14 days in A. atlanticus. By May, conditioning time for both A. atlanticus and P. ferox was less than 7 days since the hatch rate was essentially 100% for both species at Day 7.

Eventually, it should be possible to plot increasing temperature against decreasing conditioning times for A. atlanticus and P. ferox by flooding subsamples daily to determine exactly how long conditioning requires from termination of diapause to completion of the conditioning process. This information will be used to predict when heavy rains can

be expected to hatch most A. atlanticus and P. ferox eggs in the field. Conditioning for both species is probably complete by late June.

(8) Effect of refrigeration on egg hatching. To determine if eggs in soil samples could be kept refrigerated until needed, soil samples were collected in the field, divided into 2 parts each and returned to the laboratory. One set of subsamples was refrigerated for 14 days at 5°C, the other set of subsamples were conditioned at 78°F, RH 78% LD 15:9 for 4, 10 and 14 day respectively. After 74 days, the refrigerated subsamples were removed and conditioned at 78°F, RH 78%, LD 15:9 for 3, 10 and 15 days respectively. Problems with hatching medium, resulting in erratic hatching, prevented drawing many definitive conclusions at this time, but preliminary results from a subsequent experiment indicate that eggs can be stored for at least 30 days at 5°C in soil inside plastic bags and will hatch normally, provided they are conditioned for about 10 days.

If refrigeration proves effective, this might replace the presently inadequate method of egg storage in a climate chamber. Eggs can be removed from the refrigeration and served without hatching for study, or left in soil until needed for colonies or experiments.

(9) Colonization of floodwater mosquito species. As of 15 May 1974, only Psorophora ferox has been successfully established as a self-sustaining colony. Aedes canadensis is easily maintained, but must be force mated. Aedes atlanticus has not responded well to colonization attempts.

Psorphora ferox - this species has adapted very well to the laboratory. Females take blood readily from chicks, egg production is excellent on moist pans of sphagnum moss, and the hatching rate of the eggs, following a holding period of 14 days for embryonation is excellent with no indication of diapause. Larval survival and emergence rate of healthy adults are excellent. Mating occurs naturally in the large 1x1x1 m. cages.

Aedes canadensis - this species has adapted well to the laboratory, but will not mate in enclosed cages yet. Females feed readily on rabbits or guinea pigs, egg production is excellent on moist sphagnum moss. Hatch rate of eggs is excellent with no indication of diapause. Larval survival and adult emergence rates are excellent. Adults must be force mated, but eventually the goal is for a self-sustaining colony.

Aedes atlanticus - this species has responded poorly to colonization attempts. Adult survival in cages is good, females feed readily on rabbits or guinea pigs. Egg production is poor on moist sphagnum, with many eggs collapsed. Hatch rate of eggs is poor; probably most eggs are not viable. Larval survival is fair; larvae tend to be small, possibly due to overcrowding, survival rates of larval and pupae are poor. Emergence rate from pupae is very poor, with many pupae dying before emergence of the adults. Adults will not mate in enclosed cages, and

females will not respond readily to force mating as do those of A. canadensis. Sperm transfer is poor in force-mated females.

Some problems encountered in colonizing A. atlanticus may be overcome by rearing at slightly lower temperatures, careful feeding of larvae, prevention of overcrowding and other possible adverse factors, but much work remains to be done before A. atlanticus can be established as a self-sustaining colony.

2. Comparative studies of two North American mosquito species, Culex restuans Theobald and C. salinarius Coquillett.

a. Introduction

Culex restuans Theobald and C. salinarius Coquillett are 2 common Nearctic mosquitoes whose ranges largely overlap. Adults of the former species emerge earlier in the spring and persist longer into the fall. Moreover, the range of C. restuans extends farther north. Eldridge et al. 1972 has described the seasonal geographic distribution of these 2 species and presented data which showed interspecific differences in blood-feeding patterns and ovarian development in response to various combinations of temperature and photoperiod in the laboratory. C. restuans females showed a marked reduction of blood-feeding and exhibited ovarian diapause (or so-called gonotrophic dissociation) in the response to a combination of short photophase and low temperature. C. salinarius females showed a significant reduction of blood-feeding under short photophase conditions, but only at the lowest conditioning temperature (15°C.) and did not exhibit ovarian diapause. The results suggested that both species underwent true diapause permitting overwintering in the adult stage, but that qualitative and quantitative differences in their response to environmental conditions contributed to the differences in geographic range and phenology.

Reported here is an extension of the earlier study with observations on the effect of temperature and photoperiod on ovarian follicle growth in non blood-fed female mosquitoes of the 2 species as well as the influence of temperature upon rates of adult eclosion, blood digestion, and ovarian development. The objective of these experiments was to find differences between the species which would be consistent with the differences in seasonal and geographic distribution.

Culex salinarius females were obtained from our self-mating laboratory colony established in 1969 from larvae collected in a swamp near Pocomoke City, Maryland. The C. restuans females were reared from egg rafts collected in rain water-filled stainless steel pans placed outside our laboratory in Washington, D.C. during the spring months. Egg rafts were constantly present in the pans from April through June. Egg rafts were isolated after collection and maintained in individual vials until specific identification of larvae was made. Other than this, larval rearing procedures were the same for both species.

Simulated environments were produced in modified BOD incubators. Details of the light sources and temperature compensation system have been described previously (Eldridge et al. 1972).

In all cases, larvae were reared at a temperature of 27°C and a photoperiod of 16:8 L:D. Separation into experimental groups was made randomly at the time of pupation. Other experimental details are given under the results of the particular experiment involved.

b. Rate of adult eclosion at different temperatures. In this experiment, a large number of pupae of both species were collected within 24 hours of pupation. The collection of pupae was made at the mid-point of time of pupation in the larval pans. Although the pupae were a mixture of males and females the latter predominated. Each batch of pupae was randomly divided into 4 groups of 100 pupae each and placed in cages in incubators at 4 different temperatures: 10°, 15°, 20°, and 25°C. Photoperiod was 16:8 L:D throughout the experiment. Emerged adults were removed and recorded daily.

The results are shown in Fig. 4. All pupae eventually underwent ecdysis, even at 10°C. At 25°C, the peak of adult eclosion for both species occurred on day 2 post-pupation (when pupae were 48-72 hours old). At 20°C, the 2 emergence curves are similar, with the majority of pupae of both species undergoing ecdysis on day 2. However, twice as many adults of C. salinarius emerged on day 3 compared with C. restuans. At 15°C, a clearcut difference is observable between the species. The peak of adult eclosion occurred a full day later in C. salinarius. At 10°C, the median time of eclosion is approximately 9 days for C. salinarius, 6 days for C. restuans.

c. Rate of blood digestion. A large batch of 3-4 day old females of each species was provided a shaved chick as a blood meal source. Females which took a complete blood meal were randomly divided into 4 groups and placed in cages in incubators at the following temperatures: 10°, 15°, 20°, and 25°C. Photoperiod was 16:8 L:D throughout the experiment. A single female was removed from each treatment daily. The status of blood digestion was estimated by the Sella method (Sella 1920). Each female was then dissected and the length of the ovary measured as an indication of the degree of ovarian development (see below). Rates of blood digestion are shown in Fig. 5.

d. Rate of ovarian development. As mentioned above, the same blood-fed females removed for the purpose of estimating rate of blood digestion were also dissected to determine the degree of ovarian development. The results of these dissections are shown in Fig. 6. The overall pattern seen in the previous experiments is evident. Ovarian development proceeds at approximately the same rate in both species at 25°C; is slower at lower temperatures in C. salinarius. The lower the temperature, the greater the disparity in rates.

In females of C. restuans maintained under conditions of short photophase and low temperature (15°C/8:16 LD), most ovarioles remained in a diapause state (stage N). At low temperature and long photophase, however (15°C/16:8 LD) follicles of most ovaries developed to the resting stage from which full ovarian development will occur after a blood meal (stage Ib). At 25°C, C. restuans follicles developed equally under both photoperiod regimes, but did not attain as large a size, on the average, as did long photophase follicles at 15°C. Furthermore, more females maintained at 15°C/16:8 L:D had follicles reaching stage Ib than did any of the females held at 25°C at either photoperiod.

In Culex salinarius, no photoperiod influence on follicular development could be seen. The influence of temperature, however, was striking. At 15°C, 30-50% of the ovaries of females examined had follicles in the diapause state. Most ovaries had follicles in stage Ia, but none had developed past this point. At 25°C, however, all ovaries had follicles which had developed at least to stage Ia and some which had gone to stage I-II.

d. Discussion. The results presented here and those presented in an earlier study (Eldridge et al. 1972) suggest that the physiological events accompanying overwintering in these 2 species differ considerably, at least in mid-Atlantic U.S. populations. The higher rates observed at low temperatures in the 3 phenomena observed are consistent with the earlier appearance and later persistence of C. restuans. Apparently, mid-Atlantic populations of C. salinarius do not undergo ovarian diapause in response to autumn photoperiod conditions. The species does apparently overwinter in the adult stage, however, in the Maryland-Virginia area of the U.S. After intensive searching, we captured a single female C. salinarius in January of 1973 in a stable open at one end. This is one of the few instances of this species being collected during the winter months above 35°N latitude.

The differences in physiological response to photoperiod and the more northern range of C. restuans are consistent with the hypothesis than an ovarian diapause induced by a short photophase is associated with an adult overwintering mechanism which permits survival under very stringent winter conditions. Implicit in this hypothesis would be that the physiological differences would hold even in populations of C. salinarius at the northern limit of the range of the species, although one would expect some northern extension during the summer months past the point where the females could survive winter conditions. If this is the case, C. restuans would survive the winter in a dormant state classified by Mansingh (1971) as diapause; C. salinarius in oligopause. To test how well these species fit the criteria presented by Mansingh, especially that of "tolerance to adversity", samples of the species should be tested for tolerance to simulated winter conditions in the laboratory.

3. Biosystematics of mosquitoes

A monograph on the new subgenus Bothaella of the genus Aedes was published. Additional information on the taxonomy of Aedes (Diceromyia)

was published and included are new species and one species previously placed in the subgenus Aedimorphus. Aedes consonensis, a new species of the subgenus Neomacleaya was described. The complex arrangement of the male genitalia of Neomacleaya was described and elucidated.

Unnamed setae on the larval cervical membrane were reported. Their taxonomic importance and possible phylogenetic significance were discussed with special reference to Aedes subgenera Finlaya and Ochlerotatus.

A paper was published outlining the detailed procedures for preparing and dissecting the female genitalia of aedine mosquitoes for taxonomic examination. A taxonomic glossary of the female genitalia of mosquitoes was also presented and all significant structures illustrated.

Studies are virtually completed on a new interpretation of the subgenus Verrallina of Aedes which is confined to the Oriental, Australian and Oceanic Regions. Verrallina will be redescribed and the 91 included species will be assigned to section and series categories. Considerable new information on the biology, distribution, descriptions, synonomy and corrections to published species and numerous previously undescribed immature stages will be available. Four new species and numerous previously undescribed immature stages will be covered.

Work is approximately one half finished on a comparative study of the female genitalia of the 37 subgenera of Aedes. These studies indicate that the female genitalia of the tribe Aedini are of taxonomic value at the generic, subgeneric and species group levels. Descriptions have been completed for 52% of the subgenera and illustrations for 67% of the subgenera.

Some progress has been made on revisions of the subgenera Paraedes, Edwardsaedes and Ochlerotatus in Southeast Asia and adjacent areas.

Work has continued on a revision of the subgenus Christophersiomyia of Aedes. Members of this subgenus occur throughout tropical Asia and the southwestern South Pacific Islands. The subgenus consists of 6 nominal species:

annulirostris (Theobald)
brayi Knight
chionodes Belkin
gombakensis Mattingly
ibis Barraud
thomsoni (Theobald)

The medical importance of species of Christophersiomyia has not been investigated. A. gombakensis has been taken biting man in Malaysia.

All specimens, including immature stages and male genitalia mounted on slides, in the MEP/USNM collections have been examined. New distributional records have added greatly to the known geographic ranges of 4

of the species. Preliminary work is proceeding on descriptions of previously - undescribed immature stages and in writing improved descriptions for others described in the early literature.

Fourth-stage larvae and pupae are known for annulirostris, gombakensis and thomsoni. The larvae and pupae of all 3 species are being redescribed. In addition, the larva and pupa of ibis are being described for the first time.

Adult males have been described for all species except ibis. Male specimens of this species exist in good numbers in our collection and are being described for the first time. Male terminalia of all species except gombakensis show very few differences.

At this time it seems that brayi resembles ibis so closely that the former may have to be placed in synonymy. Specimens of brayi from the Philippines have the fore-and midfemora paler both anteriorly and posteriorly than specimens of ibis from Thailand. Two specimens from Cambodia are intermediate in this respect. Other characters for separating the 2 species, including those in the male terminalia, are variable. The examination of immature stages and reared specimens from the Philippines would be most helpful in solving this problem.

A bibliography of 35 entries has been compiled for Christophersiomyia. A few of these have been new additions to the extensive MEP bibliography of mosquito literature.

Illustrations of adults of 4 species have been completed. Preparation is now underway to begin illustrations of the immature stages. An illustrator has been assigned to this work.

Conclusions and Recommendations

1. A high rate of infection of Aedes atlanticus mosquito by the Keystone subtype of California encephalitis virus was detected. Jamestown Canyon subtype exists also in the Pocomoke Swamp study area, but apparently at much lower frequency. Aedes atlanticus appears to be involved only with the Keystone subtype, whereas Aedes canadensis have been found infected with both Keystone and Jamestown Canyon. The infection rate in A. canadensis for both subtypes is only a tenth to a hundredth that of Keystone in Aedes atlanticus. Further studies should be done to determine susceptibility of these 2 species for both CE subtypes.

2. The pattern of CE isolations and the population dynamics of Aedes atlanticus suggested that transovarial transmission of Keystone was occurring in this species. The isolation of Keystone from field-collected larvae and from male and female mosquitoes reared from field-collected larvae is virtual proof of its occurrence. Further studies should address the question of infection of uninfected mosquitoes in nature, as well as transmission of virus from infected mosquitoes to vertebrates.

3. Cold-blooded vertebrates were shown to comprise about a fifth of the blood meals for Aedes atlanticus collected during the year. Squirrels were also shown to be frequent hosts. The role of cold blood vertebrates in arbovirus ecology has never been satisfactorily studied.

4. Substantial differences among species of floodwater mosquitoes were found in relation to embryonic diapause; conditioning, hatching, and distribution of eggs. Aedes atlanticus and A. canadensis eggs often occur in the same woodland pool, but eggs of the former species are deposited closer to the bottom of the pools than those of the latter. Eggs of A. canadensis require no conditioning when collections are made March to June. Those of A. atlanticus and P. ferox require varying times of conditioning at spring-summer temperatures (ca. 25°C) before hatching can occur. More studies are needed with eggs collected at various times of the year, especially autumn.

5. Culex salinarius and C. restuans from the mid-Atlantic States vary considerably in their ability to function at low (ca. 10°C) temperatures. Culex restuans undergoes ovarian diapause under the influence of short photoperiod/low temperature conditions whereas C. salinarius does not. These differences suggest a differential ability to survive winter temperatures during hibernation, and thus a differing likelihood of serving as an overwinter hosts of Group A. Arboviruses. Further research should be done with infected females of these species under simulated winter environments.

6. Biosystematic studies of Aedes mosquitoes of Southeast Asia have continued. Studies are nearly completed on 2 subgenera. Verrallina and Christophersiomyia. Further research should be done on the subgenera Paraedes, Edwardsaedes, and Ochlerotatus.

TABLE 1

Adult female mosquitoes collected by light trap and tested for infection with California encephalitis virus strains, 1972

Species	Number Collected	CE Isolates
Aedes canadensis	63,216	3[1]
Aedes atlanticus	19,110	60[2]
Aedes cantator	8,559	
Psorophora ferox	6,894	
Aedes sollicitans	4,856	
Aedes taeniorhynchus	2,942	
Aedes vexans	832	
Aedes triseriatus	179	
Aedes infirmatus	24	
Psorophora columbiae (=confinnis)	8	
Psorophora varipes	1	
Total	106.621	63

1. 2 Keystone subtype, 1 Jamestown Canyon subtype

2. All Keystone subtype

TABLE 2

Data on filtration, chloroform inactivation and identification of CE virus isolates, 1971

Pool #	Filtration (Seitz S pad)	Inactivation by 20% chloroform			Identification
		Control titer*	Treated titer	Log inactivated	
4	+	7.7	3.0	4.7	Jamestown Canyon
84	+	6.7	1.2	5.5	Keystone
366	+	6.7	<1.0	>5.7	Keystone
373	+	7.0	2.2	4.8	Keystone
374	+	7.1	1.5	5.6	Keystone
414	+	6.5	<1.0	>5.5	Keystone
455	+	7.4	1.9	5.5	Keystone
464	+	7.3	1.7	5.6	Keystone
477	+	7.2	2.0	5.2	Keystone
484	+	7.2	2.1	5.1	Keystone
488	+	7.1	1.5	5.6	Keystone
505	+	6.5	1.5	5.0	Keystone
524	+	7.3	<1.0	>6.3	Keystone
525	+	7.3	<1.0	>6.3	Keystone
533	+	6.9	2.1	4.8	Keystone
534	+	7.2	2.0	5.2	Keystone
537	+	6.5	1.1	5.4	Keystone
538	+	6.7	<1.0	>5.7	Keystone
539	+	7.1	<1.0	>6.1	Keystone
541	+	6.5	<1.0	>5.5	Keystone
542	+	6.0	1.5	4.5	Keystone
544	+	6.9	1.7	5.2	Keystone
547	+	7.1	1.3	5.8	Keystone
548	+	5.7	<1.0	>4.7	Keystone
550	+	6.5	<1.0	>5.5	Keystone
553	+	7.5	<1.0	>6.5	Keystone

TABLE 2 (Continued)

Pool #	Filtration (Seitz S pad)	Inactivation by 20% chloroform Control titer	Treated titer	Log inactivated	Identification
557	+	7.2	<1.0	>6.2	Keystone
560	+	6.6	<1.0	>5.6	Keystone
561	+	6.3	<1.0	>5.3	Keystone
577	+	6.4	1.5	4.9	Keystone
578	+	6.7	<1.0	>5.7	Keystone
597	+	7.6	1.5	6.1	Keystone
598	+	6.6	<1.0	>5.6	Keystone

* Titer of virus measured as $\log_{10}$ SM ic LD_{50}/0.02 ml

TABLE 3

Virus isolation rates from A. atlanticus by date of collection, Pocomoke Cypress Swamp, Maryland, 1972

Collection date	A. atlanticus tested	Number of isolations	Isolation rate
3 July	513	3	1:171
10 July	3,393	12	1:282
11 July	4,320	8	1:540
12 July	3,643	9	1:404
16 July	1,171	5	1:234
18 July	774	4	1:193
19 July	1,150	7	1:164
26 July	431	1	1:431
31 July	2,938	3	1:979
TOTALS	18,333	52	1:352

TABLE 4

CE virus isolations made from larvae and reared adult A. atlanticus, Pocomoke Cypress Swamp, Maryland and Snow Hill, Maryland 27-31 August 1973

Location	Stage	No. collected	No. isolations	Isolation rate
Pocomoke Cypress	larvae	518	1	1:518
Swamp, Maryland	reared adult ♀	1028	3	1:343
	reared adult ♂	990	2	1:445
Snow Hill, Maryland	reared adult ♀	660	0	0:660
	reared adult ♂	1050	3	1.350

TABLE 5

Results of reisolation attempts made on 1973 CE isolations made from larvae and reared adult A. atlanticus mosquitoes, Pocomoke Cypress Swamp (PCS, Maryland and Snow Hill, (SH), Maryland, 1973

Isolation number	Source	Locality	Titer on reisolation (pfu/1.0 ml)
T 73-4	reared ♀	PCS	1750
T 73-15	larvae	PCS	2500
T 73-17	reared ♂	PCS	500
T 73-21	reared ♀	PCS	500
T 73-29	reared ♂	PCS	75
T 73-39	reared ♀	PCS	1750
T 73-63	reared ♂	SH	failed to reisolate
T 73-66	reared ♂	SH	25
T 73-34	reared ♂	SH	75

TABLE 6

Results of tests of screening antisera of engorged mosquitoes collected in Pocomoke Cypress Swamp, Maryland, 1972

Mosquito species	Number of mosquitoes tested	SCREENING ANTISERA			
		Bird positive	Mammal positive	Reptile positive	Negative
Aedes atlanticus	305	1	236	62	6
Aedes canadensis	168	0	113	55	0
Aedes infirmatus	1	0	1	0	0
Aedes taeniorhynchus	2	0	2	0	0
Aedes triseriatus	1	0	0	1	0
Aedes vexans	12	0	12	0	0
Culex salinarius	6	2	3	1	0
Culiseta melanura	2	2	0	0	0
Mansonia perturbans	1	0	1	0	0
Psorophora ferox	16	0	15	1	0
TOTAL	514	5	383	120	6

TABLE 7

Specific mammal identification of engorged mosquitoes collected in Pocomoke Cypress Swamp, Maryland, 1972

Mosquito species	MAMMAL POSITIVE															
	Bovine	Deer	Dog	Goat	Horse	Human	Opossum	Pig	Rabbit	Raccoon	Rat	Squirrel	Deer-bovine, deer-goat, goat-bovine, deer-goat-bovine	Negative	Unidentified	Double feeding
Aedes atlanticus	4	69	1	36	0	4	2	3	34	16	0	25	46	6	2	6
Aedes canadensis	3	25	0	16	5	0	1	0	8	8	0	1	45	0	1	0
Aedes infirmatus	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Aedes taeniorhynchus	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
Aedes vexans	0	1	0	8	1	0	0	0	2	2	0	0	1	0	0	3
Culex salinarius	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0
Mansonia perturbans	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Psorophora ferox	0	6	0	5	0	0	0	0	0	3	0	0	1	0	0	0

TABLE 8

Identification to bird order of engorged mosquitoes collected in Pocomoke Cypress Swamp, Maryland, 1972

Mosquito species	BIRD POSITIVE				
	Ciconiiformes	Columbiformes	Gruiformes	Passeriformes	Unidentified
Aedes atlanticus	0	0	0	1	0
Culex salinarius	0	0	0	2	0
Culiseta melanura	0	0	0	2	0

TABLE 9

Bloodmeal identifications of insects collected in Zaire, West Africa in 1973

Species	No. Tested	Blood meal		
		Human	Sheep	Bovine
Glossina palpalis	14	11		1
Anopheles gambiae complex	5	4	1	
Culex sp.	6	5	1	

TABLE 10

Hatching of floodwater mosquito egg collected 8 March 1974 and held under two different photoperiod regimes

Days conditioned before flooding	Species	Percent hatching 16:8, L:D	8:16, L:D
0	Aedes canadensis	100	
	Aedes atlanticus	0	
	Psorophora ferox	0	
7	Aedes canadensis	100	100
	Aedes atlanticus	44.8	36.2
	Psorophora ferox	100	97.4
14	Aedes canadensis	100	80.0
	Aedes atlanticus	90.3	96.9
	Psorophora ferox	100	96.4
21	Aedes canadensis	100	100
	Aedes atlanticus	100	98.5
	Psorophora ferox	100	96.4

TABLE 11

Hatching of floodwater mosquito eggs collected 2 May 1974 and held under two different photoperiod regimes

Days conditioned before flooding	Species	Percent hatching 16:8, L:D	8:16, L:D
0	Aedes atlanticus	0	
	Psorophora ferox	3.6	
7	Aedes atlanticus	91.3	100
	Psorophora ferox	100	93.1
14	Aedes atlanticus	100	100
	Psorophora ferox	100	100
21	Aedes atlanticus	80.0	100
	Psorophora ferox	100	100
28	Aedes atlanticus	95.0	100
	Psorophora ferox	85.7	68.7

TABLE 12

Hatching of floodwater mosquito eggs after 40-90 days of storage at 20-27° C. and 85% R.H.

Species	No. eggs	No. larvae	Percent Hatch
Aedes atlanticus	1,494	92	7.4
Aedes canadensis	666	81	17.8
Psorophora ferox	1,554	367	20.6

TABLE 13

Hatching of floodwater mosquito eggs after 40-90 days of storage at 20-27° C. and 85% R.H., then 14 days of moistening

Species	No. eggs	No. larvae	Percent hatch
Aedes atlanticus	1,068	235	22.0
Aedes canadensis	164	30	18.3
Psorophora ferox	1,215	411	33.8

TABLE 14

Spatial distribution of floodwater mosquito eggs in a woodland ditch (A-s) in Wicomico County, Maryland

Horizon*	No. Samples	Species	No. eggs	Eggs/ sample	Percent in Horizon
A	7	Aedes canadensis	727	61.0	49.5
		Aedes atlanticus	146	20.9	16.9
		Psorophora ferox	290	41.4	33.6
B	7	Aedes canadensis	336	48.0	29.2
		Aedes atlanticus	406	58.0	35.3
		Psorophora ferox	407	58.1	35.4
C	6	Aedes canadensis	123	20.5	18.9
		Aedes atlanticus	373	62.2	57.4
		Psorophora ferox	154	25.7	23.7
		TOTAL	2,662	133.1	

*Elevations within ditch. A Horizon is near top of ditch, B is near middle, C is close to bottom.

TABLE 1[illegible]

Percent hatch of eggs of the 3 major upland forest floodwater mosquitoes of the Pocomoke Swamp Study Area after various periods of conditioning*

Date Collected	Conditioning Time (days)	Species	Hatched	Unhatched	Percent
19 Feb 74	5	Aedes canadensis	463	13	97.3
		Aedes atlanticus	28	174	16.1
		Psorophora ferox	400	104	79.4
8 Mar 74	0	Aedes canadensis	2	0	100
		Aedes atlanticus	0	15	0
		Psorophora ferox	0	5	0
	7	Aedes canadensis	6	0	100
		Aedes atlanticus	43	53	44.8
		Psorophora ferox	9	0	100
	14	Aedes canadensis	30	0	100
		Aedes atlanticus	84	9	90.3
		Psorophora ferox	12	0	100
	21	Aedes canadensis	6	0	100
		Aedes atlanticus	69	0	100
		Psorophora ferox	12	0	100
2 May 74	0	Aedes canadensis	-	-	-
		Aedes atlanticus	0	9	0
		Psorophora ferox	1	28	3.6
	14	Aedes canadensis	-	-	-
		Aedes atlanticus	21	2	91.3
		Psorophora ferox	58	0	100
	21	Aedes canadensis			
		Aedes atlanticus	11	0	100
		Psorophora ferox	22	0	100
	21	Aedes canadensis	-	-	-
		Aedes atlanticus	8	2	80.0
		Psorophora ferox	24	0	100

*Conditioning accomplished by holding eggs at 25°C before flooding

Captions for Figures 1 - 6, following

Figure 1. Adult female Aedes atlanticus as sampled by CDC miniature light traps with dry ice, Pocomoke Cypress Swamp, Maryland, 1971, and CEV isolations made.

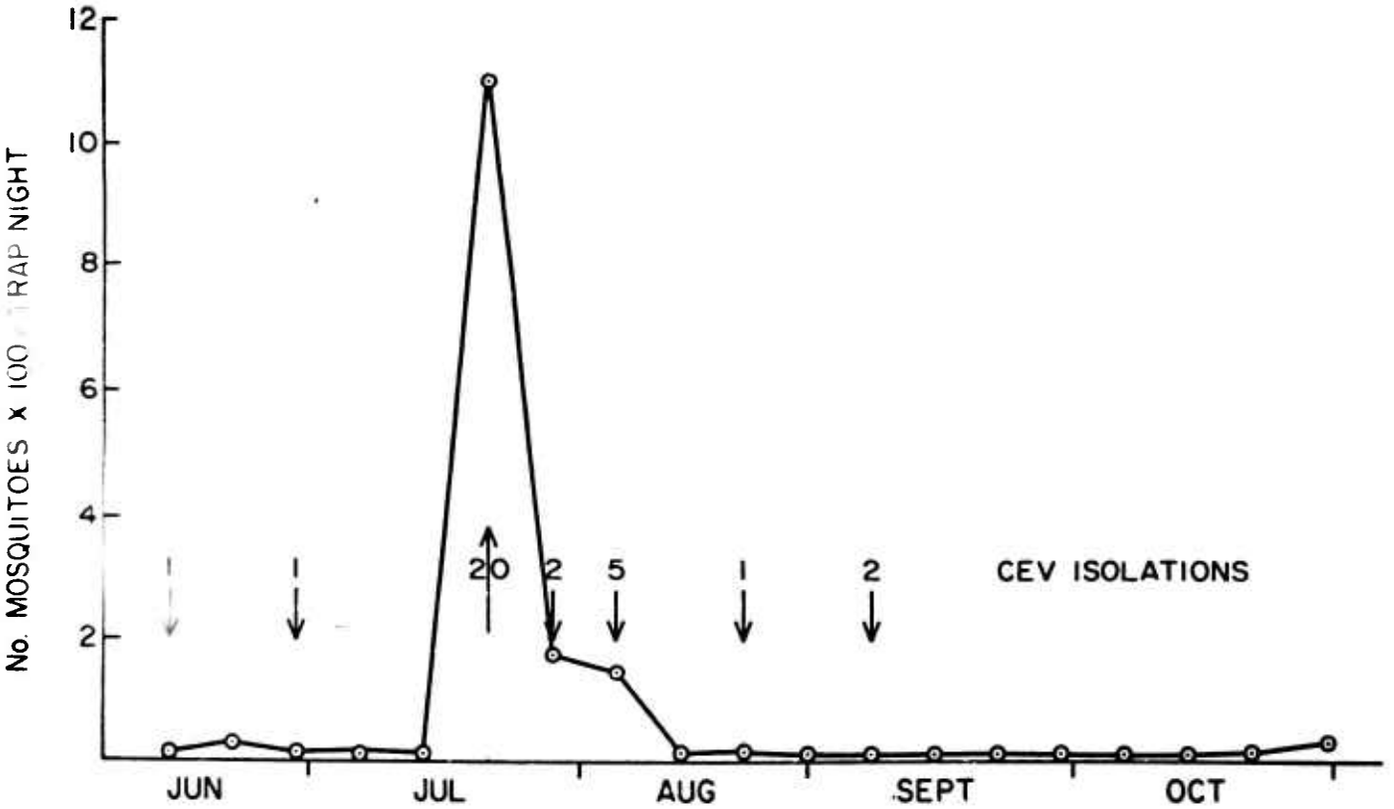
Figure 2. Adult female Aedes atlanticus as sampled by CDC miniature light traps with dry ice, Pocomoke Cypress Swamp, Maryland, 1972, and CEV isolations made.

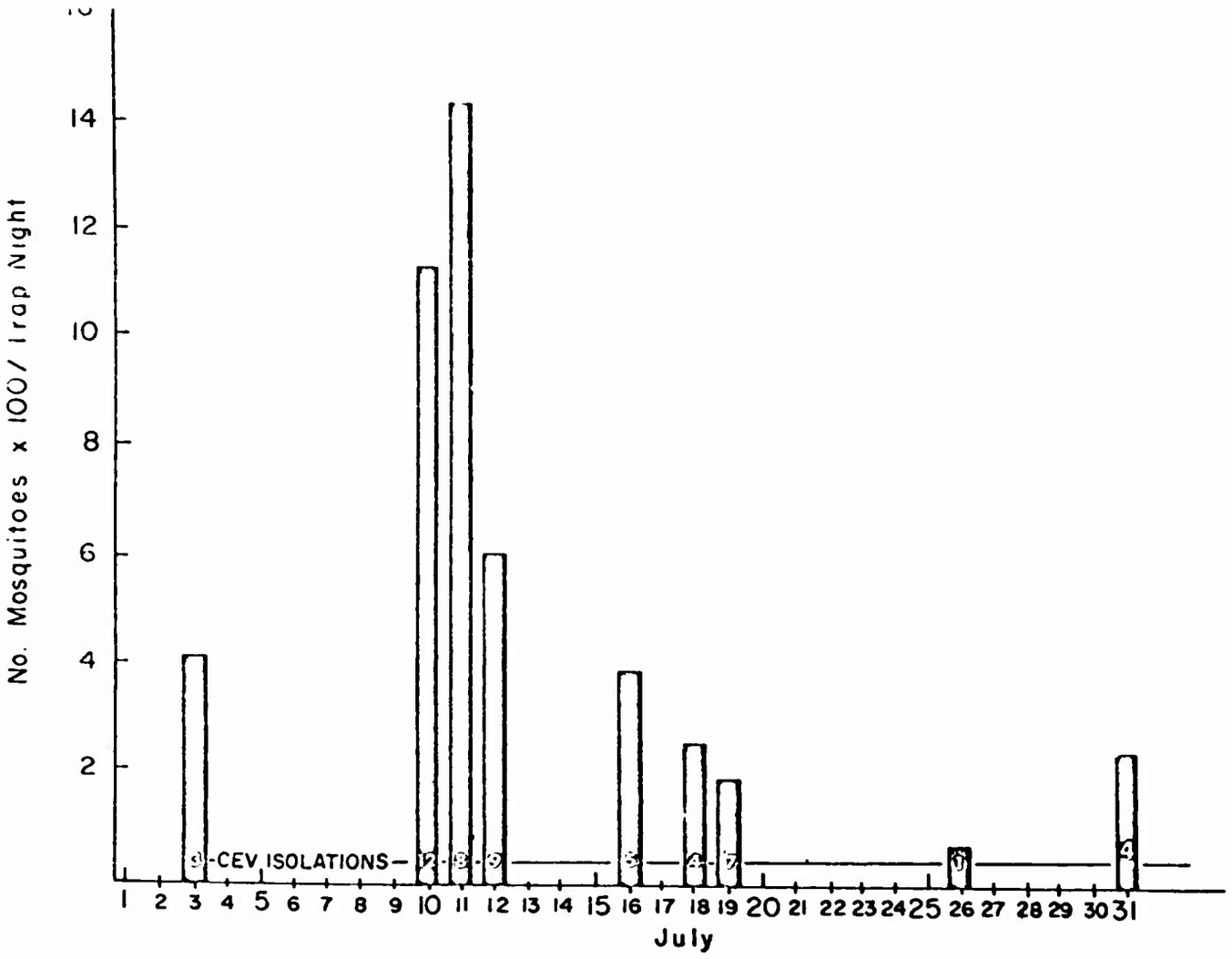
Figure 3. Comparison of feeding patterns of Aedes atlanticus and A. canadensis as determined by bloodmeal identification using the capillary tube precipitin test, Pocomoke Cypress Swamp, 1969-72.

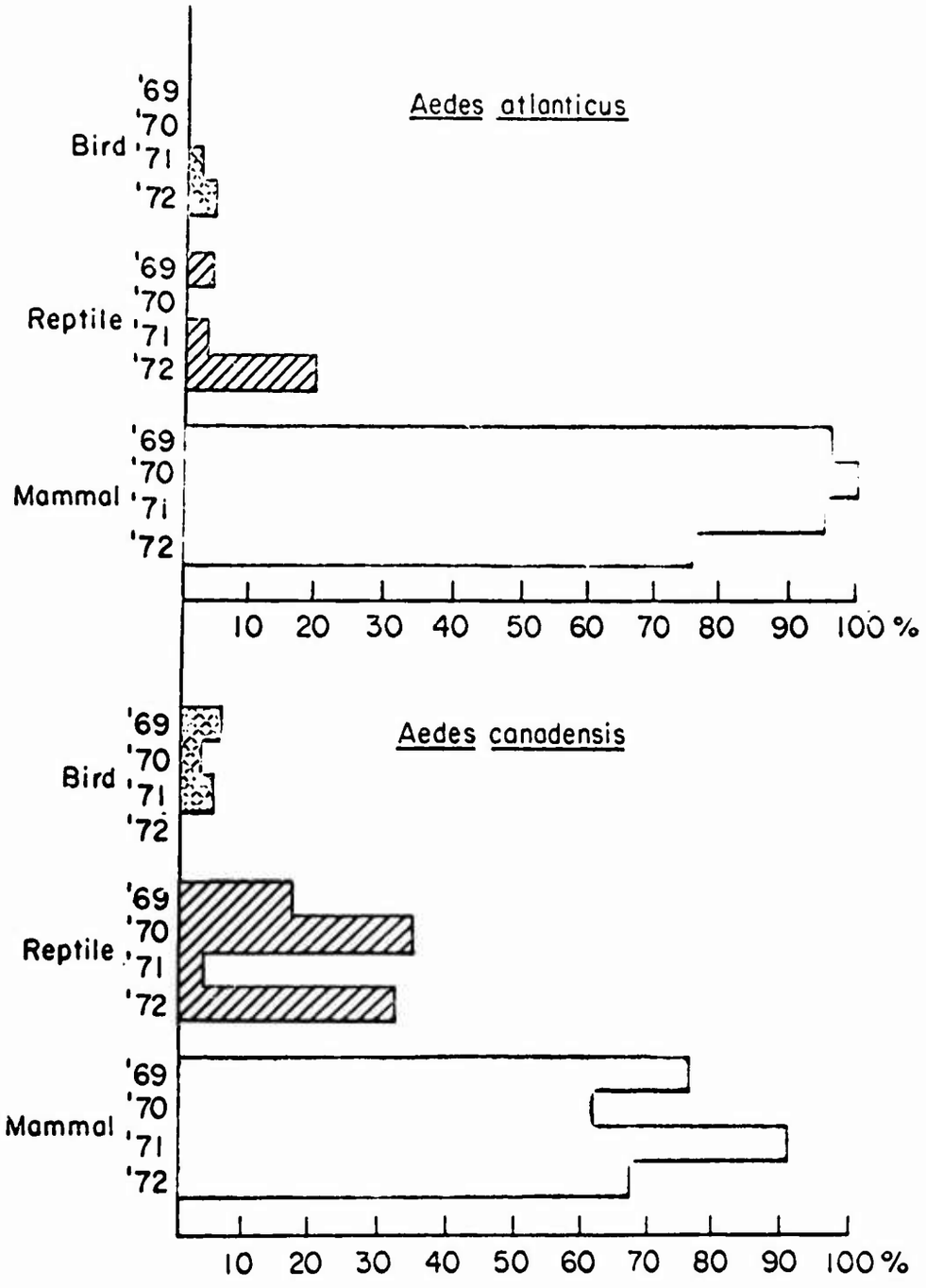
Figure 4. Pattern of adult emergence of Culex salinarius and C. restuans at 10°, 15°, 20°, and 25°C.

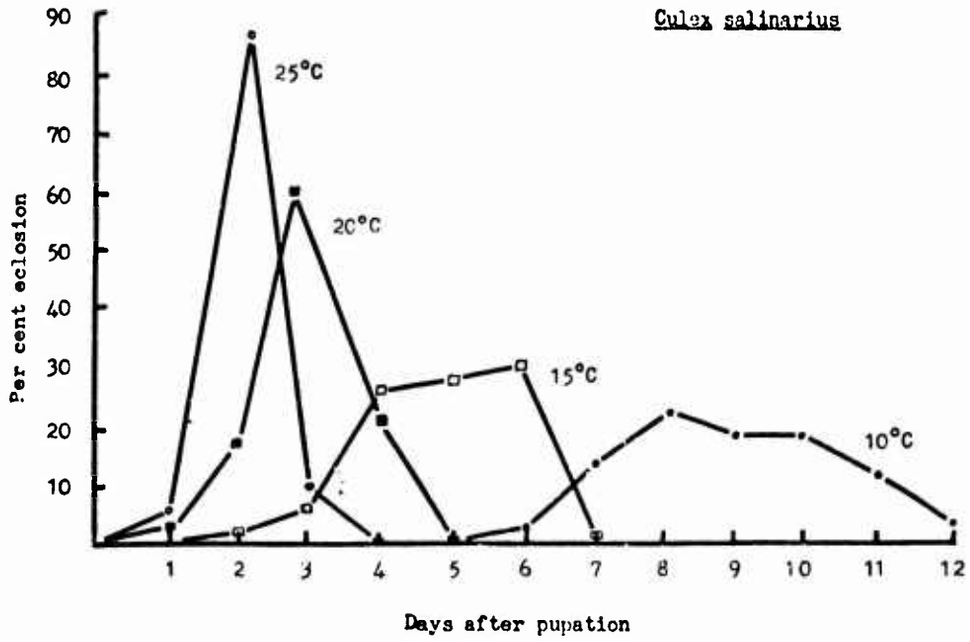
Figure 5. Pattern of blood digestion of Culex salinarius and C. restuans at 10°, 15°, and 25°C.

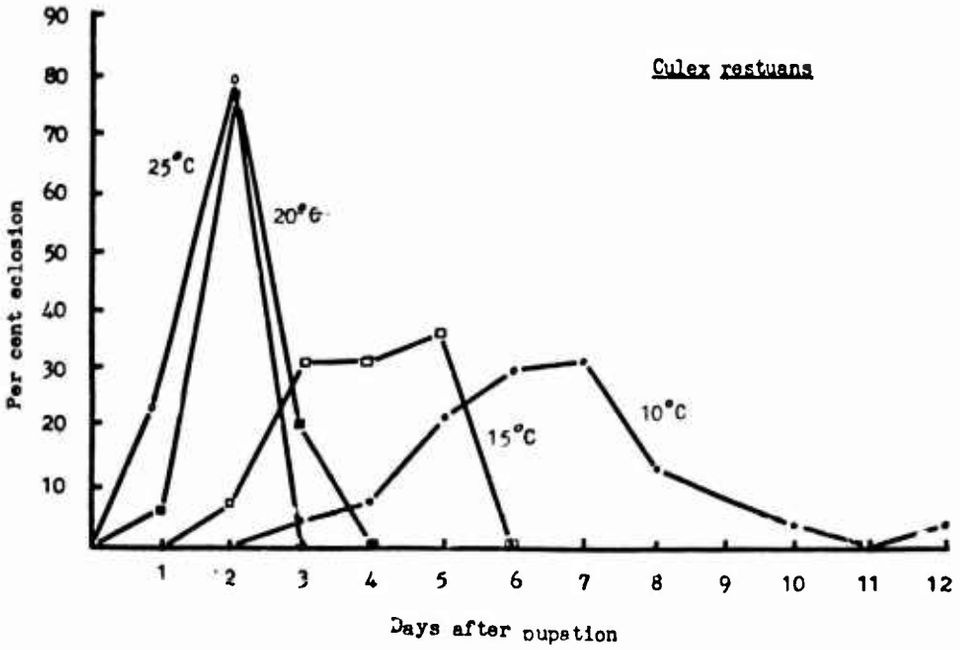
Figure 6. Pattern of ovarian development of Culex salinarius and C. restuans at 10°, 15°, and 25°C.


No. MOSQUITOES x 100 / TRAP NIGHT
12
10
8
6
4
2
1
1
20
2
5
1
2
CEV ISOLATIONS
JUN
JUL
AUG
SEPT
OCT


No. Mosquitoes x 100/ Trap Night
14
12
10
8
6
4
2
CEV ISOLATIONS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
July


Aedes atlanticus
Bird
Reptile
Mammal
'69
'70
'71
'72
10 20 30 40 50 60 70 80 90 100 %
Aedes canadensis
Bird
Reptile
Mammal
'69
'70
'71
'72
10 20 30 40 50 60 70 80 90 100 %


Culex salinarius
Per cent eclosion
90
80
70
60
50
40
30
20
10
25°C
20°C
15°C
10°C
1
2
3
4
5
6
7
8
9
10
11
12
Days after pupation


Culex restuans
Per cent eclosion
90
80
70
60
50
40
30
20
10
25°C
20°C
15°C
10°C
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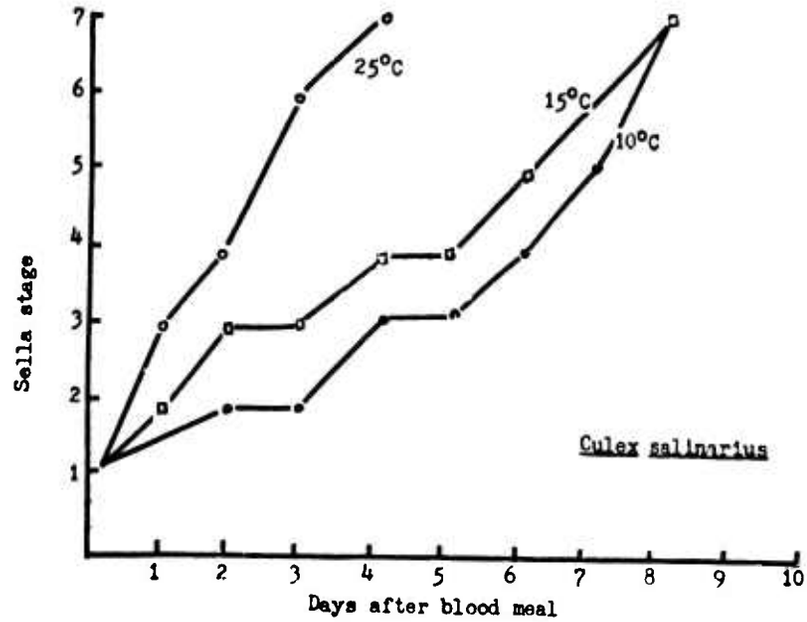
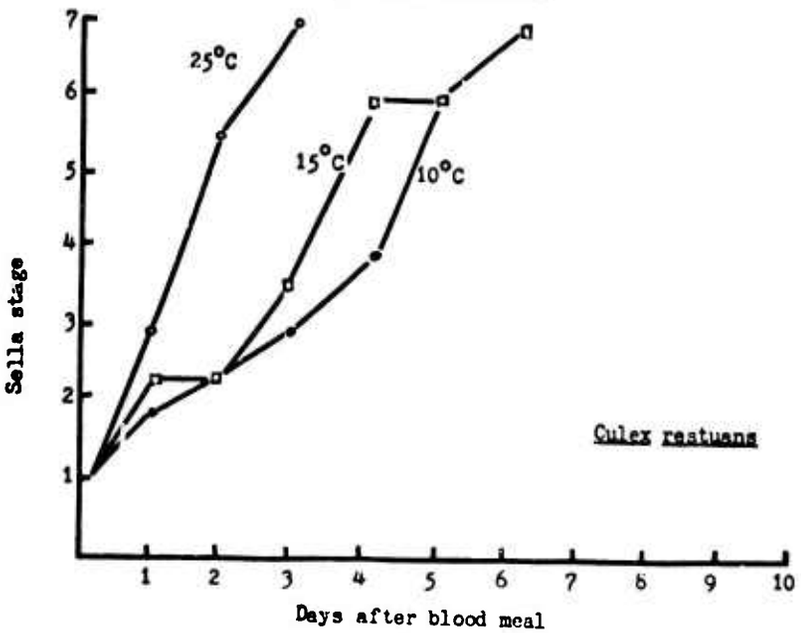
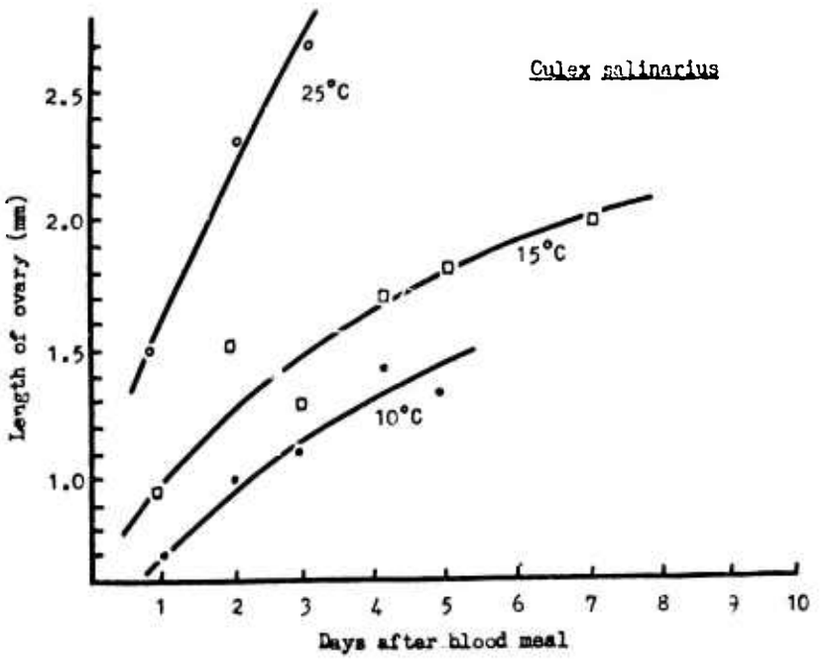
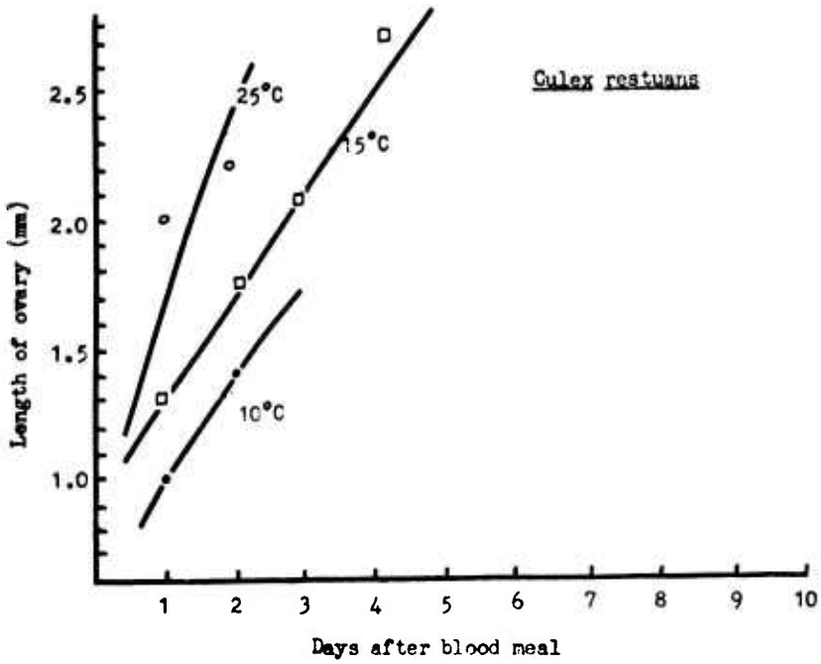
2
3
4
5
6
7
8
9
10
11
12
Days after pupation


25°C
15°C
10°C
Culex salinarius
Sella stage
Days after blood meal


25°C
15°C
10°C
Culex restuans
Sella stage
Days after blood meal


Culex salinarius
25°C
15°C
10°C
Length of ovary (mm)
2.5
2.0
1.5
1.0
1
2
3
4
5
6
7
8
9
10
Days after blood meal


Culex restuans
25°C
15°C
10°C
Length of ovary (mm)
2.5
2.0
1.5
1.0
1
2
3
4
5
6
7
8
9
10
Days after blood meal

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03 Human Ecology

Work Unit 035 Ecology and control of disease vectors and reservoirs

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA QA 6438	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D.CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A762758A823	00	033
b. CONTRIBUTING	CH FR	3A161102B71P	03	036
c. [redacted]	CARDS 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Anatomical & Physiological Correlates of Brain Function in Stress & Disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002400 Bioengineering 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	6	178
	CURRENT 75	6	178

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

PII Redacted

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Neurophysiology; (U) Neuropsychiatry; (U) Neuroanatomy; (U) Combat Shock; (U) Automamic Nervous System; (U) Biomedical Engineering; (U) Stress

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The principal research objectives are to identify the anatomical and physiological mechanisms by which the central nervous system maintains vital functions during or following physiological stress, disease or trauma in the military environment.

24. (U) The approaches employed include neurophysiological recording of intracellular and extracellular bioelectric potentials; the descriptive and experimental neuroanatomical techniques of light microscopy, electron microscopy and histochemistry. Tissue culture methods permit explanted brain cells to be studied physiologically and anatomically in environmentally controlled circumstances.

25. (U) 73 07 - 74 06 Studies of the corticospinal system were continued. The findings tended to discredit prevailing theories that discrete areas of the motor cortex synapse upon discrete spinal loci which control individual limb muscles, and that the cortex is the site of origin of voluntary movements. A study was initiated to identify the source of gap junctions in the lateral vestibular nucleus using radioactively labelled amino acids injected into vestibular ganglion cells. The morphology of habenular neurons was studied to determine whether cells of the nucleus possess reuroendocrine properties comparable to other brain neuroendocrine sites. Research on the anatomy of autonomic cell groups was continued. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03 Human Ecology

Work Unit 036 Anatomical and physiological correlates of brain function in stress and disease

Investigators.
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DESCRIPTION.

The research program of the Department of Neurophysiology attempts to: (1) provide fundamental neuroanatomical and physiological information, both basic and applied, regarding behavioral functions of the limbic system and the physiological and behavioral functions of the autonomic nervous system acting to integrate and regulate the body's vital functions, particularly as these functions may be related to the medical, surgical or psychiatric care of military patients; (2) define the environmental or physiological circumstances contributing to or causing trauma, stress, disease, and shock; (3) suggest applied methods of corrective therapy for stress avoidance and recovery from diseases and surgical or medical shock. These studies have applied physiological, neurological, and neurosurgical implications.

The knowledge and research methods of neuroanatomy, neurophysiology, neuroendocrinology, physiology, tissue culture, and experimental psychology are utilized in the department's studies. In some cases the expertise of one discipline is applied to a particular research task, but whenever possible, multidisciplinary approaches are utilized to study stress. The following problem areas are under study:

Neuroanatomy.

(1) The functional anatomy of the corticospinal tract;
(2) Anatomy of autonomic nuclei;
(3) An ultrastructual study of the medial habenular nucleus;
(4) *In vitro* studies of the hypothalamus-pituitary axis.

Neurophysiology.

(1) Corticospinal system projections;

(2) Reticular formation receptive fields and projections to the cerebral cortex;
(3) Synaptic transmission in the vestibular nuclei;
(4) Recovery of function after section of the dorsal roots of the spinal cord;
(5) Electrical activity in the brain related to inhibition of movement;
(6) Biomedical engineering and electronics in support of research in military medicine.

PROGRESS.

Neuroanatomical Studies.

Functional Anatomy of the Corticospinal Tract.

We have continued to study the anatomy of the corticospinal tract and its relationship to fine motor movements. The findings in the California sea lion, Zalophus californianus, follow:

Cerebral cortical ablations made with suction were placed unilaterally in the motor cortex. The resulting fiber degeneration to the spinal cord was mapped utilizing the Nauta and Fink-Heimer methods. Corticospinal fibers were found bilaterally, in all spinal cord segments. The principal descending pathway appears to be located in the contralateral lateral funiculus. In cervical, thoracic, lumbar, and sacral regions fiber degeneration in the gray matter is: (1) massive contralaterally in the external basilar region of the dorsal horn, the zona intermedia, and among nucleus proprius cornus ventralis neurons located along the dorsal and medial borders of the appendicular motoneuronal cell population; (2) abundant ipsilaterally in the nucleus proprius cornus ventralis; (3) abundant bilaterally in nucleus cornucommissuralis dorsalis. Corticospinal fibers appear not to be present in the nucleus posteromarginalis (Waldeyer), the nucleus sensibilis proprius (substantia gelatinosa of Rolando), the nucleus proprius cornus dorsalis, the column of Clarke, the nucleus cervicalis centralis, and the nucleus cervicalis lateralis.

Additional corticospinal fibers are found among the large somatic motor neurons which innervate forelimb and hindlimb skeletal muscles. Fewer pyramidal tract fibers appear to be distributed among somatic motor neurons which innervate axial skeletal muscles. Comparative neuroanatomical observations suggest that the monosynaptic proprioneuronal and somatic motoneuronal connections in Zalophus more closely resemble the motor cortical projections in New World and Old World monkeys and anthropoid apes (Petras, 1968) than the same cortical system of carnivores such as the cat, dog, raccoon, and kinkajou (Petras and Lehman, 1966; Petras, 1969).

The anatomy of the corticospinal system of Zalophus was compared with carnivores and primates and reviewed in the context of the anatomy of corticocortical circuitry of the mammalian cerebral hemispheres. An approach was developed to explain species-typical movements, graded on a hypothetical scale of fixed-action responses to variable-action skilled motor behavior.

Techniques were successfully acquired and applied for the restraint, tranquilization and surgical anesthesia of another species of aquatic mammal, Phoca vitulina (the Harbor seal). This species possesses different habits of ambulation on land and of swimming movements in the water. The results of these experiments are now being analyzed.

Parasympathetic Nuclear Groups in the Spinal Cord.

Studies are in progress to determine the nuclei of origin for the outflow of the parasympathetic division of the nervous system. In man these cells innervate the gastrointestinal tract distal to the splenic flexure and supply efferent fibers which innervate the pelvic viscera and the external genitalia. Axons of the parasympathetic neurons are selectively cut to produce central chromatolysis of the nerve cell body. The axonal reaction displayed by the nerve cell bodies will permit identification of the nuclei of origin for parasympathetic fibers. Some evidence was found which confirms our earlier description of a sacral autonomic cell group in the zona intermedia of the rhesus monkey.

Ultrastructural Study of the Medial Habenular Nucleus.

As part of a project aimed at understanding the possible role(s) of the medial habenular (MH) nucleus in neuroendocrine and visceromotor functions, a descriptive scanning (SEM) and transmission (TEM) electron microscopic study was undertaken in the rat. By SEM, the ependymal surface contained regularly spaced cilial tufts interspersed with microvilli and a network of cylindrical processes with varicosities. Correlative TEM showed that these processes were unmyelinated nerve fibers making synaptoid contacts with ependymal cells. The subependymal region contained: 1) capillaries and fine neuropil surrounded by glial processes; 2) MH neurons in direct apposition with ependyma; and 3) occasional cisternae with homogeneous electron dense centers. Large and small neurons were found. Their cell bodies were arranged in glomeruli with a central neuropil. Neighboring cell bodies were either in direct apposition or separated by glial processes. This glomerular arrangement was more evident in myelin free areas. The neuronal nuclei had a polar invagination facing the origin of the dendrite. Each nucleus contained a prominent nucleolus and a homogeneously dispersed karyoplasm. An extensive Golgi apparatus faced the region of nuclear infolding. Numerous

smooth and coated vesicles were associated with Golgi cisternae. Lysosomes and multivesicular bodies were common. Large neurons had moderate stacks of rough endoplasmic reticulum (RER). In small neurons, the RER was dispersed in the perikarya. The neuropil consisted of oligodendroglial, fibrous astrocytic and neuronal cell processes with axo-dendritic, dendro-dendritic and axo-somatic synapses. The sub-junctional dense bodies reported by Milhaud and Pappas (1966) in the cat MH nucleus were not observed.

In vitro studies of the hypothalamic-pituitary axis.

Hypothalamic explants and pituitary tissue of newborn rats have been maintained for up to 30 days. This *in vitro* model will be used to study neuroendocrine events, particularly the trophic influences of the median eminence upon the development and differentiation of endocrine tissue.

Neurophysiology.

Corticospinal system projections.

During the past year, the first part of a combined anatomy and physiology project on the cat corticospinal system was completed and published (Tyner, 1974). This work involved making very discrete lesions in the somatic sensorimotor cortex of cats and tracing the axons and terminals in the spinal cord, using silver stains. A substantial body of earlier work had suggested that the corticospinal pathway in mammals is organized very discretely, and that this pathway can serve as a "voluntary" motor pathway which, among other things, exerts control over individual muscles. The present study has thus far yielded the rather surprising conclusion that the corticospinal axons arising from even a very restricted part of cortex distribute their terminals over a remarkably wide part of the cord. These findings may necessitate partial revision of the earlier views of the corticospinal system's organization and function.

Reticular formation receptive fields and cortical projections.

Data collection for this single-neuron electrophysiological study in the cat has continued successfully. The preliminary results demonstrate: cells in the medullary reticular formation are sensitive to gentle mechanical stimulation of large body areas, but for each cell there is a peripheral area of particular sensitivity; cells in the reticular formation are organized in a dorsal-ventral fashion, with the ventral cells most sensitive to contralateral body stimulation, and the dorsal cells to ipsilateral body stimulation; and that the majority of these cells probably cannot serve as inputs to cells of the motor cortex.

Substantial progress has been made in development of the computer system for storage and analysis of single-neuron data and the data base from this study and others.

Synaptic Transmission in the Vestibular Nuclei of the Rat.

Previous data from this laboratory indicate that in the rat, at least some vestibular afferents are electrically coupled to their target cells in the vestibular nuclei (Wylie, 1973). This implies that axonal terminals forming gap junctions with cells of the vestibular nuclei (Sotelo and Palay, 1970) must originate from vestibular afferents. However, lesions of the vestibular ganglion are reported not to cause degeneration of the gap junction-bearing terminals (Sotelo, personal communications). The apparent discrepancy between the anatomical and electrophysiological observations may indicate that the anatomical or physiological results are wrong, or that the gap junction plays a special role in allowing the terminals to survive after they have been severed from their parent cell bodies. To test this latter hypothesis, we are beginning a study to resolve the question of whether gap junctions are formed by vestibular afferents. We plan to label the terminals with a tritium label by injecting the label into the vestibular ganglion. We will use the technique of electron microscopic autoradiography to determine whether the label is found in terminals with gap junctions or in terminals which do not form gap junctions. We have thus far obtained electron micrographs of the normal vestibular nuclei and have confirmed the presence of terminals with gap junctions. We are currently working out the approach to the injection of the radioactive label and in the near future expect to carry out the autoradiography.

Recovery of Function After Section of Dorsal Roots of the Spinal Cord.

Our earlier study (Wylie et al., 1973) indicated that the recovery of function after bilateral section of the dorsal roots innervating the limbs of a monkey does not depend upon regeneration of the dorsal roots. We are now investigating the physiology of synaptic transmission between the corticospinal tract and motoneurons. We have found that stimulation of the corticospinal tract in chronically deafferented monkeys is capable of activating large pools of motoneurons, in spite of the absence of facilitating influences from the periphery. These findings may indicate that the loss of peripheral afferents consequent to dorsal rhizotomy is followed by collateral sprouting of pyramidal tract fibers. One of the events underlying the recovery of function in these animals may then be a shift in control of motoneuronal activity from the influences of peripheral afferents to those of the corticospinal system. The motoneuronal response to corticospinal stimulation in deafferented monkeys is at least as large or perhaps larger

than that obtained from normal animals.

The recent report (Coggeshall et al., 1973) that there are large numbers of unmyelinated fibers of presumed afferent function in the ventral roots raises a fundamental question of whether these presumed afferent fibers may provide important proprioceptive feedback which may contribute to the recovery of function following dorsal rhizotomy. We have begun a study of motor skills in normal monkeys, using an arm flexion task to obtain baseline data with which to compare the motor skills of deafferented monkeys. By altering the performance requirements, we will observe whether the motor performance breaks down when the task requires peripheral feedback from the performing limb. This will allow us to determine whether the presumed ventral root afferents provide the central nervous system with information about muscle tension, fatigue, length or other parameters of the motor performance.

Electrical Activity in the Brain Related to Inhibition of Movement.

Studies have continued to determine the role of the cerebellum in inhibiting movement. Olivocerebellar fibers are stimulated while simultaneously recording climbing fiber evoked potentials from the cerebellar cortex. These bioelectric potentials are recorded from cats which are asleep, awake but motionless, and awake and ambulating.

A FABRI-TEK, Model FT-1050, signal averager was incorporated into the existing system to enhance the resolution of the averaged evoked potentials. The raw data were collected in the form of evoked potential plots which were recorded via an x-y plotter. Two papers describing the instrumentation for this project are in preparation for press. In addition, a third paper, "A System for Biofeedback Conditioning of Electroencephalograpic Activity" (A. T. Pryzyblik and R. C. Howe), has been accepted for publication (J. Med. Biol. Eng., 1975).

Biomedical Engineering and Electronics in Support of Research in Military Medicine.

In our biomedical engineering laboratory, during the reporting period, the following projects were completed:

(1) An AC power alarm to indicate power failure and to interrupt that particular circuit, so that when power is reinstated, measures can be taken by the operator to protect the instruments on that circuit against the sudden inrush of current.

(2) A digital designing aid for our own electronics laboratory. The device is a quick-connect breadboard system to be used in

the design of digital circuits. It contains its own signal source and power supply.

(3) An analog designing aid. A breadboard similar to the description in (2) above, but for analog circuits. It contains a plus and minus 15 volts power supply and a signal source.

(4) Modification of 18 Gerbrandts cumulative recorders to prevent inductive spikes caused by in and out switching of the motor to get into solid state programming equipment and causing logic errors. The special circuit, consisting of two Triacs, was developed in the electronics laboratory. The circuit works on the principle of sensing when the AC current goes through zero and then, and only then, allowing the motor to be switched. The method is highly effective and foolproof.

(5) A DC lesion maker. To replace a tube-type model, a solid state model was developed. Subsequently, several models were built, every one smaller in size, taking into account the limited space available in an operating room. The last model is powered by rechargeable batteries, thus avoiding dangling cords and increasing operating safety.

(6) A ten-channel constant-current AC stimulator. Our formerly developed six-channel stimulator was copied in a ten-channel version. The device allows ten animals to be run simultaneously, each channel current regulated, sharing one meter.

(7) An input-output panel for the PDP 8/E computer.

(8) A strain gauge to measure respiration of small animals. The animal has a belt attached to its chest. The belt contains a strain gauge, which will bend according to expansion and contraction of the chest.

(9) A device to detect the small distances between an electrode and the skull of an experimental animal during surgery. Formerly, this distance had to be evaluated optically, resulting in many bent and destroyed electrodes. The electrical method gives a more reliable forewarning of immediate approach.

(10) Design, construction and installation of pump connection alarms for ten baboon chambers. During cleaning of the baboon chambers or even due to violent movement of the baboons themselves, the electrical connection to their injection pump systems often was disabled, resulting in interruption of the ongoing experiment. This interruption went unnoticed by the researcher and showed up only after a data

analysis. A buzzer and a flashing light now warn the researcher in case of electrical connection failure.

(11) On the data collection device for the Department of Neurophysiology, nine component cards were built. For one card, a printed circuit is in preparation, as it is envisioned to build ten or more copies of this particular component card. On unique cards, it is not economically feasible to develop a printed circuit. Three card cages have been prepared as part of a test set-up consisting of an oscilloscope, a test generator, and various power supplies.

(12) A four-channel electronic system was designed for recording deep body temperature in rats.

(13) Preliminary work has been initiated on the design and construction of a system for recording EEG and other physiological variables in the laboratory rat. An electrical noise-free environmental chamber and a recording device have been incorporated in the system.

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03 Human Ecology

Work Unit 036 Anatomical and physiological correlates of brain function in stress and disease

Literature Cited.

References:

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4. Petras, J. M.: Some efferent connections of the motor and somatosensory cortex of simian primates and felid, canid and procyonid carnivores. Ann. N. Y. Acad. Sci. 167: 469-505, 1969.

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10. Wylie, R. M., Barro, G., Perrella, P. N., Weinberg, S. G., and Taub, E.: An electrophysiological study of the spinal cords of deafferented monkeys. Proc. Soc. Neurosci., 1973. (Abstract #1.10)

Publications:

1. Coidan, R. S.: A study of developing human cerebral cortical tissue in vitro. Anat. Rec. 178: 332, 1974.

2. Koob, G. F., Annau, Z., Rubin, Robert J., and Montgomery, Mark R.: Effect of hypoxic hypoxia and carbon monoxide on food intake, water intake, and body weight in two strains of rats. Life Sci. 14: 1511-1520, 1974.

3. Neely, W. E., Wardlaw, S., and Swinnen, M. E. T.: Design and operation of a signal comparator to increase efficiency of continuous-flow analyzers. Clin. Chem. 20: 78-80, 1974.

4. Tyner, C. F.: Anatomic specificity in the feline corticospinal system. Brain Res. 69: 336-340, 1974.

5. Wylie, R. M., Barro, G., Perrella, P. N., Weinberg, S. C., and Taub, E.: An electrophysiological study of the spinal cords of deafferented monkeys. Proc. Soc. Neurosci., 1973. (Abstract #1.10)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6439	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A762758A823	00	034
b. CONTRIBUTING	CH FR	3A161102B71P	03	037
c. CONTRIBUTING	CARDS 114(f)			

11. TITLE (Procede with security Classification Code): (U) Influence of Stress on Hormone Response, Performance & Emotional Breakdown in Military Personnel

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 013400 Psychology 016200 Stress Physiology 005900 Environmental Bio

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METH.
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	6	236
	CURRENT 75	4	236

19. RESPONSIBLE DOD ORGANIZATION
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PII Redacted

21. GENERAL USE
Foreign Intelligence Not Considered

22. KEYWORDS (Procede EACH with Security Classification Code): (U) Stress; (U) Emotions; (U) Homeostasis; (U) Psychophysiology; (U) Neuroendocrinology; (U) Psychoendocrinology; (U) Human Volunteer

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) Principal objective is to study the integrating influences of the central nervous system in controlling and coordinating the organs of the body and their metabolic functions under environmental and emotional stresses which are likely to produce casualties due to psychiatric or psychosomatic disease.

24. (U) This involves measurement of plasma and urinary hormone levels in humans and monkeys in a variety of acute and chronic stress situations. One important conceptual approach is that the organization of endocrine regulation can only be understood by viewing "overall" hormonal balance, or multihormonal patterns. A second approach requires intensive study of individual endocrine systems in order to establish the significance of multihormonal patterns for the individual exposed to stress. Continuous updating of endocrine assay methodology is also required.

25. (U) 73 07 - 74 06 Collaborative studies of physical stress with ARIEM (Natick) have continued. Marked and consistent plasma testosterone elevations have been observed during heavy muscular exertion. Preliminary observations also suggest elevated androgen excretion as a function of long-term physical training. Studies of heat stress indicate no elevation in urinary 17-OHCS levels. Urinary epinephrine and norepinephrine are also not elevated. These data strongly suggest a need to reexamine generality of these measures as indicators of stress as it is generally conceptualized. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*Available to contractors upon originator's approval

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Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03 Human Ecology

Work Unit 037 Influence of stress on hormone response, performance and emotional breakdown in military personnel

Investigators.

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Description.

This program is concerned primarily with the role of the central nervous system in the control and especially the coordination of endocrine regulation. As this program has gradually developed, some general concepts have emerged which appear to have major and far-reaching implications for the field of stress research. Included among such concepts which have been particularly important in opening up productive new avenues of stress research are: 1.) The neuroendocrine apparatus represents a third effector system of the brain (along with the autonomic and skeletal-muscular systems) providing sensitive and objective reflections of central integrative processes, such as emotions and psychological defenses, which are of key importance in human performance under stress, but which have so far been extraordinarily difficult to bring under rigorous experimental investigation. 2.) There is now serious doubt concerning the validity of Selye's "non-specificity" concept in stress theory, because of considerable recent research indicating that psychoendocrine reactions are frequently and inadvertently elicited during experiments designed for the study of physical stimuli. There is a pressing need, therefore, for a thoroughgoing reevaluation of past research on neuroendocrine responses to the physical stresses, with closer scrutiny of independent variables and special attention to the possible contamination of physical stress experiments by psychoendocrine reactions reflecting attendant discomfort, pain, or emotional reactions. Our recent studies of muscular exertion, fasting, and heat, for example, indicate that erroneous conclusions concerning the effects of these stimuli upon neuroendocrine systems are likely to be drawn unless rigorous and systematic efforts are made to evaluate and to

minimize interfering psychoendocrine reactions. 3.) While it has long been the prevailing practice in stress research to study isolated endocrine systems, usually one at a time, it is increasingly clear that the many neuroendocrine systems are closely interdependent in their functions and that a key to the understanding of the principles underlying the integration of these systems lies in the study of relative changes between interacting endocrine systems, as manifested in the organization of patterns or profiles of multiple hormonal responses to various stressful stimuli. In recent years, we have learned that relatively distinctive, broadly organized patterns of hormonal changes, involving many hormones in addition to those of the adrenal systems, occur in response to various types of psychological and physical stress. A major immediate goal, therefore, is to define as conclusively as possible the characteristic hormone response profiles for various stressful stimuli, with principal emphasis on psychological stimuli, but also including exercise, heat, cold, fasting, hypoxia, infection, and other physical stimuli encountered in military stress situations. Such basic knowledge of the organization of integrative machinery is an essential foundation for more complex neuroendocrine approaches to the study of stress-related clinical and field problems concerned with such parameters as endurance, fatigue, host resistance, performance, and the pathogenesis of some psychosomatic disorders. It is clear that this approach logically must move eventually through a series of successive stages, beginning with 1.) basic definition of response profiles for the various stressful stimuli, 2.) determination of the degree of response profile specificity for diverse, discrete stimuli, 3.) evaluation of factors which determine response profile priorities when there are various natural admixtures of multiple, concurrent stimuli, 4.) evaluation of the physiological significance of differing neuroendocrine response profiles by extending the approach to the concurrent study of their metabolic or physiological concomitants or consequences, and 5.) evaluation of the degree to which both acute and chronic hormone response profiles may be adaptive or maladaptive. It is evident that each of the above projected stages in the development of this conceptual approach is in large measure dependent upon establishment of prerequisite knowledge in the preceding stage, so that the stages must generally best be pursued in logical sequence. Our efforts at present, therefore, are still largely limited to the sizeable task involved in just the first two stages of this approach. The amount of stress response profile data accumulating, however, is already quite substantial and is providing an increasingly useful basis for the clarification and revision of stress concepts, as discussed in some detail in a recent

overview of our approach currently in press (Toronto Neuroscience Symposium). During the past year, a major portion of our effort has been devoted to continued collaboration in physical stress with the Army Research Institute for Environmental Medicine (ARIEM) at Natick, MA. Developmental work has also continued on new or refined hormone assay procedures in order to provide the necessary, up-dated methodological foundation for this stress research program.

Progress

1. Organization of Neuroendocrine Responses to Psychological Stress Profile of Acute Hormonal Responses to Capture and Chair Restraint in Monkeys: The development of highly sensitive and reliable new methods for the measurement of plasma levels of certain hormones has now made possible, for the first time, in our laboratory, the study of a relatively detailed profile of neuroendocrine responses during acute emotional reactions. The selection of hormonal indices, in our research program is based primarily on the rationale that it is logical in the study of neuroendocrine organization to begin with endocrine systems that have well-established neuroendocrine linkages, that is, systems in which endocrine cells articulate with nerve cells, either via neural or neurohumoral connections. The battery of methods used in this study includes those for the measurement of plasma cortisol, epinephrine, norepinephrine, total thyroxine, thyrotropin, testosterone, growth hormone, insulin, prolactin, and glucagon. Chair restraint and capture was selected as a particularly suitable situation for response profile study, since previous research with the adrenal systems had shown it to be an especially potent, reliable, and convenient psychoendocrine stimulus. Following a period of at least one week of cage housing in a quiet, stable environment, monkeys were captured as rapidly as possible and blood samples obtained by saphenous venipuncture at intervals of 1, 3 and 5 minutes. The monkey was then installed in the restraining chair and additional samples obtained at 20, 40 and 60 minutes, 2, 4, 6, 24 and 48 hours. Results are largely complete on four monkey studies so far as follows: Plasma cortisol levels show about a three-fold elevation, peaking usually between 4 to 6 hours after restraint onset. Plasma TSH levels show nearly a two-fold rise in the first hour and tend to remain elevated thru the second day. Plasma thyroxine levels rise more slowly to about a two-fold elevation at the 48-hour point. Plasma growth hormone levels show a greater than fifteen-fold elevation with a peak at about 40 minutes, followed by a fairly sharp decline towards baseline values. Plasma prolactin levels also appear surprisingly labile, showing about a four-fold increase

with a peak usually at 20 minutes. In one monkey studied so far, plasma glucagon levels proved even more responsive, rising from 300 to 1300 pg/ml in 5 minutes, remaining near 1500 pg/ml for the first 6 hours, before declining close to the baseline at 24 hours. Plasma testosterone levels, after a mild and brief initial rise, show a rather slow decline over the first 24 hours and remain about 70% to 80% below pre-restraint baselines for at least one week. Plasma insulin levels tend to be suppressed at the 4- to 6-hour points, with a tendency towards brief elevations during the first hour in some instances. In general the overall pattern of acute neuroendocrine responses observed following capture and chair restraint is closely similar to that observed earlier in conditioned avoidance experiments and chair restraint experiments which were of longer duration and dependent largely upon 24-hour urinary hormonal excretion measurements. In these earlier studies it was difficult to evaluate in some instances the extent to which such factors as altered food intake, altered sleep patterns, prolonged postural changes, or altered muscular activity might have been determinants of the neuroendocrine response pattern along with psychological factors. The present study, however, using plasma hormone measurements during the initial minutes and hours of restraint, before the above non-psychological variables become significant factors, provides a much stronger basis for interpreting these data as representing primarily a psychoendocrine response pattern. The data on prolactin and glucagon provide some of the most striking data available so far, particularly with regard to control of non-psychological variables, which indicate that these two hormones should be included among the growing assemblage of hormones which are sensitively responsive to psychological stimuli. Finally, control experiments incorporated in this study include experiments in which blood samples were taken by saphenous venipuncture on exactly the same schedule after one month of restraint and three subsequent control experiments performed in each monkey at weekly intervals in which blood samples on the same schedule were withdrawn remotely through chronic indwelling venous catheters in order to minimize psychological disturbance of the monkeys. Hormone analyses on these control experiments are still in progress.

2. Organization of Neuroendocrine Responses to Physical Stress.

a. Profile of Acute Hormonal Responses to Heat Exposure in Human Subjects: Earlier pilot neuroendocrine studies of heat exposure in monkeys indicated that heat per se elicits a distinctive pattern of neuroendocrine responses in which the pituitary-adrenal cortical system is suppressed rather than stimulated, as was thought on the basis of

early experiments in the stress field. In order to pursue these observations to a more conclusive level, a collaborative study with Dr. John Maher at ARIEM in Natick, MA, has been conducted in which neuroendocrine measurements were made before, during, and after 3-hour periods at exposure at four different ambient temperature levels: 74°F (Control), 95°F, 100°F, and 105°F in a group of 8 normal young men. Experiments were performed at weekly intervals and relative humidity was maintained constant at 50%. Measures employed to minimize or evaluate possible interfering psychoendocrine reactions were: 1.) "Sham," or pre-experimental, exposure to the experimental setting and procedures, excluding heat exposure, on an occasion during the initial week, in order to minimize well-known novelty effects during the subsequent heat experiments; 2.) Gradual temperature change from 74°F to the respective heat exposure levels over a 30-minute period, in order to avoid sudden discomfort effects apparent in our pilot monkey experiments; 3.) The use of indwelling venous catheters to avoid repeated venipuncture and the allowing of a two-hour recovery period from possible psychoendocrine reactions to I.V. catheter insertion, which were sometimes observed in earlier exercise studies in human subjects; 4.) Multiple blood samples at intervals during the hour immediately prior to onset of heat exposure in order to assess possible anticipatory psychoendocrine reactions to the impending procedures, again as were often observed in previous exercise studies in human subjects.

Hormonal measurements completed so far have yielded the following findings. First, on the basis of successive 3 hour urine samples collected before (0800-1100 hours), during (1100-1400 hours) and following (1400-1700 hours) heat exposure, it is clear that there is no 17-hydroxy-corticosteroid elevation in relation to heat exposure under these conditions. At all three heat exposure levels, mean urinary 17-OHCS levels were lower than during the control experiment at the 74°F level. Urinary epinephrine and norepinephrine also consistently showed the same tendency to be mildly suppressed during all three levels of heat exposure. Urine volume was not decreased during heat exposure because of a liberal water intake schedule designed to minimize the significance of residual urine in the bladder following voiding at sample close-out times. These findings add substantial additional support to our growing body of data indicating that the "non-specificity" and "general adaptation syndrome" concepts formulated by Selye are probably erroneous and that neuroendocrine responses are organized on a considerably more specific or selective basis, depending upon the nature of the "stressful" stimulus in question. These results do appear to fit generally with Cannon's view

of the homeostatic principle of bodily organization from which it might be predicted that levels of a thermogenic hormone such as epinephrine, for example, should not be increased at a time when homeostatic needs would presumably be best served by mechanisms promoting either increased loss of body heat or decreased production of body heat. It should be pointed out that the graded heat exposure levels used in this study produced the expected graded increases in body temperature, ranging up to a mean rectal temperature of 100.2°F at the 105°F ambient temperature level. Plasma hormone analyses in the study are still only partially complete and permit only a few general comments at this point. There appears to be a tendency for most of the trends observed in different hormonal levels so far to be relatively mild, whether they are in the direction of elevation or suppression. Plasma testosterone determinations so far suggest a mild and rather consistent elevation at the two higher heat exposure levels. However, some occasional instances in which elevations of testosterone levels during the pre-exposure period or following difficulty with I.V. catheters have also occurred suggesting that psychoendocrine reactions unrelated to heat itself could conceivably be occurring in this situation and that this possibility should be carefully considered in evaluating the relationship of heat to testosterone secretion. When completed, our data will permit evaluation of a profile of neuroendocrine responses to heat which includes plasma cortisol, thyroxine, TSH, testosterone, growth hormone, prolactin, and insulin levels, in addition to urinary 17-OHCS, epinephrine, and norepinephrine levels. Plans are currently in progress with Dr. Maher to extend our heat exposure studies to the study of heat acclimatization so that we will have a view of the organization of neuroendocrine responses to both acute and chronic heat exposure. Acclimatization studies present greater technical difficulties, but are of considerable interest in relation to field stress research of heat-related medical or performance problems.

b. Profile of Acute Hormonal Responses to Muscular Exercise in Human Subjects: Although most of the major findings in this study have been reported previously, some additional parameters have been explored during the past year, since frozen plasma is still available from these experiments. A striking finding in this study was the discovery of marked and consistent elevations in plasma testosterone levels during 3 hour bicycle ergometer sessions in normal young men performing at 40% and 70% maximal oxygen uptake workload levels. MAJ Monroe has recently made further studies of the plasma LH and FSH levels in these experiments and finds that the gonadotropins do not rise as might be expected in view of the testosterone elevations. This observation is similar to several others in the literature in indicating that a dissociation

of LH and testosterone levels can apparently occur. Additional work is needed to evaluate this finding, particularly to determine whether the explanation may be related to such factors as differing dynamics between the two hormonal response curves requiring a different blood sampling schedule for each, or inadequate specificity of the LH radioimmunoassay, or simply elevation of testosterone levels through some mechanism other than LH stimulation. It is also hoped that some plasma prolactin and plasma glucagon data may yet still be gleaned from this study to extend the scope of the neuroendocrine response profile defined so far.

3. Neuroendocrine Responses to Electrical Stimulation of the Brain in Monkeys: To gain further insight into the neural mechanisms responsible for alterations in hormonal balance, a study utilizing electrical stimulation of the brain has been initiated. Although this laboratory has clearly demonstrated that a variety of physical and psychological stresses can alter hormone balance, little information regarding the neural pathways responsible for these hormone changes in man or non-human primates is available. As an initial attempt to identify some of these pathways, bipolar electrodes were chronically implanted in the hypothalamus and amygdala of rhesus monkeys. These two sites were selected for our first studies because of the role presumably played by the hypothalamus in the synthesis of releasing factors and the known connections between the amygdala and the hypothalamus.

Results obtained to date are incomplete because many of the hormone measurements have not been completed and anatomical verifications of electrode placements have not been made. Electrical stimulation of the hypothalamus and amygdala in some instances has resulted in marked increases in plasma growth hormone concentration with only minimal changes in plasma prolactin levels. Conversely, one animal following electrical stimulation of the hypothalamus showed increased serum prolactin concentrations with no change in serum growth hormone levels. These data suggest that growth hormone and prolactin may be regulated, in part at least, at different neural loci. Serum luteinizing hormone in these same studies has shown little change after electrical stimulation. Serum testosterone and cortisol levels have not yet been measured.

4. Developmental and Methodological Research.

a. Steroid Radioimmunoassay Development: During the past year, radioimmunoassay for plasma testosterone, which has been used for human samples, was modified so that rhesus monkey testosterone concentrations could be measured. This modification was necessitated by

the low plasma testosterone level observed in many monkeys, and consists of purification of the plasma sample by LH-20 column chromatography prior to quantitation in the radioimmunoassay.

In order to improve our radioimmunoassay capability, rabbits have been immunized with three conjugates: testosterone-11 -bovine serum albumin, estradiol-11B-bovine serum albumin, and estrone-11B-bovine serum albumin. Animals immunized with these derivatives have, on occasion, produced highly specific antibodies which have been used in radioimmunoassay methods.

b. Polypeptide Radioimmunoassay Development: The radioimmunoassay for human prolactin described last year has required additional developmental work. Through the use of milder iodination conditions as well as extensive purification of the radioactively labeled prolactin, a number of difficulties which were encountered have been alleviated. In addition, this radioimmunoassay system has been validated for the measurement of rhesus monkey prolactin.

c. Plasma Insulin and Growth Hormone: Refinement of the methods for insulin and growth hormone was undertaken. This involved the testing of new insulin antibodies which would give equal or better sensitivity and specificity than the existing antibody and testing a double isotope method which would combine both insulin and growth hormone determination in the same radioimmunoassay and modifying a computer program for the calculation of the data. This permits a saving of the amount of plasma required to perform the assay and reduces the counting and bench work time in half.

d. Plasma Glucagon: Since a suitable I-125 glucagon preparation was not commercially available, procedures for the labeling of glucagon were developed using the Chloramine-T method and purifying over a sephadex column. For purposes of comparisons, five different I-125 glucagon labelings prepared by another investigator, Anne Eisentraut, Dallas, TX, were compared with our preparations. This helped also in establishing sensitivity and cross-reactivity to six different glucagon antibodies prepared previously in our laboratory. A search is underway for a highly specific, sensitive and higher titer glucagon antibody. We have on hand an antibody which was prepared in rabbits to beef-pork glucagon with a titer of 1:100. Therefore, two purified antigens, one derived from pork and the other a mixture of beef-pork are being employed for immunization. Rabbits are immunized by multiple intracutaneous injections of antigen emulsified in complete Freund's adjuvant. Each

animal receives 750 micrograms of antigen every 6 to 8 weeks. The rabbits are bled every two weeks and the blood tested for antibody production.

e. Cortisol: A unique BSA conjugate of cortisol was synthesized. This was effected through the 3 position using the carboxymethoxime intermediate. Antibodies produced in rabbits against this material were tested for titer and specificity. The purpose of this was to develop a more specific assay for plasma cortisol than has been previously reported. These antibodies proved to be no more specific than those raised against the cortisol-21-BSA conjugate which are commercially available. A radioimmunoassay procedure for the measurement of plasma cortisol was developed and set up using commercially available antibodies raised against the cortisol-21-BSA conjugate. Efforts are underway to produce our own supply of cortisol-21-BSA antibody. This will alleviate the expense of having to purchase this relatively expensive material.

f. Serum Triiodothyronine: A commercial kit for the radioimmunoassay of circulating serum triiodothyronine was tested and evaluated and found to be unacceptable for routine use due to lack of day-to-day reproducibility. Efforts will continue to make this valuable thyroid test available to the department.

g. Urinary Cortisol (RIA): A major part of our method development effort has been directed toward replacing the urinary 17-OHCS procedure, which is currently being contracted out at great expense, with a suitable radioimmunoassay procedure for the urinary cortisol metabolites. To this end, three unique BSA conjugates of tetrahydrocortisone and tetrahydrocortisol were synthesized. These have been used to immunize rabbits for the purpose of raising antibodies against the steroid moieties. We are presently in the process of testing the rabbit bleedings for suitable antibody levels.

Project 3A161102B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03 Human Ecology

Work Unit 037 Influence of stress on hormone response, performance and emotional breakdown in military personnel.

Literature Cited.

Publications:

1. Mason, J.W., Mougey, E.H., and Kenion, C.C.: Urinary epinephrine and norepinephrine responses to chair restraint in the monkey. Physiol. and Behav. 10: 801-804, 1973.

2. Mason, J.W., Hartley, L.H., Kotchen, T.A., Wherry, F.E., Pennington, L.L., and Jones, L.G.: Plasma Thyroid-Stimulating Hormone Response in Anticipation of Muscular Exercise in the Human. J. Clin. Endocrinol. and Metab. 37(3): 403-406, 1973.

3. Mason, J.W., Hartley, L.H., Kotchen, T.A., Mougey, E.H., Ricketts, P.T., and Jones, L.G.: Plasma Cortisol and Norepinephrine Responses in Anticipation of Muscular Exercise. Psychosom. Med. 35(5): 406-414, 1973.

4. Mason, J.W.: The Integrative Approach in Medicine - Implications of Neuroendocrine Mechanisms. Perspect. in Bio. and Med. 17(3): 333-347, 1974.

PROJECT 3A161102B71Q
COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00
Communicable Diseases and Immunology

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6440	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D.CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71Q	00	165
b. CONTRIBUTING				
c. [redacted]	CARDS 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Parasitic Diseases of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	6	129
	CURRENT 75	6	129

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS NAME: Moon, A. P. DA

NAME:

NAME:

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Parasite; (U) Schistosomiasis; (U) Pathology; (U) Primate; (U) Chemotherapy; (U) Immunology; (U) Trypanosomiasis; (U) Filariasis

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) The purpose of this research is to study various physiological, immunological and ecological aspects of parasitic diseases of military importance toward the goal of gaining a better understanding of natural susceptibility, acquired resistance and effectiveness of therapeutic agents for the prevention, suppression and treatment of these infections.

24(U) Through careful perusal of pertinent literature and discussion with other scientists both classical and new methods are used to set up controlled experiments.

25(U) 73 07 - 74 06 The Wellcome strain of T. rhodesiense which has been passaged in laboratory rodents for many years has retained its capability for antigenic variation. Guinea pigs given repeated doses of irradiated T. congolense were successfully immunized against challenge of infected Glossina morsitans flies. Neutron irradiation was found to be more effective than gamma rays in attenuating trypanosomes for use in immunizing animals against African trypanosomiasis. Studies on the immunologic responses of rabbits infected with T. rhodesiense showed that sensitized lymphocytes and antibody producing cells were detected one week after infection and continued to be present in the spleen and lymph nodes throughout the infection suggesting that cellular immunity develops along with humoral immune responses early in the course of infection. A mosquito flight mill study using various quantitative parameters showed that Brugia pahangi-infected Aedes aegypti flew significantly shorter distances and for shorter periods of time than did control mosquitoes over a 24 hour period. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 165 Parasitic Diseases of Military Importance

Investigators.

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1. Protection induced in mice against Trypanosoma rhodesiense by inoculations of homologous parasite products.

Rodents, bovines and sub-human primates have been immunized against trypanosomiasis by inoculating them with attenuated living trypanosomes exposed to ionizing radiation. Irradiated parasites were more effective as immunizing agents than vaccines prepared from dead parasites or their extracts. Attempts by various investigators to induce resistance to trypanosomiasis by artificial immunization have often given poor and conflicting results, although Thillet and Chandler successfull immunized rats against Trypanosoma lewisi by injecting them with metabolic products of the parasites. Partial protection against T. brucei in mice was reported by Miller who used trypanosome homogenates prepared in a Hughes press. Some success has also been attained by disrupting the organisms under controlled pressure and temperature. This method was used by Gonzales Cappa et al to protect mice against T. cruzi and by Corradetti et al to immunize rats against T. lewisi. Goble used the French pressure cell, ultrasonication and shaking with glass beads to produce homogenates for immunizing mice against T. cruzi. In another study with T. cruzi, freeze-dried culture forms inoculated into mice conferred resistance to subsequent infection with virulent blood forms.

Theoretically, immunization with attenuated trypanosomes may approach that produced by living, nonattenuated parasites in an infection which has been arrested by chemotherapy. This would indicate that the excretions and secretions of the trypanosomes are the antigens most important in inducing protective immunity. Until recently a serious handicap in using excretion and secretion antigens or disintegrate trypanosomes for immunization purposes was the difficulty in obtaining large numbers of organisms in suspensions relatively free of host blood components. This difficulty was obviated by the introduction of an efficient method for isolating trypanosomes from infected blood by adsorbing particulate blood components onto DEAE cellulose columns.

As a part of a continuing effort to develop effective vaccines against African trypanosomiasis, experiments were designed to determine if antigens from pressure-disrupted T. rhodesiense or from excretions and secretions of the living trypanosomes could be used to immunize mice against this parasite.

Trypanosoma rhodesiense (Wellcome strain) maintained in albino rats (Walter Reed WI, BR strain) was used as the source of immunizing material. The trypanosomes were collected from the blood of heavily parasitized rats on the third or fourth day of infection. Heparin was used as an anticoagulant. The parasites were separated from the blood in a DEAE cellulose column and washed twice by centrifugation at 600 g for 20 minutes in phosphate-buffered saline-glucose, pH 8.0 (PSG).

The procedure used for fractionation and extraction of the parasites was as follows: The trypanosomes were separated from the blood and lyophilized at .005 mm Hg. At a later date 270 mg of the lyophilized parasites in 100 ml PSG were placed in a Sorvall-Ribi Cell Fractionator (Ivan Sorvall Inc., Norwalk, Connecticut 06856) in which they were ruptured by passage through a needle valve under pressure of 40,000 p.s.i. at $5^{o}C$. The resulting suspension, centrifuged at 27,000 g for 40 minutes, yielded 80 ml of a soluble fraction (S) for use in immunizing mice. The sediment at the bottom of the tube was mixed with PSG in a tissue grinder to suspend a particulate fraction (P) of the trypanosomes for immunizing purposes. The volume of this fraction was brought up to 15.0 ml in PSG.

For collection of excretion and secretion (ES) antigens, washed trypanosomes from the column were suspended in Medium 199 (Microbiological Associates Inc., Bethesda, Maryland 20014), to which 1.5 gm glucose/100 ml of the medium had been added, and kept for 20 hours in a refrigerator at $5^{o}C$. All the parasites were intact and most of them remained motile at the end of this time. The concentration of parasites was approximately 6×10^{7}/ml. On the following day the suspension was processed in a refrigerated centrifuge at 1000 g for 30 minutes; the supernatant fluid was removed and centrifuged again at 17,000 g for 20 minutes. This supernatant solution (200 ml) was subjected to pressure dialysis in 1/4 inch cellulose tubing at 19 p.s.i. at $5^{o}C$ and reduced to 11.0 ml. Three types of immunizing inoculations were prepared from this material; 6.0 ml were used as crude excretion-secretion antigens (ESC) and 5.0 ml was centrifuged at 200,000 g for 3 hours to produce a supernatant fluid (ESS) fraction and a particulate (ESP) fraction. The ESP fraction was resuspended in 12.0 ml of Medium 199, placed in an ultrasonicator (Raytheon, well type) and given 2 exposures of 1 1/2 minutes each at 10 kc.

Four groups of mice (Walter Reed IC, BR strain, 20-25 gm) were inoculated intraperitoneally with 0.1 ml of the S fraction, the P fraction, the ESC fraction or crude lyophilized T. rhodesiense (10 mg/ml) in saline. Some of the mice in each group were inoculated subcutaneously with 0.4 ml

aluminum hydroxide as an adjuvant along with the immunizations. A second immunizing inoculation was given 3 weeks after the first. These mice and a group of healthy control animals were challenged 2 weeks after the second immunization with 1000 T. rhodesiense of the same strain used for immunization. The same schedule of immunizing and challenging inoculations was followed in mouse protection tests with the ESS and ESP fractions. The effectiveness of the immunizations was determined by examining blood from the tail veins of all the mice 5 days per week for 30 days. The surviving animals were kept for several months for rechallenge and other experimental use.

In two experiments which included 170 mice the animals were immunized with the S and P fractions obtained in the Sorvall-Ribi Cell Fractionator, with lyophilized T. rhodesiense and with crude excretion-secretion products (ESC) of the trypanosomes. The results of the two experiments, combined and summarized in Table 1, show that a high degree of acquired resistance was produced in the mice by all four types of immunizing inoculations. In contrast to the controls, all of which died as a result of the infection after an average of 4.8 days, a majority of the treated mice survived the challenging infections and remained parasite-free throughout the experiment. Among the mice inoculated with the two fractions obtained in the Sorvall-Ribi Cell Fractionator, 70% of those that received the P fraction were protected as compared to 48% of the group immunized with the S fraction. There was 95% survival among the mice inoculated with lyophilized T. rhodesiense and 100% survival in the group that received inoculations of the ESC antigens. Partial protection, indicated by prolonged survival time, was obtained in the mice that failed to survive challenge. The mice of Groups II, III and IV that died after challenge had lived an average of 6.1, 9.3, and 7.4 days, respectively, as compared to 4.8 days for the controls in Group I. The mice in the first experiment had been separated into 2 groups, one which received inoculations with aluminum hydroxide adjuvant and one which did not. Results with the 2 groups were essentially the same and therefore the use of the adjuvant was discontinued. The results were pooled in Table 1.

Table 1

Development of Immunity against Trypansoma rhodesiense *(Wellcome) in Mice Inoculated Twice with Homologous Parasite Fractions, and Challenged 2 Weeks after Immunization*

Group no.	No. mice	Immunization	% survival	Mean survival time (Days)
I	30	Buffered saline	0	4.8
II	40	Disrupted trypanosomes (S fraction)	48	6.1
III	40	Disrupted trypanosomes (P fraction)	70	9.3
IV	40	Lyophilized trypanosomes	95	7.4
V	20	Excretion-secretion (ESC) antigens	100	—

Because of the promising results obtained with ESC antigens in the first two experiments, a third experiment was set up to include supernatant fluid (ESS) and particulate antigens (ESP) separated from the ESC by ultracentrifugation. Forty mice were separated into 4 equal groups to test the immunizing effects of these fractions. Groups II, III and IV were given two 0.1 ml intraperitoneal inoculations of ESC, ESS and ESP, respectively, at one week intervals. Group I, the controls, was inoculated according to the same schedule with 0.1 ml Medium 199. All the the mice were challenged with 1000 T. rhodesiense of the homologous strain 2 weeks after the second immunization. The results (Table II) revealed that all the untreated control animals died from the infection after an average of 4.5 days. Complete protection from the challenging inoculation was induced in the mice tht had been immunized with the ESC (Group II) and with the ESP (Group IV). A lesser survival rate, 40%, was obtained in the mice of Group III that received immunizations of ESS. However, even in the mice of this group that died, the mean survival time was increased almost 2 days over that of the controls (Group I).

Table 2

Development of Immunity against Trypanosoma rhodesiense *(Wellcome) in Mice Inoculated Twice with Excretion-Secretion (ES) Antigens, and Challenged 2 Weeks after Immunization with the Homologous Strain*

Group no,	No. mice	Immunization	% survival	Mean survival time (Days)
I	10	Medium 199	0	4.5
II	10	ES (crude)	100	—
III	10	ES (supernatant fluid)	40	6.3
IV	10	ES (particulate)	100	—

In an effort to find out whether or not cross-protection could be induced by this method, 10 mice were immunized with ESC and challenged as in the previous experiment except that the challenging inoculations were made with 1000 T. rhodesiense of a different strain (EATRO #1886). All the mice died as a result of this heterologous challenge (Table III). Again the mean survival time of the immunized mice (20.6 days) was greater than that of the controls (15.3 days).

Table 3

Development of Immunity against Trypanosoma rhodesiense *(Wellcome) in Mice Inoculated Twice with Excretion-Secretion (ES) Antigens, and Challenged 2 weeks after Immunization with a Different Strain of Trypanosoma rhodesiense (EATRO 1886)*

Group no.	No. mice	Immunization	% survival	Mean survival time (Days)
I	10	Medium 199	0	15.3
II	10	ES (crude)	0	20.6

Mice were immunized against Trypanosoma rhodesiense (Wellcome strain) with whole lyophilized trypanosomes, with antigens produced by disrupting lyophilized trypanosomes after pressure, and with excretions and secretions of the living parasites. The survival rate in groups of 40 mice inoculated with disrupted trypanosmes and challenged with the homologous strain was 48% with a soluble fraction and 70% with a particulate fraction of the parasites. There was 95% survival after challenge in a group immunized with lyophilized trypanosomes; none of the controls survived. Results were essentially the same whether or not an aluminum hydroxide adjuvant was used. In subsequent experiments complete protection was obtained with either crude excretion-secretion (ES) antigens or the particulate fraction of the ES antigen, while 40% of the mice survived challenge after inoculations of ES supernatant fluid. Mice immunized with crude ES antigen failed to survive challenge with a heterologous strain, although their mean survival time was prolonged several days beyond that of the controls.

2. Relative effectiveness of neutron and gamma radiation of trypanosomes for immunizing mice against African trypanosomiasis.

Immunity against African trypanosomiasis has been produced in rodents, cattle and monkeys by inoculating them with irradiated blood form trypanosomes. Until now the immunizing inoculations have been prepared by exposing the parasites to low linear energy transfer (LET) radiations such as X-rays and gamma rays. Since for several biological end points, high linear energy transfer radiation, e.g. neutrons, has been shown to have a greater biological effectiveness than low LET radiation, two experiments were designed to compare these two types of irradiation for use in immunizing mice against Trypanosoma rhodesiense.

T. rhodesiense (Wellcome strain) in mouse blood were exposed at different dose levels to gamma rays from a 60 cobalt source and to neutrons from a TRIGA reactor. To reduce experimental variation, the two irradiation procedures were carried out under the same conditions and at the same time. Dose rates and total absorbed doses varied only slightly between the two systems. A total of 200 mice were given intraperitoneal inoculations of 2×10^6 trypanosomes irradiated at 5 to 80 K rads. The animals in the first experiment were challenged with 1000 non-irradiated trypanosomes of the homologous strain 7 days after a single immunizing inoculation. In the second experiment the mice were challenged with the same number of trypanosomes 7 days after the completion of 2 immunizing inoculations (given 7 days apart).

The results (Table 4,5) indicate that neutrons are relatively more effective than gamma rays in reducing the infectivity of T. rhodesiense. With gamma radiation, only 4 of 20 mice survived inoculations of trypanosomes irradiated at 10 K rads in the 2 experiments; with neutron-irradiated trypanosomes 18 of 20 mice survived. A dose of 15 K rads was sufficient to assure 100% survival of the mice when neutron radiation was used, but 20 K rads was required with gamma radiation. Neutron bombardment of T. rhodesiense was also more effective in immunizing

Table 4

Mice Surviving One Inoculation with
2×10^6 Irradiated Trypanosoma rhodesiense

	Gamma-Irradiated		Neutron-Irradiated	
K rads	Survivors	MST* (Days)	Survivors	MST (Days)
5	0/10	5.0	0/10	5.0
10	0/10	6.4	8/10	10.0
15	0/10	6.6	10/10	-
20	10/10	-	10/10	-

Mice Surviving Challenge After One Inoculation
with Irradiated T. rhodesiense

	Gamma-Irradiated		Neutron-Irradiated	
K rads	Survivors	MST* (Days)	Survivors	MST (Days)
0	0/9	4.4	0/10	4.0
5	-	-	-	-
10	-	-	6/8	9.0
15	-	-	9/10	8.0
20	4/10	5.5	9/10	7.0

*MST = Mean survival time of mice that died.

Table 5

Mice Surviving Two Inoculations with
2×10^6 Irradiated Trypanosoma rhodesiense

	Gamma-Irradiated		Neutron-Irradiated	
K rads	Survivors	MST* (Days)	Survivors	MST (Days)
5	0/10	4.4	0/10	6.4
10	4/10	11.8	10/10	-
15	10/10	-	10/10	-
20	10/10	-	10/10	-
40	10/10	-	10/10	-
80	10/10	-	10/10	-

Mice Surviving Challenge After Two Inoculations
with Irradiated T. rhodesiense

	Gamma-Irradiated		Neutron-Irradiated	
K rads	Survivors	MST* (Days)	Survivors	MST (Days)
0	0/10	5.0	0/10	5.0
5	-	-	-	-
10	4/4	-	10/10	-
15	9/10	7.0	10/10	-
20	10/10	-	10/10	-
40	10/10	-	10/10	-
80	10/10	-	10/10	-

*MST = Mean survival time of mice that died.

against a challenge with the homologous parasite. As shown in Table 4 and 5 the mice immunized with trypanosomes exposed to high LET radiation had a greater survival rate when challenged. In addition, the mean survival time of the mice that died after challenge was longer in those immunized with neutron-irradiated parasites than in those inoculated with trypanosomes exposed to gamma rays.

These findings may have application in immunization studies with other parasites, particularly with multicellular organisms where the selection of optimum radiation dose levels is even more critical than with protozoa.

3. Glomerulonephritis involving the alternate pathway of complement activation in monkeys infected with Trypanosoma rhodesiense.

Immune complex glomerulonephritis in man is thought to be an immunologic complication following a variety of infections, such as, nephritogenic streptococcal infections, quartan malaria, acute staphylococcal endocarditis, and secondary syphilis. Immunoglobulins and components of complement have been found in renal glomeruli of patients who developed glomerulonephritis as a result of such infections. Experimental work has implied that antigens interact with specific antibodies forming complexes in the circulation which are deposited in the glomerular capillaries. These complexes activate complement through the classic pathway leading to the release of mediators some of which have the capacity to cause local inflammation which results in glomerular injury. Endogenous antigens may also react with antibodies and lead to the development of glomerulonephritis. Thus, in the glomerulonephritis seen in patients with systemic lupus nephritis, antinuclear antibodies have been found in the kidneys, and host immunoglobulins, nuclear antigens and complement have been demonstrated in a characteristic lympy pattern in the glomeruli. Recently properdin, a component of the alternate pathway of complement activation, has also been demonstrated in the glomeruli of patients with acute post-streptococcal glomerulonephritis, membranoproliferative glomerulonephritis, and the glomeruli of lupus erythematosus patients, implying that activation of complement and subsequent damage may be initiated by other less-understood mechanisms.

Immunopathologic studies of protozoan diseases have proven to be of considerable interest in regard to the pathogenesis of glomerulonephritis in man and monkeys infected with Plasmodia, and in rats infected with Babesia. In malaria and babesiosis, the glomerulonephritis is associated with glomerular deposits containing immunoglobulins, antigens originating from the infective agent, and complement proteins. This is compatible with the concept that in each case the glomerulonephritis is induced by immune complexes entrapped from the circulation.

The present studies were begun after finding that monkeys infected with Trypanosoma rhodesiense, developed chemical and histologic evidence of renal failure and glomerulonephritis. Morphologic changes similar to those described in human cases of membranoproliferative glomerulonephri-

tis were observed in the kidneys of some of the infected animals.

Animals: Nineteen young male monkeys (Macaca mulatta) originating from India were used in this study. After a 30 day quarantine and period of conditioning, the animals were placed in individual cages and given a diet of Purina Monkey Chow, fresh fruit and water. Sixteen monkeys were each inoculated intravenously with approximately 10,000 Trypanosoma rhodesiense (EATRO #1886 strain) contained in 0.5 ml of phosphate-buffered saline-glucose (PSG) solution. Three control animals received 0.5 ml of phosphate-buffered saline-glucose solution only. The EATRO #1886 strain was isolated from a human patient in Uganda in 1971 and has since been maintained in the laboratory either by storage at -70°C or by passage in laboratory rats. The trypanosomes for the inocula were separated from infected rat blood in a DEAE-cellulose column and washed twice by centrifugation in PSG solution. Fresh preparations of blood obtained by ear puncture were examined 5 times each week to determine the course of parasitemia. Blood for serum collection was taken from the femoral veins of the monkey after they had been sedated with intramuscular Sernylan (0.5 mg/kg). The sera were stored at -70°C until used.

Biopsies: Renal wedge biopsies were taken at 14, 30 and 50 days following inoculation of trypanosomes. Uninfected control monkeys were subjected to biopsy at the same time intervals. For biopsy, the animals were anesthetized with intravenous pentobarbital (50 mg/kg) and an incision was made through the flank using sterile operating procedures. Wedges of renal cortex were removed and immediately divided into three parts. One portion was fixed for electron microscopy (EM) by dicing the piece into 1 mm cubes and immersing them in cold one-half strength Karnovsky's fixative. A second portion was quick frozen for immunohistochemical studies by placing the tissue in isopentane quenched in liquid nitrogen. A third portion was fixed for light microscopy (LM) in 10% neutral buffered formalin.

Morphologic Studies: Paraffin sections were cut at 3-4 and stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS), and Avallone's modification of the methenamine siliver stain (MS). Tissues from seven animals was studied by EM. Pieces of renal cortex were fixed for 4 hours in one-half strength Karnovsky's fixative at 4°C and then washed overnight in 0.1M cacodylate buffer (pH 7.2) containing 7.5% sucrose. The tissues were treated in osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon. Sections cut with glass knives at 1 micron were stained with toluidine blue. Thin 100m sections were cut with a diamond knife, doubly stained with uranyl acetate and lead citrate and examined with an electron microscope (RCA EMU-3G).

Immunofluorescent Studies: Fresh frozen renal cortical tissue was processed according to earlier descriptions. Gel double diffusion tests showed antibodies to human IgG, IgA, IgM and C4 properdin gave a strong cross-reaction to the heterologous monkey serum protein. Accordingly, antibodies to the human proteins were used for immunofluorescent studies.

The only exception was the use of antibodies to monkey C3 prepared in rabbits as previously described. In IF studies the direct technique was employed for all proteins except properdin; the tissue sections were incubated with the fluorescein-labelled antibody and then washed. For the detection of properdin the indirect IF technique was employed. The tissue sections were first incubated with rabbit antibody to human properdin, then washed and incubated with fluorescein tagged sheep antibody to rabbit IgG. Additional details of the technique of IF are given elsewhere.

Serologic Measurements for CH_{50}, C3 and C4: Serum CH_{50} levels were assayed with sensitized sheep red cells according to the technique of Kent and Fife. Assays of complement components C3 and C4 were based on the cross-reactions of antibodies between human and monkey proteins. The single radial immunodiffusion technique of Mancini et al as modified by Yount et al was used. Human antibodies and standards for C3 and C4 were obtained from Hyland Laboratories (Los Angeles, Ca.) and Meloy Laboratories (Springfield, Va.). Serum albumin was determined by protein-electrophoresis in cellulose acetate.

a) Serum complement and renal deposits in non-infected monkeys: The serial serum complement levels in three control animals had a mean value of 138 + CH_{50} units. Renal biopsies from the control animals obtained at three different intervals (days 14, 30, 50) failed to show the presence of any protein deposits in the kidneys.

b) Serum complement and renal biopsies in infected monkeys: The details of serum complement as well as the normal range in each of four infected animals are given in Figures 1-4 (Numbers 801, 997, 812 and 970 respectively). Between 20 and 30 days after inoculation with the infective agent (15-25 days after detectable parasitemia), serum complement levels began to fall in each of the animals. In animals 801, 812 and 970 there was no detectable hemolytic activity on at least one occasion during this period. The CH_{50} levels in monkey #997 persisted at a 50% level for at least 10 days (Fig. 3). There was a persistent parasitemia during this time. In one infected animal (#970) (Fig. 5), the CH_{50} level fell to undetectable levels at 18 days, but then returned to hypernormal or normal range. Also demonstrated in Figures 1-4 are the findings of the renal biopsies. Positive deposits consisting of one or more of the proteins listed above were found at some time during the hypocomplementemic period.

c) Morphologic changes in glomeruli: Renal biopsies obtained from the three control animals revealed normal cellularity without evidence of exudation, proliferation, or sclerosis (Fig. 5, 6).

Fifteen of the sixteen infected animals showed glomerular abnormalities. These changes consisted of mesangial cell proliferation, endothelial swelling, variable degrees of exudation, variable mesangial sclerosis, and focal basement membrane thickening and duplication. In

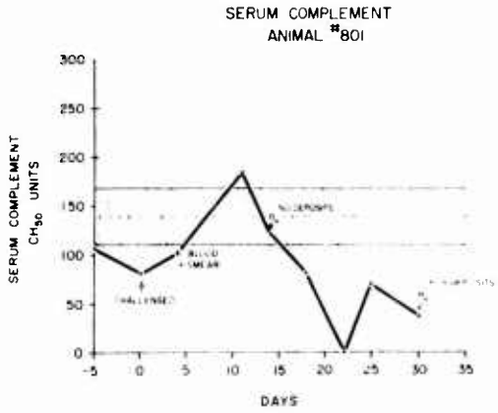


FIGURE 1. Sequential serum complement levels in animal No. 801. Arrows indicate time of renal biopsy (14 and 30 days post-inoculation of trypanosomes) and immunohistochemical results. Interrupted line and shaded area indicates mean ± S.E. of the control values for serum complement (CH_{50}).

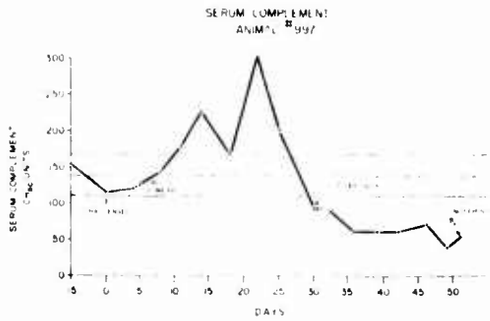


FIGURE 2. Sequential serum complement levels in animal No. 997. Arrows indicate time of renal biopsy (30 and 51 days post-inoculation of trypanosomes) and the immunohistochemical results. Interrupted line and shaded area indicates mean ± S.E. of the control values for serum complement (CH_{50}).

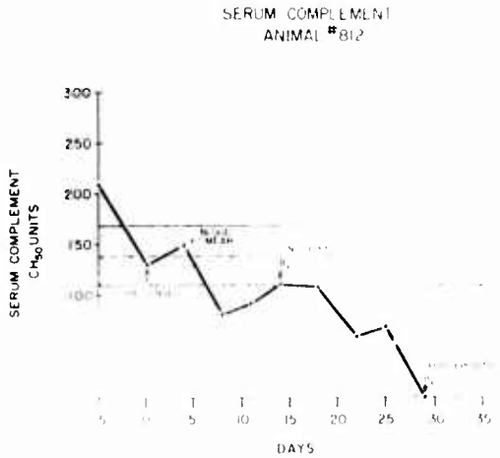


FIGURE 3. Sequential serum complement levels in animal No. 812. Arrows indicate time of renal biopsy (14 and 29 days post-inoculation of trypanosomes) and immunohistochemical results. Interrupted line and shaded area indicates mean ± S.E. of the control values for serum complement (CH_{50}).

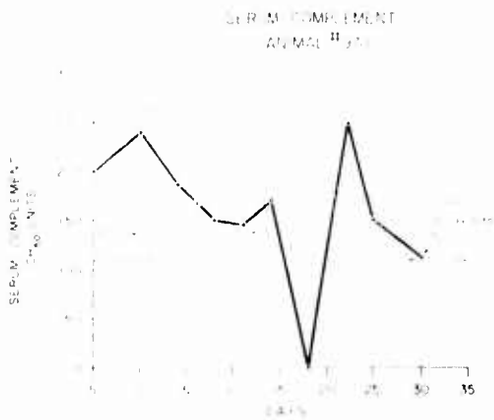


FIGURE 4. Sequential serum complement levels in animal No. 970. Arrows indicate the time of renal biopsy (30 days post-inoculation of trypanosomes) and immunohistochemical results. Interrupted line and shaded area indicates the mean ± S.E. of the control values for serum complement (CH_{50}).

four infected animals in which serologic studies were done, three (801, 997, 812) showed glomerular changes associated with depression of serum complement and protein deposits within the glomeruli.

Animal 801 was first subjected to biopsy on the 14th day (Fig. 1). The LM observations of this biopsy were essentially normal except for rare focal areas of mesangial sclerosis. EM observations confirmed this finding and revealed essentially normal capillary loops without evidence of abnormal deposits. The second biopsy obtained on the 30th day, at a time when the serum CH_{50} was 41% of the pre-infective level, showed definite focal mesangial hypercellularity with increased mesangial sclerosis and focal basement membrane thickening (Fig. 7). Electron microscopy revealed widening of the central mesangial areas which contained increased numbers of mesangial cell processes (Fig. 8). Surrounding the mesangial cells, the mesangial matrix was characterized by numerous electron lucent defects. Within these areas of rarefaction were irregular electron dense deposits (Fig. 8). Focally this change extended out into the basement membrane of the capillary loops at a point where the capillaries attach to the central mesangium.

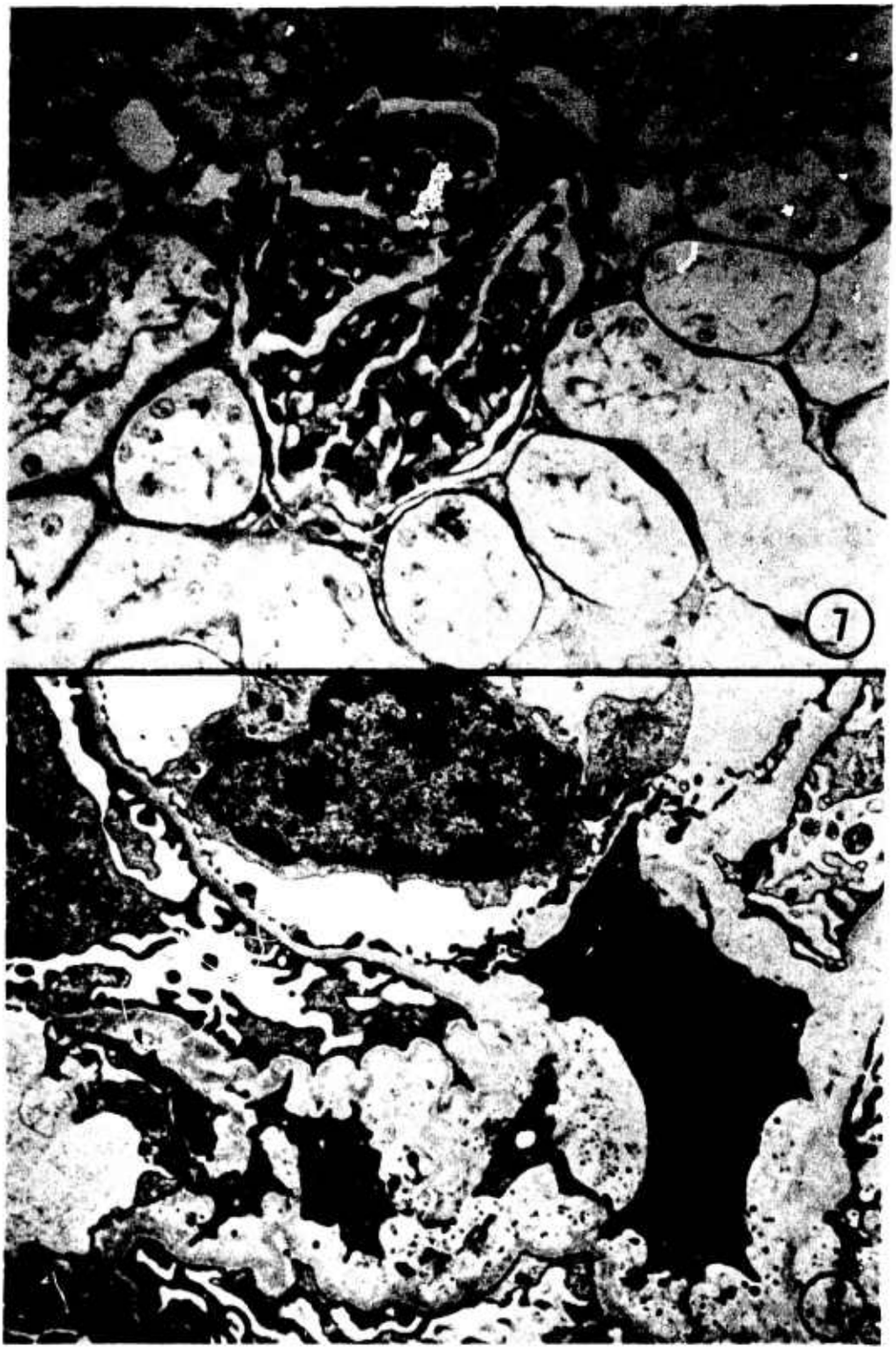
Animal 997 was first biopsied on the 30th day at a time when the CH_{50} level was depressed to 68% of preinfection values (Fig. 2). This biopsy revealed diffuse glomerular hypercellularity, numerous polymorphonuclear leukocytes in the capillaries and obliteration of the capillary lumina (Fig. 9). EM studies revealed focal swelling of the endothelial cells, and plugging of the glomerular capillary lumina with polymorphonuclear leukocytes, mononuclear cells, and occasional macrophanges containing secondary lysosomes. The mesangial areas were widened and contained electron lucent areas similar to those described in animal 801. A second biopsy obtained on the 51st day following 15 days of depression of the CH_{50} serum levels exhibited a persistent hypercellularity of the mesangium which, in addition, showed increased PAS positive mesangial mature material (Fig. 10). Methenamine silver and PAS stains showed that the capillary membrane was thickened and focally duplicated. Many capillary loops appeared obliterated (Fig. 10, 11). EM observations disclosed focal areas of capillary wall thickening with processes of the

TABLE 6

Percent changes in selected serum proteins and deposits in glomeruli 30 days after inoculation

		Serum			Renal deposits					
Animal no.	Albumin (%)*	CH_{50} (%)*	C4 (%)*	C3 (%)*	Properdin	C3	C4	IgG	IgM	IgA
801—infected	46	41	135	45	4+	3+	0	0	Trace	0
997—infected	49	68	122	27	4+	3+	0	0	2+	0
812—infected	51	12	19	22	4+	3+	0	0	2+	0
970—infected	62	57	29	72	0	0	0	0	0	0
952C—noninfected	91	135	208	115	0	0	0	0	0	0
966C—noninfected	84	104	95	98	0	0	0	0	0	0
976C—noninfected	ND†	95	112	106	0	0	0	0	0	0

* Percentage of preinfection value.
† ND, not determined.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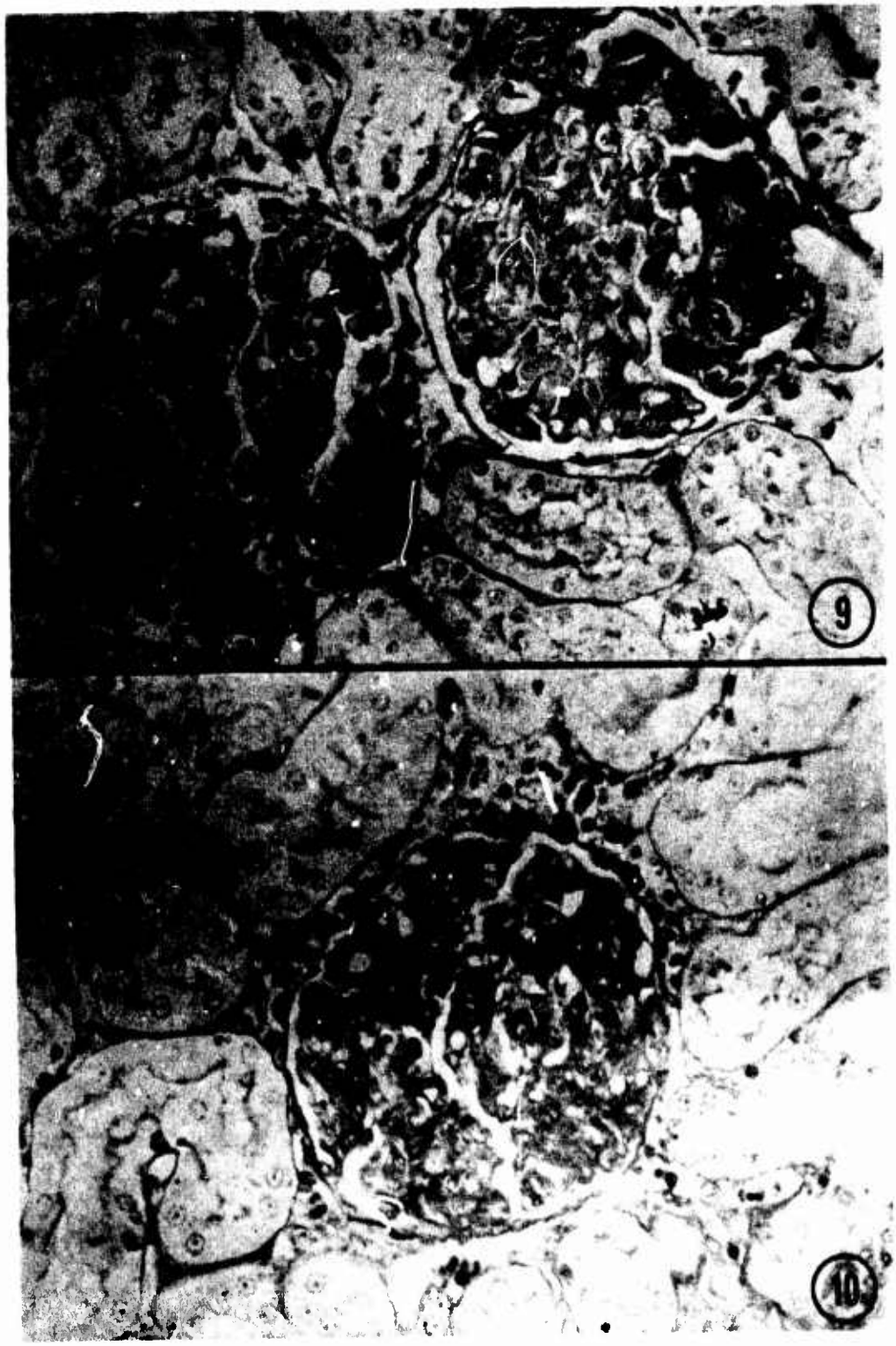
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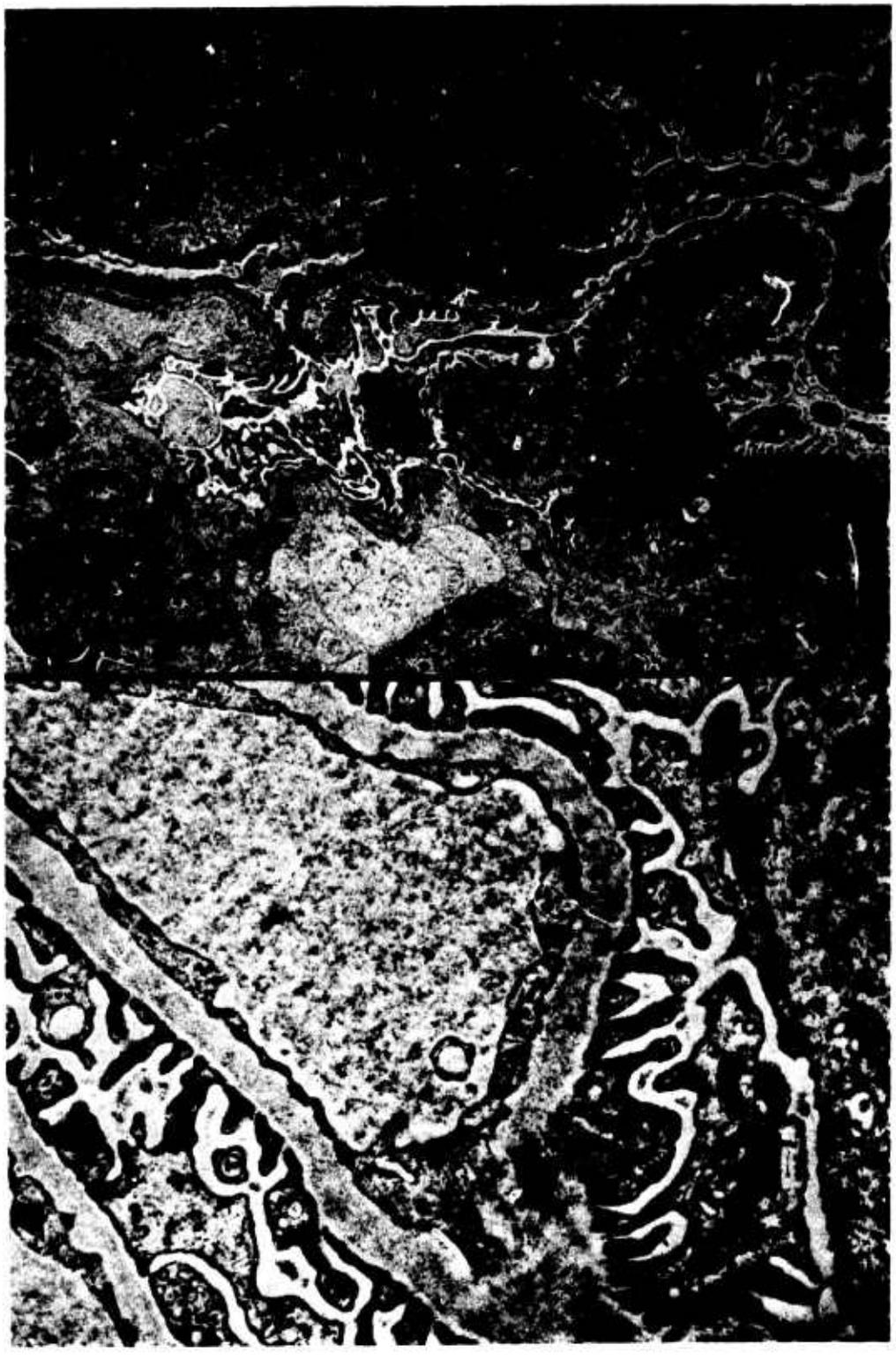

mesangial cells extending out into the peripheral capillary walls (Fig. 11). In these areas, there was production of new basement lamina which corresponds to the picture of peripherally duplicated basement membranes seen with the PAS and MS stains by LM. The capillaries were focally occluded by swollen endothelial cells, mononuclear cells and an occasional polymorphonuclear leukocyte (Fig. 11). Occasional electron dense subepithelial deposits were seen (Fig. 11, 12) as well as rare subepithelial deposits.

The first biopsy obtained from animal 812 on the 14th day at a time when the CH_{50} level was in the normal range showed a rare focus of mesangial sclerosis but no evidence of active disease. A second biopsy obtained on the 29th day when the CH_{50} level had been depressed for 7 days, revealed a definite, diffuse mesangial hypercellularity and wrinkling and focal thickening of basement membrane. E/M confirmed widening of the mesangial regions and increased numbers of mesangial cellular processes. In addition, the mesangial matrix contained electron lucent areas which contained irregular electron dense material.

A biopsy obtained on the 30th day of infection from animal 970 revealed essentially normal glomeruli with focal areas of slight mesangial hypercellularity. The CH_{50} level was within the normal control range at this time although it was 72% of this animals preinfective value. No glomerular deposits were seen.

d) Correlated studies of serological and renal biopsy observations: Four infected monkeys and three uninfected animals were studied in detail with respect to serological changes of whole complement, levels of C3 and C4 as determined by radial immunodiffusion, and protein deposits in renal glomeruli. The data are summarized in Table 6. The four infected animals were biopsied on the 30th day (after inoculation with trypanosomes) at which time their CH_{50} values were 41%, 68%, 12% and 72% of the pre-in-

FIGURES 5 and 6. **5**. Biopsy from control rhesus monkey showing normocellular glomerulus with open capillary loops limited by delicate basement laminae. PAS, × 520. **6**. Electronmicrograph of normal control. Note appearance of mesangial areas containing mesangial cells (arrows) surrounded by a compact mesangial matrix with an electron density similar to that of the basement membrane. × 1,300.

FIGURES 7 and 8. Renal biopsy of animal No. 801 taken 30 days post-inoculation of trypanosomes. **7**. Note general hypercellularity of the glomerular lobules and obliteration of capillary lumens. 3 μ section stained with PAS, ×550. **8**. Note rarefraction of mesangial matrix in areas surrounding mesangial cells. Numerous fine cytoplasmic processes of the mesangial cells extend into these areas. In addition, numerous electron dense, punctate material is seen within the mesangial area. × 2,900.

FIGURES 9 and 10. Renal biopsy of animal No. 997. **9**. Taken 30 days post-inoculation of trypanosomes, at time of depressed serum complement. Note marked hypercellularity of glomerular tufts, polymorphonuclear leukocytes, and obliteration of capillary lumina. PAS, × 500. **10**. Taken 51 days post-inoculation of trypanosomes. Note thickened and duplicated basement membranes (arrow), mesangial sclerosis, and obliteration of capillary lumina. Note also the focal interstitial inflammatory infiltrates. PAS, × 500.

FIGURES 11 and 12. Renal biopsy obtained in animal No. 997 51 days post-inoculation with trypanosomes. **11**. Note obliteration of capillary lumina, some of which contain polymorphonuclear leukocytes (p). There is focal reduplication of basement membrane (arrow). There is swelling of podocytes with loss of filtration sites focally and rare focal subepithelial deposits (d). × 1,700. **12**. Arrow indicates subepithelial deposit in area of slightly tangential section of capillary wall. × 22,000.

fection values determined in the same animals. Three uninfected control animals sampled also on the 30th day had complement levels of 135%, 104% and 95% of pre-infection values. The antigenic assay for C3 indicated severe depression in the C3 levels (45%, 27% and 22%) in three infected animals (Table 6). This was correlated with depressed CH_{50} levels and C3 deposits in the glomeruli as determined by immunofluorescence. Two animals showed depression of C4 levels but C4 deposits were not present in the glomeruli. The C3 and C4 levels in the uninfected controls were close to the preinfection values.

e) Immunofluorescent patterns of glomerular deposits: The various proteins detected in glomerular deposits have been summarized in Table 6. The pattern of fluorescence was one of a diffuse stain, limited to glomeruli, involving both glomerular capillary loops and mesangial areas. In some cases discrete granular patterns of fluorescence were seen. There was no evidence of smooth, linear fluorescence. Typical patterns of fluorescence are shown in Figure 13; the properdin reaction (Pro) was completely blocked (xPro) by previous absorption of the antiserum with purified human properdin. Deposits of IgM were seen in one monkey (Fig. 14). The granular appearance of the deposits was particularly apparent. Considerable deposition in mesangial areas was evident.

Glomerulonephritis develops in the course of experimental trypanosomiasis in monkeys. The earliest lesions are characterized as proliferative and show variable degrees of increased numbers of mesangial cells, swelling of endothelial cells, and variable margination of polymorphonuclear leucocytes. Later in the evolution of the process there is mesangial sclerosis and duplication of capillary basement membranes which in several cases clearly resemble a membranoproliferative type of glomerulonephritis.

Glomerulonephritis has been implicated in at least two other parasitic diseases. Kibukamusoke reported a high incidence of diffuse proliferative and membranoproliferative glomerular lesions in adults and children with nephrotic syndrome in Uganda and provided extensive evidence suggesting a malarial etiology in many of the cases. A spectrum of glomerular abnormalities has been found in selected patients with hepatosplenic schistosomiasis resembling the lesions of membranoproliferative glomerulonephritis.

The immunohistochemical evidence presented in this study points to and immunopathologic basis for the glomerulonephritis. The granular pattern of immunofluorescence would indicate the likelihood of an immune complex-type of nephritis rather than a nephrotoxic mechanism. The presence of properdin and C3 in the glomerular deposits of all animals studied suggested that the alternate (properdin) pathway was activated. The serologic data indicated that the basis for the acquired hypocomplementemia is in some animals related to a change in the C3 but

not the C4 levels. The explanation for a low C4 in two animals but absence of C4 deposits in the glomeruli in these animals was not apparent. A second study has indicated that in at least some animals C4 deposits were in the glomeruli. It is probable that both the classic and alternate pathway operate in the pathogenesis of this lesion. The agent(s) responsible for activating complement has not been defined. Indeed, it is not evident if the activating agent resided in the glomerular deposits, or if the complement pathway was activated within the blood stream with subsequent deposition of altered proteins in glomeruli. On the basis of these studies it would seem that trypanosomal infection in monkeys is particularly predisposed to initiating activation of the alternate pathway of complement.

In the context of glomerulonephritis in man it should be pointed out that there are two general situations in which reactant proteins of the alternate pathway (properdin) have been demonstrated in glomerular deposits. On one hand, a combination of reactant proteins of both the classical (immunoglobulin and C4) and the alternate (properdin deposits) pathways have been described in lupus nephritis, suggesting that both pathways have been activated. This agrees with the current concept that activation of the classical pathway can result in activation of the alternate pathway through release of the C3b fragment from C3 and sequential interactions of complement proteins beyond this point.

A second disease reported in humans which seems relevant to these studies is the syndrome of hypocomplementemic glomerulonephritis. This disease is associated with a membranoproliferative glomerulitis, deposits of properdin in renal glomeruli, and persistently low C3 levels in serum

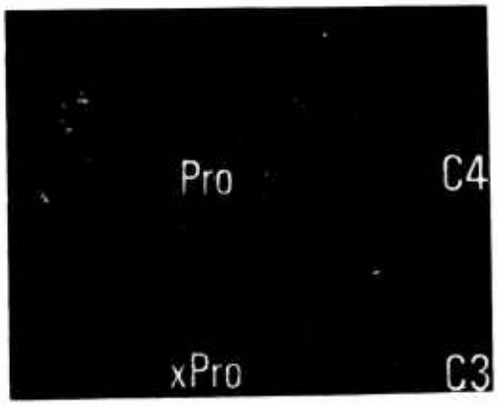

FIGURE 13. Immunohistochemical findings in a renal biopsy from animal No. 997, 30 days post-inoculation of trypanosomes. Upper left panel reveals a 4+ reaction with indirect technique using rabbit serum containing anti-properdin antibody (Pro). Lower left shows blockage of the reaction by prior absorption with purified human properdin (xPro). Upper right shows negative staining in C4. Lower right reveals 3+ staining for C3.

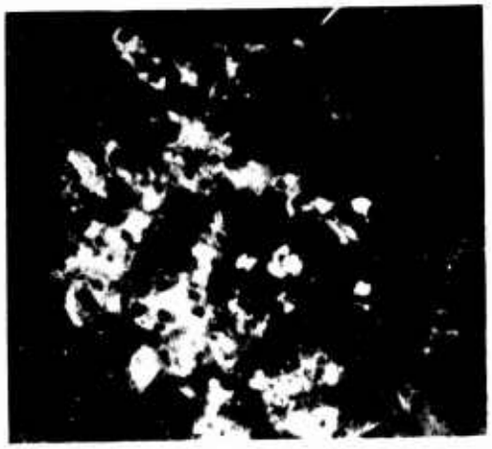

FIGURE 14. Renal biopsy from animal No. 997 showing deposits of IgM in a granular distribution.

were monitored daily and parasites (designated M-1) were obtained from monkey 505 during the first peak of parasitemia. Trypanosomes isolated from this monkey at a later date were designated M-2. Trypanosomes designated M-3 were obtained from monkey 614. On the days these parasites were collected, the trypanosomes were counted in a hemacytometer and diluted with PSG to a concentration of 2 x 10^4/ml. Each of these isolates was then injected I.P. into 5 immune and 5 normal rats. Immunized rats also were challenged with trypanosomes derived from the stock rat strain a second time (S-2). Each isolate of trypanosomes (S-1, M-1, M-2, M-3, S-2) also was tested by neutralization against the serum collected from rats 7 days after immunization. Five concentrations of trypanosomes from each isolate were tested against 3 dilutions of immune serum and against normal rat serum as the control. Trypanosomes were diluted in PSG and immune serum was diluted with normal rat serum. The mixtures were incubated at 25°C for 2 hr and then 5 mice were each inoculated I.P. with 0.2 ml of each dilution.

Rats which received 1 immunizing injection of irradiated trypanosomes of the stock strain and subsequently challenged with viable homologous parasites (S-1) exhibited a strong resistance to infection (Table 7). Both rhesus monkeys developed patent infections; parasitemias are depicted in Fig. 15. When immunized rats were challenged with parasites isolated from monkey 505 during the initial peak of parasitemia (M-1), no deaths occurred in the immunized group whereas all controls died in 6 to 7 days (Table 7). However, when immunized rats were challenged with trypanosomes isolated from monkey 505 (M-2) or monkey 614 (M-3) later during the infection, little resistance was detected; 4 of the immunized rats in each group died within the range of the control animals' survival times. Subsequently a second challenge of immunized rats with a second isolate of the stock strain (S-2) was made to retest homologous immunity. Again a strong resistance was apparent in the immunized rats while controls died in 5 to 6 days. Some differences in survival times among control rats were noted. The control animals challenged with the stock rat strain (S-1, S-2) died on days 5 and 6, but animals inoculated with parasites derived from monkeys survived for periods varying from 6 to 7 days with M-1 and from 7 to 8 days with M-2 and M-3.

When neutralization tests wereperformed, all dilutions of immune serum collected from rats 1 week after immunization reacted positively with parasites of the stock strain (both S-1 and S-2). The serum also had a marked effect on trypanosomes isolated from monkey 505 (M-1) during the initial peak of parasitemia. Subsequent isolates of trypanosomes from monkeys 505 (M-a) and 641 (M-3) were not neutralized by the immune serum and their infectivity appeared to be enhanced when the osolates were mixed with immune rat serum at a 1:2 dilution.

Rats immunized with irradiated Trypanosoma rhodesiense resisted infection with the homologous strain. When similarly immunized rats were challenged with parasites obtained from rhesus monkeys infected with the same strain, the response depended on the number of peaks of parasitemia were solidly immune; immunized rats challenged with trypanosomes obtained

with relatively normal levels of C1, C4 and C2. More recent investigations suggest that this disease may be a unique syndrome insofar as the renal disease appears to be associated with a more or less specific activation of the alternate complement pathway. There is, as yet, no information that would pinpoint the reaction product responsible for the morphologic and functional alterations in the glomeruli.

T. rhodesiense infection in monkeys results in glomerulonephritis which in certain respects resembles the hypocomplementemic glomerulonephritis of humans. In veiw of these findings it would be of interest to carefully study the renal structure and function of patients infected with African trypanosomiasis. Likewise this experimental infection could prove useful in studyin, the evolution and pathogenesis of glomerulonephritis involving the alternate pathway (properdin) of complement activation.

4. Variant specificity of immunity induced by irradiated Trypanosoma rhodesiense.

Sanders and Wallace induced a protective response to Trypanosoma lewisi in rats by the administration of irradiated trypanosomes. Subsequently, Duxbury and Sadun reported immunity in rats produced by irradiated parasites against a virulent strain of T. rhodesiense. Cattle also developed immunity which persisted for at least 8 months after this treatment. Recently Duxbury et al have shown that primates may be similarly immunized against a recently isolated strain of T. rhodesiense. In the experiments reported here, the specificity of immunity induced by irradiated trypanosomes has been explored, taking into account the well known capability of trypanosomes to evade the host response by changes in antigenic character.

Ninety-five random bred Wrm:WPC(WI)BR rats (140-200 gms) and 500 random bred Wrm:(ICR)BR mice (20-25 gms) were used in these studies. One male (No. 505) and one female (No. 614) Macaca mulatta monkey weighing 4 and 2 kg, respectively, were infected and served as sources of parasites. The Wellcome strain of T. rhodesiense was used throughout the experiment.

Five heavily infected rats were anesthetized and bled by cardiac puncture. This heparinized blood was then irradiated at 70 kr in a Gammacell, 60 cobalt irradiator and diluted with phosphate buffered saline with glucose (PSG), pH 7.8, to a concentration of 1×10^9 trypanosomes per ml. Forty-five rats were inoculated intraperitoneally (I.P.) with 1 ml of this suspension. One week later serum was obtained from 20 of theimmunized rats and from 20 nonimmunized animals. One the same day, 5 of the remaining immunized rats and control rats were each challenged I.P. with 1×10^4 viable trypanosomes from the stock rat strain (designated as S-1) the 2 monkeys, (505 and 614), also were injected intravenously with 1×10^5 and 5×10^4 trypanosomes, respectively, from an aliquot of the challenge inoculum S-1. Parasitemias

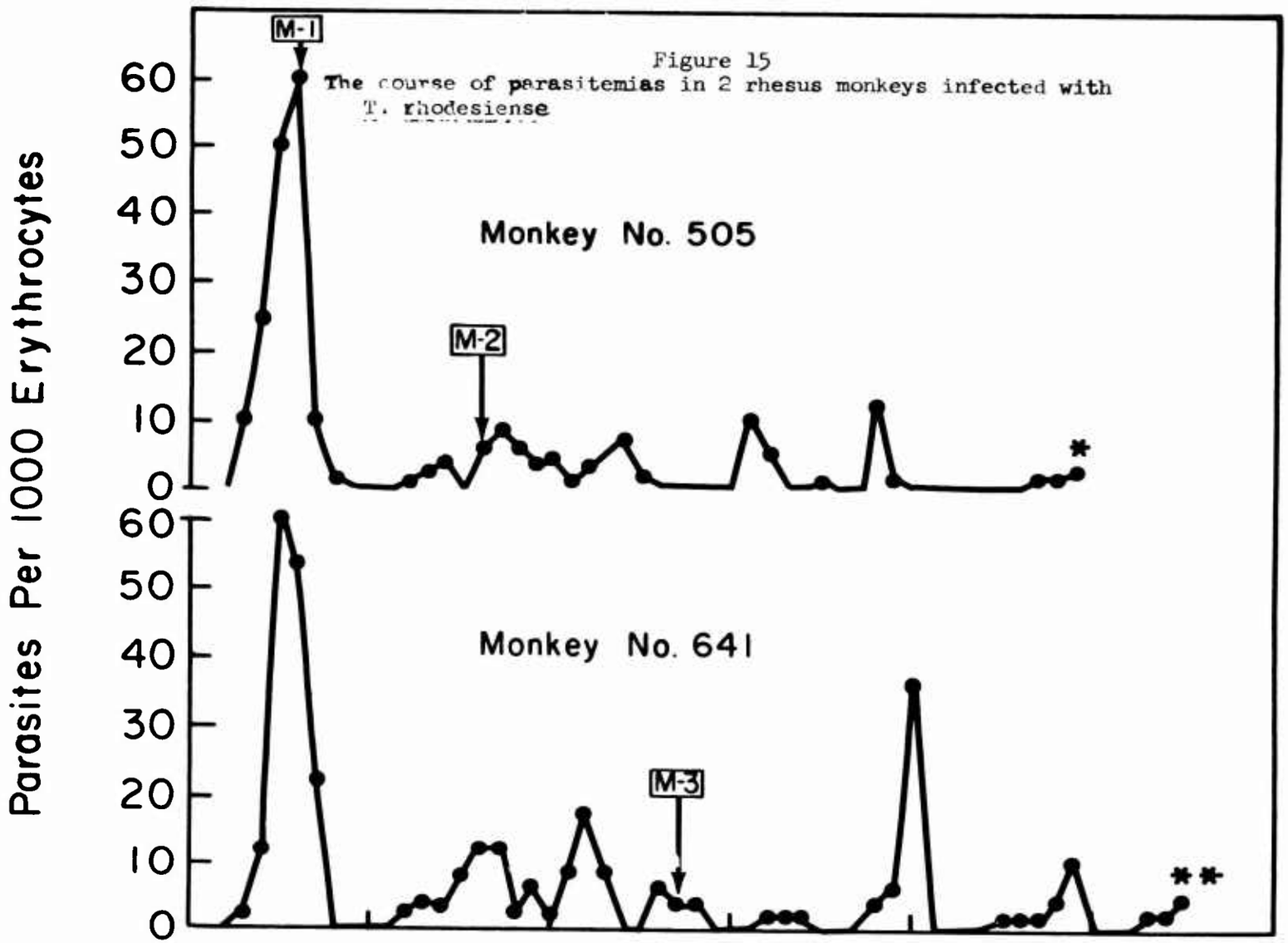

Figure 15
The course of parasitemias in 2 rhesus monkeys infected with T. rhodesiense

Table 7

Specificity of the Immunity Produced in Rats by Irradiated Trypanosoma rhodesiense

Source of challenge parasites	Group	Mortality[a]	Day of death (No. animals)
S-1	Immunized	1/5	8(1)
	Control	5/5	5(3), 6(2)
M-1	Immunized	0/5	--
	Control	5/5	6(2), 7(3)
M-2	Immunized	5/5	6(2), 7(2), 12(1)
	Control	5/5	7(3), 8(2)
M-3	Immunized	5/5	7(4), 11(1)
	Control	5/5	7(3), 8(2)
S-2	Immunized	1/5	8(1)
	Control	5/5	5(2), 6(3)

[a]No. rats dead/no. injected

from monkeys after their initial peak of parasitemia all succumbed to the challenging infection. These observations indicate that parasites of a variant antigenic specificity arose during the course of the monkey infections. Neutralization tests performed on the various isolates from rats and monkeys using antibody obtained from immunized rats confirmed that the immunity produced by irradiated trypanosomes was variant specific.

5. Lymphatic dynamics in experimental filariasis monitored with ^{99m} Technetium-sulfur colloid.

Progressive alteration of lymphatic function is a common sequela in human filariasis and often leads to lymphedema and elephantiasis. Previous investigators have used various animal models to study changes in lymph nodes and vessels due to experimental filarial infections. Radiographic lymphangiograms commonly have been utilized in these experimental

infections to visualize anatomical changes of lymphatics. This technique, though very useful, has numerous disadvantages and does not provide any direct information how efficiently the lymphatics are functioning in the infected animal.

This report describes the results of a pilot study to develop an isotopic technique for quantitating lymphatic function in a filaria-infected host using the radiopharmaceutical ^{99m} Technetium-sulfur colloid (Tc-SC). The model used was the African patas monkey (Erythrocebus patas) infected with subperiodic Brugia malayi.

Four patas monkeys were each inoculated subcutaneously on the dorsum of both feet with 174 third stage larvae of B. malayi. The source of the parasites was Aedes aegypti mosquitoes (black eye strain) which had been membrane fed 11 days previously on infective patas monkey blood. All exposed monkeys developed patent infections 10-12 weeks post-exposure and remained patent throughout the experiments. Radionuclide experiments were performed during the 36th to 48th week of infection.

Monkeys were prepared for radionuclide studies by anesthetizing them with mg/kg Sernylan^{R} (phenyclidine hydrochloride) given intramuscularly. An additional 1 mg/kg of the drug was given 1 hr later during studies requiring 2 hr to complete.

The radiopharmaceutical, ^{99m} Technetium-sulfar colloid (Tc-SC), was prepared according to Dunson et al. Each monkey received approximately 1 mCi of Tc-SC suspended in 0.2-0.3 ml saline per foot. Injections were given subcutaneously in the distal third of the 2nd-3rd metatarsal space. Gamma emissions were counted at the injection sites from time zero for 1 min using a high performance scintillation camera. Hinds limbs then were exercised passively for 5 min to stimulate lymph flow. At various times from 10-120 min post-injection, the pedal sites were recounted; serial counts also were obtained from popliteal and abdomino-pelvic node areas. All counts represented the combined emissions from the right and left side of each animal.

Monkeys were placed in a supine position beneath the gamma sensor during counting. Lead shields were placed appropriately during each count to insure specific counting with maximum uniformity. These shields were placed proximally to the malleolus during pedal counts, distally and proximally from the knee for popliteal counts, and distally to the inguinal region and immediately proximally to the navel for abdominopelvic counts. Scintophotos, when desired, were taken simultaneously with counting.

Because of the short half-life of the isotope (T 1/2 = 6 hr, 4 min) all counts were standardized to the time of the first count taken during each experiment using the standard isotope decay formula.

Radiographic lymphangiograms were performed using Evans blue dye and the radio opaque contrast medium Ethiodol^{R} after Kinmonth.

Four hr prior to necropsy, all monkeys received a total of 0.1 ml Evans blue dye subcutaneously between the 2nd-3rd and 4th-5th toes for delineation of lymphatics. Leg skin was removed from all infected animals at necropsy and soaked overnight in normal saline at 26 C. The saline washings then were examined for adult worms with a dissecting microscope. Standard histologic procedures were followed with tissues fixed in 10% neutral buffered formalin, sectioned at 6 microns, and stained with hematoxylin and eosin.

A radiographic lymphangiogram of a normal patas monkey detailing the lymphatic vessels and nodes in the lower extremities is shown in Figure 16. Figure 17 is a scintophoto using Tc-SC which images the lymphatics in the popliteal and abdomino-pelvic area of a normal patas. The radiographic technique clearly illustrates lymph nodes and vessels in greater detail than does the isotopic technique. The Ethiodol is seen, however, only after a sufficient amount is injected under pressure into the lymphatic system to fill the nodes and vessels to be image. In contrast, the amount of Tc-SC in the nodes and vesels is entirely a function of the ability of the lymphatic system to passively transport the radionuclide from the point of subcutaneous injection to those locations.

The results of 3 to 5 individual isotope exposures per monkey showing relative isotope migration rats and popliteal and abdomino-pelvic appearance rates are demonstrated at 20 min post-injection in Figure 18. These data indicate that the infected monkeys on the average are not as efficient as the control monkey in clearing the isotope from the injection sites and moving it up to nodes into which lymph drains from the pedal area. Pedal migration rates in the 3 infected monkeys (925, 927, 928) averaged 55, 39, and 20%, respectively, of the control animal's average migration rate. Likewise, popliteal and abdomino-pelvic appearance rates of the 3 infected animals averaged 96 and 46%, 67 and 60%, and 33 and 17%, respectively, of the control animal's average appearance rates. In spite of these differences, however, infected monkeys showed no signs of lymphedema in the affected limbs.

The flow rate of lymph in patas #928 appeared to be the most altered of any monkey by its filarial infection. Therefore a number of 2 hr studies was initiated with this monkey and control #924 to determine whether the isotopic technique could be used to meaningfully quantitate lymph flow rates during time course studies of filaria-infected animals. A representative 2 hr study is shown in Figure 19. These comparative migration and appearance curves suggest that B. malayi infection in #928 resulted in a significant decrease in the efficiency of the lymphatic system as showed by the inability of the infected animals to quickly clear Tc-SC from the injection sites to the draining popliteal and abdomino-pelvic nodes as compared to the control monkey.

Histopathologic examination of all monkeys after necropsy substantiate the results of the isotopic experiments. The lymphatics in both legs of the control animal (#924) appeared normal. The isotope

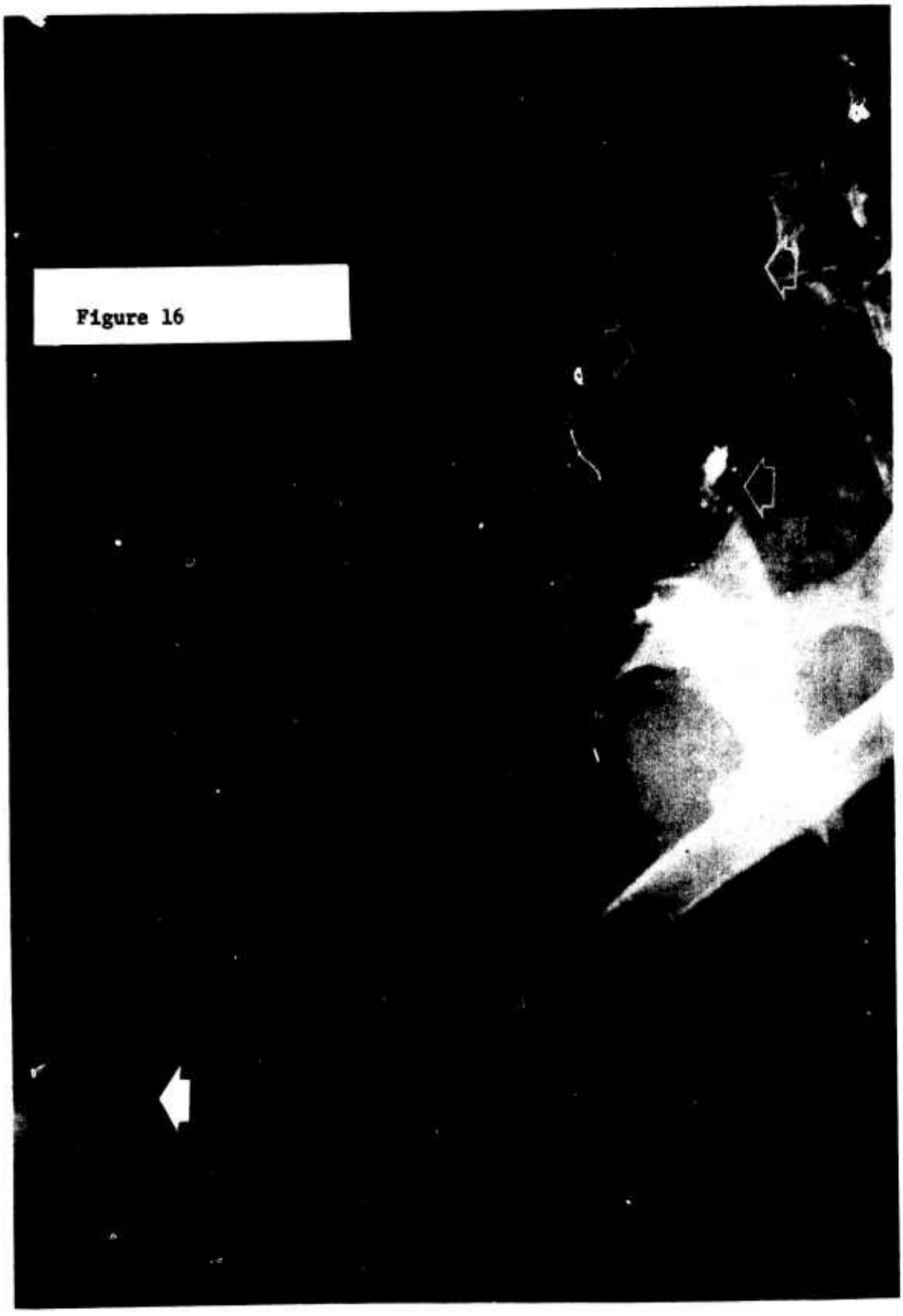

Figure 16

flow rates in monkeys #927 and #925 were only moderately altered compared to the control animal. Monkey #927 exhibited the least pathologic involvement of any exposed animal. Histologic sections of a few adult worms were seen in the afferent vessel to the left popliteal node with only slight occlusion of the vessel evident. Extensive gross and histopathologic examination revealed no other adult worms in this animal. The pedal lymphatics of monkey #92[illegible] were partially occluded bilaterally with numerous adult worms, most of [illegible]hich were viable. Numerous lymphatic varices were noted in both feet. The afferent lymphatic vessel to the right popliteal node was dilated. No other adult worms were found in this animal except for 2 females recovered from saline washings of the leg skin.

Histopathologic examination of patas #928 revealed the most extensive and severe pathologic involvement of all infected monkeys. These findings correlate directly with the severe reduction in lymphatic flow rates seen in the isotopic studies. The afferent lymphatic to the right popliteal node was observed to be completely obstructed due to the presence of a dead worm 1 cm distal to the node. Sections taken from the remainder of the right afferent vessel showed both viable and dead worms with associated moderate to severe lumenal blockage. The system as showed by the inability of the infected animal to quickly clear Tc-SC from the injection sites to the draining popliteal and abdominopelvic nodes as compared to the control monkey.

Histopathlogic examination of all monkeys after necropsy substantiate the results of the isotopic experiments. The lymphatics in both legs of the control animal (#924) appeared normal. The isotope flow rates in monkeys #927 and #925 were only moderately altered compared to the control animal. Monkey #927 exhibited the least pathologic involvement of any exposed animal. Histologic sections of a few adult worms were seen in the afferent vessel to the left popliteal node with only slight occlusion of the vessel evident. Extensive gross and histopathologic examination revealed no other adult worms in this animal. The pedal lymphatics of monkey #925 were partially occluded bilaterally with numerous adult worms, most of which were viable. Numerous lymphatic varices were noted in both feet. The afferent lymphatic vessel to the right popliteal node was dilated. No other adult worms were found in this animal except for 2 females recovered from saline washings of the leg skin.

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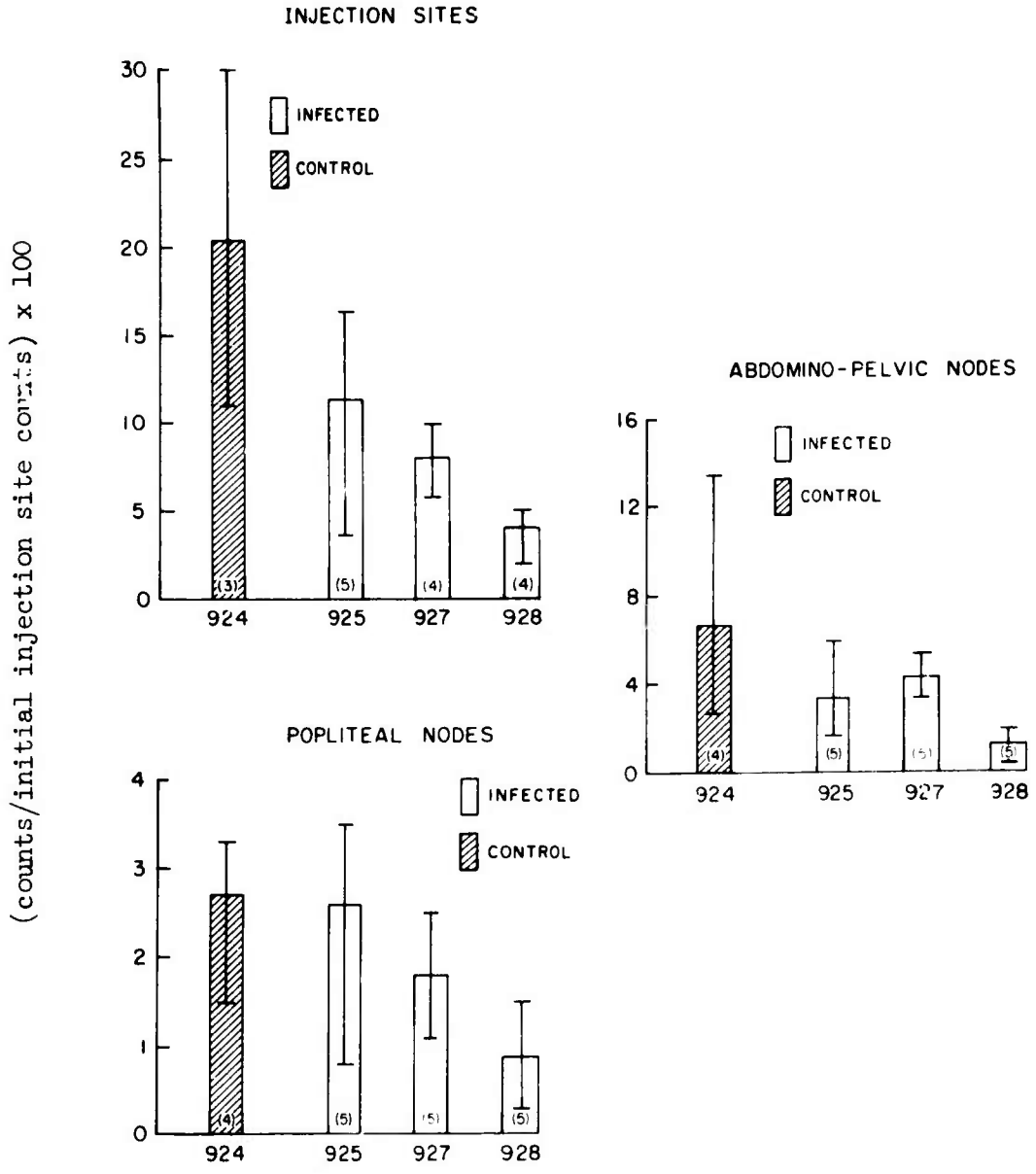
Figure 17

dilated and partially obstructed with numerous viable worms present. Gross appearance of the pedal lymphatics of #928 showed marked lymphangitis with dilated, tortuous, and beaded lymph vessels (Figure 20). In contrast, in the control monkey a normal pedal plexus showing only delicate filamentous vessels was visible (Figure 21). No other adult worms were seen in #928 except for 3 females recovered from leg skin washings.

Three general types of lymphatic vessel lesions were seen microscopically in the infected animals. First, the reaction associated with the presence of viable adult worms was dilatation with endolymphangitis and perilymphangitis characterized by endothelial swelling and subendothelial proliferation. Second, lesions associated with dying or recently dead adult worms were more severe with pooling of eosinophils and neutrophils around the worms. The associated lymphatic vessel wall was diffusely infiltrated by an admixture of inflammatory cells. These factors together resulted in moderate vessel obstruction. The most severe lesions were granulomatous and associated with partially or completely calcified adult worms. Histiocytes and foreign body giant cells often surrounded these dead worms. Obliterative lymphangitis with perilymphatic fibrosis was often the sequelum to this chronic inflammatory process.

Radiographic lymphangiography has been a very useful tool in describing detailed morphologic changes of lymphatic vessels and nodes in animal and human filarial disease. The technique, however, has numerous drawbacks: frequently repeated exposures are not possible because of long lasting residual which may result in severe tissue reactions, the proce-

Figure 18


INJECTION SITES
(counts/initial injection site counts) x 100
INFECTED
CONTROL
30
25
20
15
10
5
0
(3)
(5)
(4)
(4)
924
925
927
928
ABDOMINO-PELVIC NODES
16
12
8
4
0
(4)
(5)
(5)
(5)
924
925
927
928
POPLITEAL NODES
4
3
2
1
0
(4)
(5)
(5)
(5)
924
925
927
928

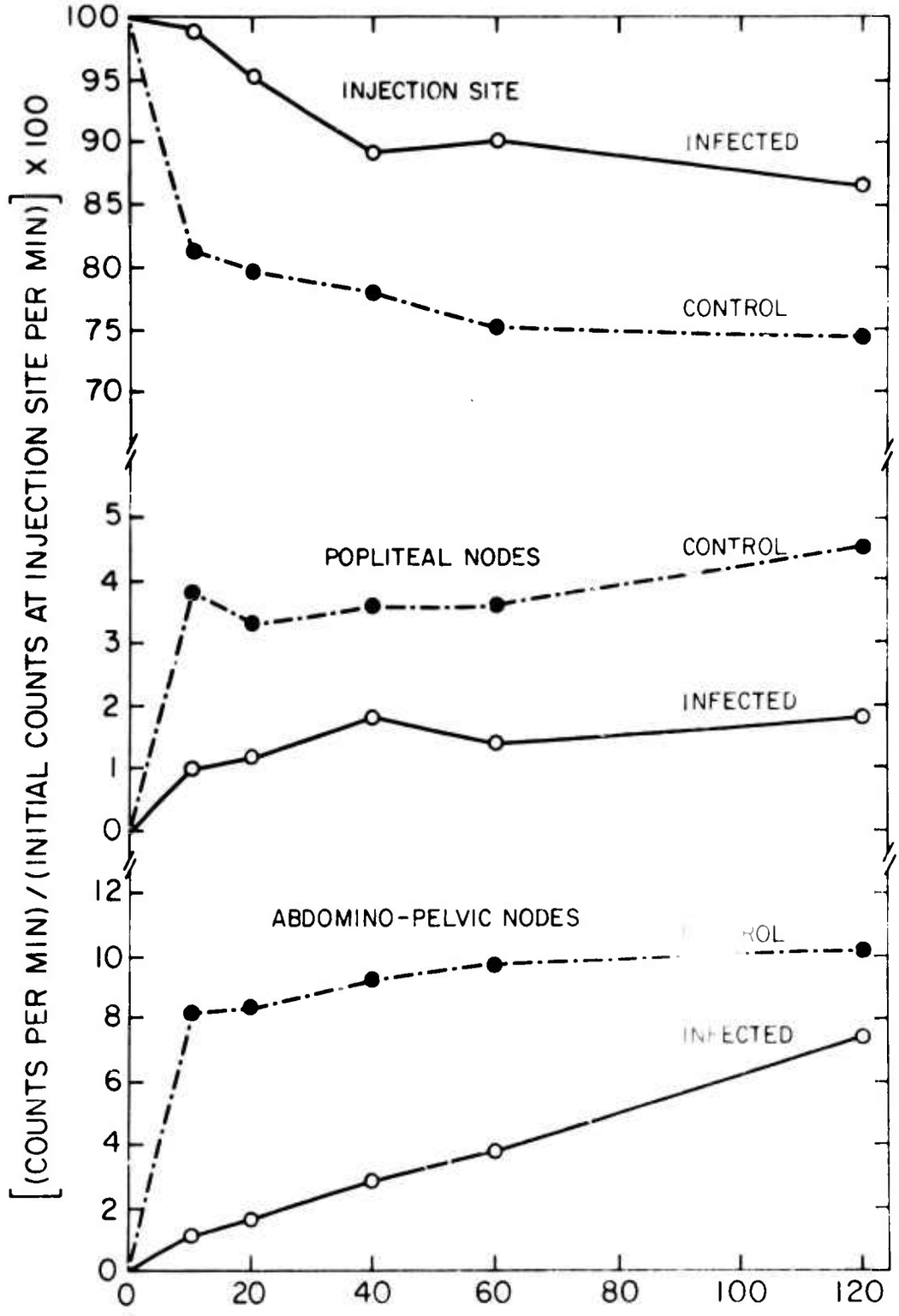

[(COUNTS PER MIN) / (INITIAL COUNTS AT INJECTION SITE PER MIN)] X 100
INJECTION SITE
INFECTED
CONTROL
POPLITEAL NODES
CONTROL
INFECTED
ABDOMINO-PELVIC NODES
INFECTED
0
20
40
60
80
100
120

Figure 19

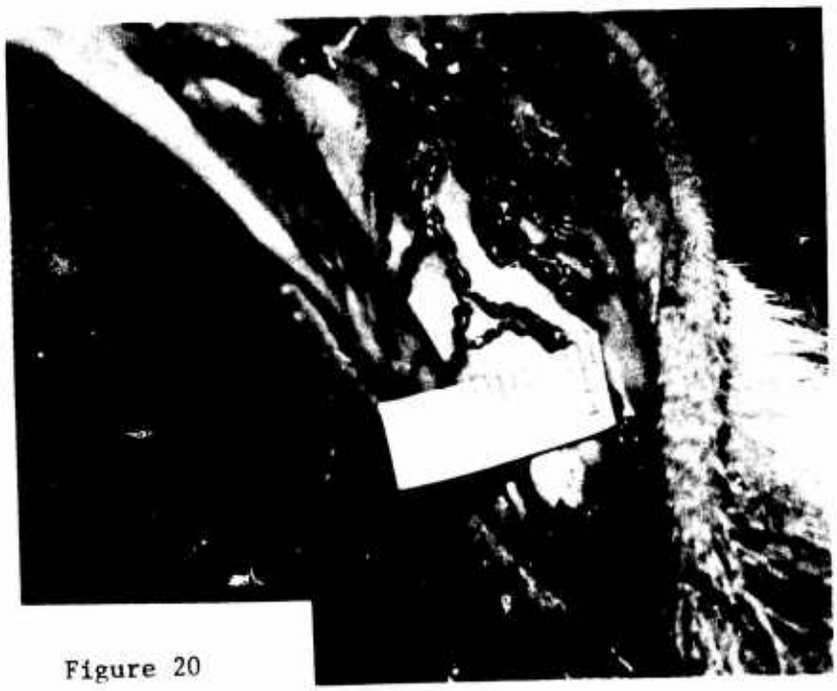


Figure 20

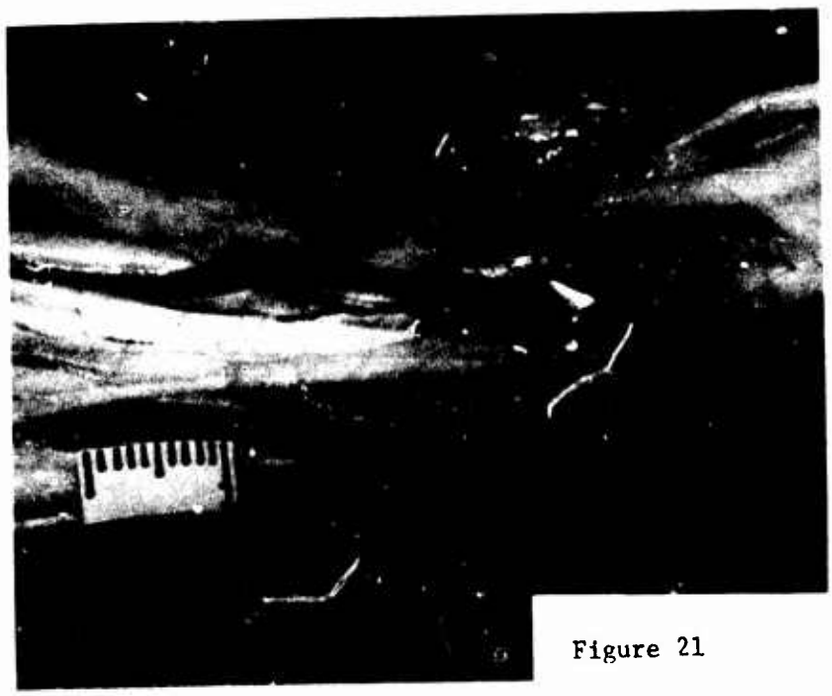


Figure 21

LEGENDS FOR FIGURES

Figure 16 - Radiographic lymphangiogram (lateral view) of a normal patas monkey detailing the popliteal node (closed arrow) and the abdomino-pelvic nodes (open arrows). The afferent lymph vessel to the popliteal node which originates from the pedal area is clearly visible.

Figure 17 - Scintophoto (frontal view) of a normal patas monkey imaging the popliteal nodes (bottom) and the abdomino-pelvic nodes (top).

Figure 18 - Relative Tc-SC injection site disappearance rates, and popliteal and abdomino-pelvic appearance rates in B. malayi - infected patas monkeys at 20 min post-injection. (Numbers in parentheses equal individual experimental readings per animal; vertical brackets represent their range).

Figure 19 - Tc-SC injection site disappearance curves and popliteal and abdomino-pelvic appearance curves for B. malayi - infected patas (#928) versus control patas (#924) over a 2 hr period.

Figure 20 - Dorsal pedal lymphatic plexus of right foot of B. malayi - infected patas #928. Note the highly dilated, tortuous, and beaded lymph vessels.

Figure 21 - Dorsal pedal lymphatic plexus of left foot of control patas #924 showing delicate, filamentous lymph vessels.

dure is relatively difficult and has a significant failure rate, and contrast medium must be forced into the lymphatics under pressure. Furthermore, numerous complications may result from this procedure. Because of Technetium's short half-life, the isotope technique described in this study can be performed as often as every 2nd or 3rd day if desired. The technique requires only a single subcutaneous injection of the saline-suspended colloid which is passively absorbed into the lymphatic system. There are no known complications resulting from the procedure.

Radiographic lymphangiograms showed that the location of the popliteal node in the patas was in the primary lymphatic drainage chain from the dorsum of the foot. This condition is similar to that of the cat and dog but not the rhesus monkeh. Histopathologic examination of patas lymphatics in the present study demonstrated a worm migration pattern most closely resembling that of experimental infections of B. malayi in the cat where with very few exceptions worms migrated only as far as the popliteal node when the host was exposed on the volar surface of the foot. This situation makes possible the use of the patas as a unilaterally infected animal where the contralateral leg can be used as an autocontrol during isotopic and radiographic studies. The major pathologic effects of B. malayi upon the patas monkey lymphatic system appear to occur distal to the popliteal node where essentially all adult worms are recovered. These effects were manifested in this study by decreased flow rates of Tc-SC-labeled lymph.

In spite of a marked decrease in lymphatic efficiency, particularly with patas #928, there was no evidence of clinical edema of the feet or legs. This suggests that significant impairment of the lymphatics may occur in the absence of clinical symptoms. Bosworth et al showed that cats unilaterally exposed in one hind leg to B. malayi were made susceptible to a secondary Group G beta hemolytic streptococcal infection which caused lymphangitis, abscesses, and edema in the Brugia-infected leg. The contralateral leg which was exposed only to the streptococci showed no signs of bacterial infection. Cats showed no clinical symptoms in the Brugia-infected legs before streptococcal challenge. These data along with the results of the present work suggest that filarial infection by itself may not be entirely responsible for the edema and elephantiasis. As Drinker et al postulated, secondary bacterial infections which cannot be adequately resisted and cleared by the host due to lymphatic blockage may contribute significantly to the onset and progression of lymphstasis and elephantiasis. The isotopic technique described herein could be very useful in detecting filarial disease before such clinical symptoms become evident. Because of these encouraging preliminary results a larger and more definitive experiment using unilaterally and bilaterally exposed patas monkeys has been initiated to further study changes in lymphatic dynamics during long term experimental filarial disease.

6. Immunolcgical reactions in rabbits experimentally infected with Schistosoma japonicum.

Allergic manifestations are an important component of schistosomal infections. Skin reactions of the immediate hypersensitivity type have been readily detected in people and experimental animals and reagin-like antibodies have been demonstrated in vivo and in vitro. However, attempts to detect cell mediated immunity in infections established by the normal route produced conflicting results. Increase of lymphoblastogenesis was demonstrated in chimpanzees experimentally infected with Schistosoma japonicum by incorporation of tritiated thymidine into lymphocyte DNA following an antigenic stimulus. However, no delayed skin hypersensitivity was observed in these animals. The skin tests were conducted with only one antigen considerably after the peak of blastogenesis on a limited number of chimpanzees. Therefore, these results did not provide definitive evidence of delayed hypersensitivity.

Therefore, rabbits were exposed to S. japonicum cercariae and the presence and extent of hypersensitivity reactions were monitored by passive cutaneous anaphylaxis, by antigen induced histamine release and by determining the degree of stimulation of lymphocytes to antigen at frequent intervals. Before necropsy each animal was given intradermal inoculations of egg, cercarial, somatic adult and metabolic adult antigens, and their reactions were observed grossly and histologically. The effects of S. japonicum infection on serum complement activity were also studied.

Albino rabbits weighing 3.5 - 4.5 kg were used in these experiments. Sixteen rabbits were each exposed to 150 S. japonicum cercariae and 4 were left as uninfected controls. All animals were fed a standard diet. They were bled from the central ear artery before infection and at regular intervals for 18 weeks.

Cercariae of the Japanese strain of S. japonicum were obtained from pools of approximately 50 infected Oncomelania nosophora snails which had been exposed 3 - 4 months earlier to miracidia obtained by hatching eggs from lives of infected albino mice. The rabbits were exposed percutaneously as described earlier.

Soluble antigen fluorescent antibody (SAFA) tests and radioactive microprecipitin (RAMP) assays were done according to the described methods.

The presence of immediate hypersensitivity reactions was monitored by passive cutaneous anaphylaxis and by antigen induced histamine release. The degree of stimulation of lymphocytes to antigen was studied at frequent intervals with strict adherence to the technique described earlier.

Seventy-two hours before necropsy each rabbit was given intradermally 0.1 ml of one or more of the cercarial, egg, metabolic or so-

amounts of thymidine incorporated was ten fold greater in the infected animals than the noninfected animals.

An increase in complement level occurred in the infected rabbits beginning 6 weeks after infection.

Examination of skin sections revealed mild to moderate lesions with focal to diffuse infiltrated dermis with apparent vessel and perifollicular affinity. Predominantly mononuclear cellular infiltrates were present. Lesions were primarily in middle and deep thirds of the sections, increasing in severity as they approached the underlying musculature but without musculature involvement.

Necrosis was of a cellular nature limited to the deep dermal third. Edema was generally found in the deeper dermal sections tending to separate collagen bundles.

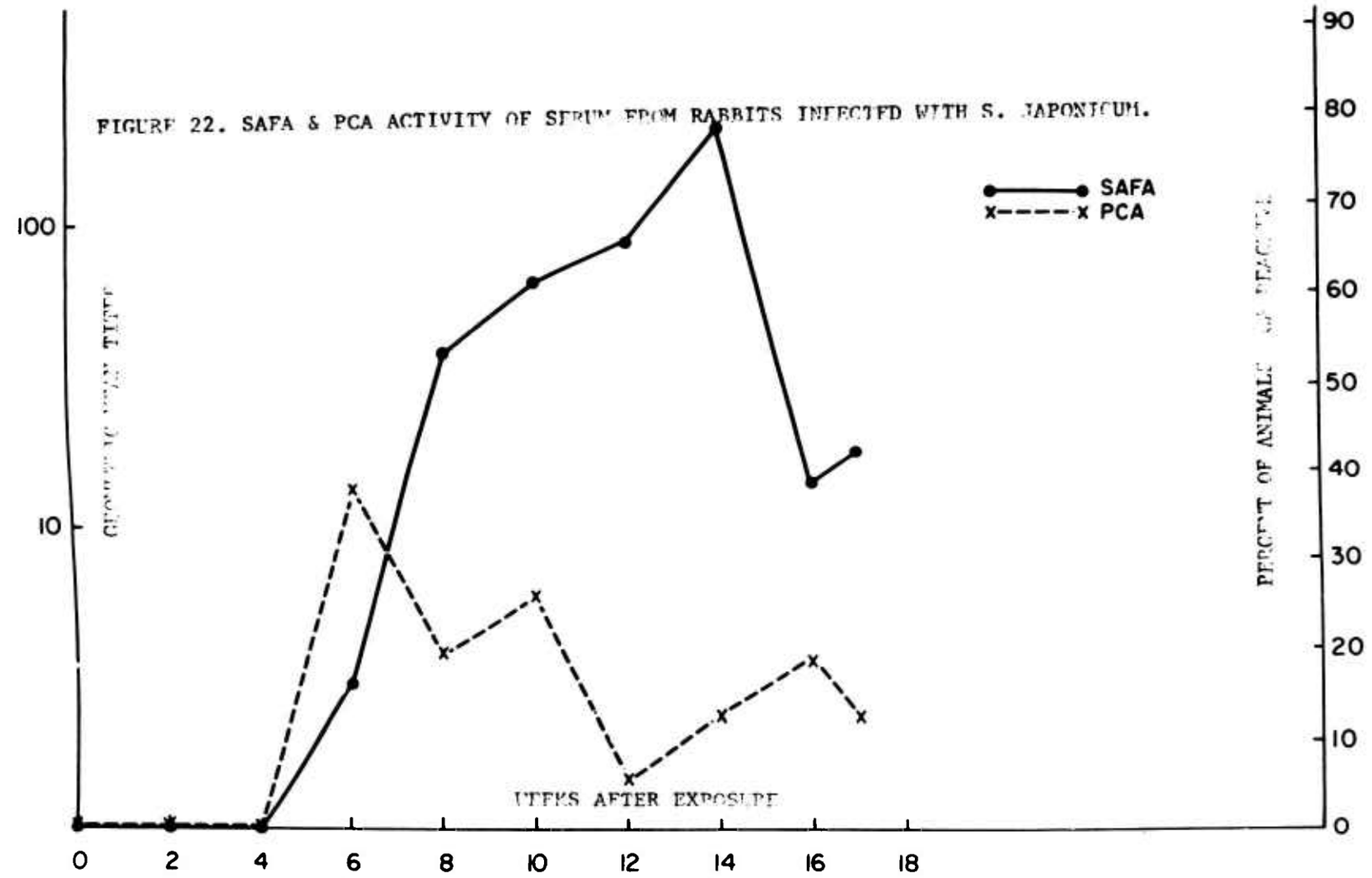
There were no significant differences among the several antigens. True Arthus phenomenon was not observed. Pure delayed hypersensitive response was not observed. The observations were consistent with a mixture of immediate and delayed response along with an inflammatory reaction.

The results of these investigations support and extend those previously observed in chimpanzees. Immediate hypersensitivity was demonstrable using in vitro and in vivo tests. As reported previously although less than one-half of infected animals developed detectable PCA reactions in vitro antigen-induced histamine release was detectable in all of the infected animals both in the presence and absence of passive cutaneous anaphylactic antibodies. Similarly, both homocytotropic antibodies (as detected by the RAMP test) and blastogenesis were demonstrated in all infected animals.

Since there is some evidence that antigen-induced lymphocyte transformation may not correlate with delayed hypersensitivity, confirmatory evidence was to be derived by the histologic examination of skin biopsies at various intervals following introduction of 4 different antigens. However, no clear indication of skin reaction of the delayed type was observed with any of these 4 antigens employed at any of the times studied. It is possible that the immediate reaction might have interfered with the ensuing skin reactions of the delayed hypersensitivity type.

It has been suggested that some of the lesions of schistosomiasis may result from immune complexes. In the studies conducted in chimpanzees, although no significant differences were noted in the mean values of complement levels among infected and noninfected groups, there was a general decline was observed coincides with the massive release of eggs near the beginning of patency, the possibility of fixation of the complement components to antigen-antibody complexes with consequent depletion from the serum was suggested. However, in the present studies

FIGURE 22. SAFA & PCA ACTIVITY OF SERUM FROM RABBITS INFECTED WITH S. JAPONICUM.



matic adult antigens along with a normal saline control. The resulting lesions were excised and fixed in 10% buffered formalin for light microscopy. Histologic sections were prepared, stained with hematoxylin and eosin and examined.

The complement levels were determined with sheep red cells which had been stored no longer than 3 weeks in modified Alsever's solution. They were washed and spectrophotometrically standardized to contain 5×10^8 cells per ml. The preparation of sensitized cells and the spectrophotometric method for complement assays used were described previously.

All the rabbits exposed to S. japonicum cercariae became infected and began excreting eggs in the feces between 8 and 12 weeks after exposure. Six of 16 infected animals produced reaginic antibodies detectable by passive cutaneous anaphylaxis 6 weeks after exposure. Reaginic activity was still detectable in two of the 16 animals 17 weeks after exposure. In 10 infected animals and all four uninfected controls, no reaginic activity was demonstrated by PCA. No relationship was observed between the presence of PCA reactions and the intensity of infection as determined by egg counts. All of the infected animals and none of the uninfected controls developed fluorescent antibody titers using cercariae as antigen beginning 4 weeks after exposure to infection. Antibody produced as measured by the SAFA test followed a different time course of development. Fluorescent antibodies were detected at low titers in the sera of a few animals 4 weeks after the exposure to infection. The titers increased rapidly and remained elevated for the duration of the experiment. The SAFA test results and PCA reactions of serum from S. japonicum infected rabbits are shown graphically in Figure 22.

Previous studies had indicated that the RAMP assay can reliably measure activity due to antibodies primarily of the IgE class and can be used to demonstrate the binding of antigen by reaginic antibodies in schistosomiasis. Therefore, all the sera from the rabbits were assayed every two weeks for RAMP activity. As shown in Figure 23, all animals gave negative results (<20 percent precipitation) before two weeks and 4 weeks after exposure. However, 6 weeks after exposure the sera of infected animals precipitated increased amounts of radioactive antigen and these values remained elevated for the duration of the experiment.

The most reproducible and quantitative technique for measuring the response of lymphocytes to an antigenic stimulus is an assay of the total tritiated thymidine uptake by sensitized lymphocytes. This quantitative measure of total DNA synthesis by lymphocytes is a sensitive indicator of their transformation. Blast transformation was studied in lymphocytes from the peripheral blood of rabbits at monthly intervals after exposure to infection. The amount of thymidine incorporated by lymphocytes obtained from noninfected rabbits remained low throughout the experiment. A significant increase in DNA synthesis was observed in the infected animals 6 weeks after exposure. The difference in

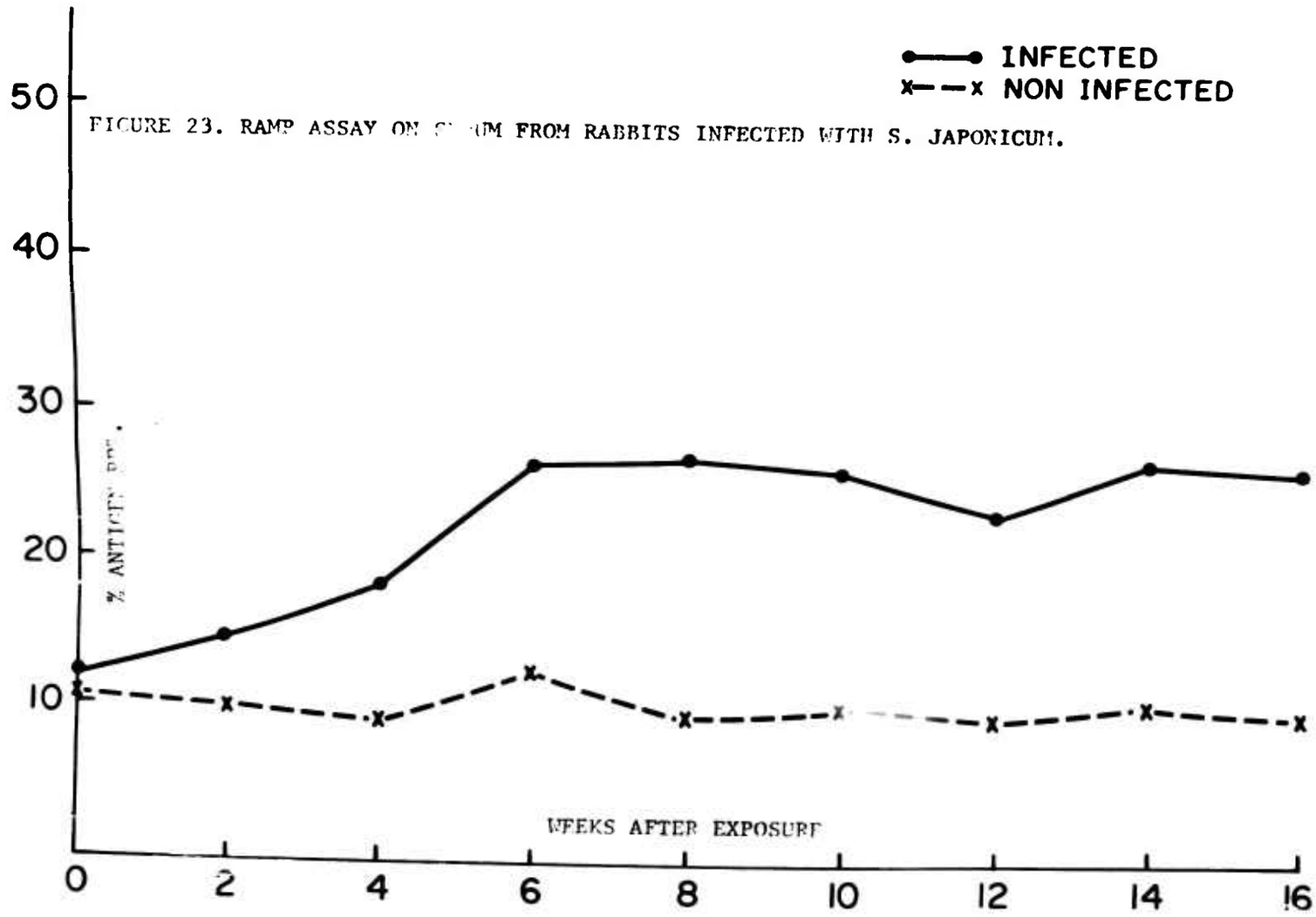


FIGURE 23. RAMP ASSAY ON SERUM FROM RABBITS INFECTED WITH S. JAPONICUM.

complement levels in the infected animals were higher than those of the uninfected controls at all times. These differences between the results in the chimpanzees and those in the rabbits may be in line with previous observations of marked glomerulonephritis observed in infected chimpanzees and the relative absence in rabbits.

These experiments confirm the presence of immediate hypersensitivity, demonstrable by in vitro and in vivo tests. However, although blastogenesis was demonstrated in all infected animals no clearcut indication of skin reaction of the delayed type was observed with any of the four antigens employed at any of the times studied.

7. Effects of chemotherapy on the evolution of schistosomiasis japonica in chimpanzees.

Studies conducted on 15 chimpanzees experimentally infected with Schistosoma japonicum have shown that pipestem fibrosis and its associated nephropathy can be reliably reproduced in this animal. Therefore, studies of pathogenesis, treatment, and management in this model are likely to be applicable to human schistosomiasis. Information can be obtained on the favorable or unfavorable effects of schistosomicidal treatment at various stages of infection and, in particular, on whether established bilharzial lesions in various organs are persistent or reversed after treatment. Such information would be difficult to collect in a clinical setting where the time and number of exposures, the intensity of infection, and the role of other pathogenic factors are usually unknown.

Recent studies have shown that administration of a nitrovinylfuran derivatives (SQ 18,506) to Rhesus monkeys infected with either S. mansoni or S. japonicum resulted in marked chemotherapeutic activity and no apparent host toxicity. The number of worms recovered from the treated monkeys was reduced considerably and the few surviving worms were stunted. Damage to these worms was also revealed by a very marked decrease in the number of eggs present in the tissues and feces of the host.

Most clinicians have found that treatment, apart from the immediate toxicity of the drug itself, is beneficial. While it is true that worms killed by treatment elicit severe local reactions, these are gradually resorbed leaving small residual lesions and the attempts to relate lesions surrounding dead worms to the pathogenesis of pipestem fibrosis are unconvincing. Other investigators have suggested that liver damage is caused primarily by dead adult schistosomes. Sadun et al concluded that, at least in mice, the time at which chemotherapy is instituted is an important determining factor. When chemotherapy was begun shortly after egg deposition, it prevented the establishment of hepatic fibrosis. Conversely, if given when liver damage was already extensive, irreversible lesions persisted. Warren found that portal hypertension in schistosome-infected mice decreased rapidly after treatment. In more chronic infections, slight portal hypertension and considerable fibrosis persisted after treatment.

Pipestem fibrosis of the liver is the principal cause of morbidity and mortality in S. mansoni and S. japonicum infections, and nothing is known concerning the reversibility of this lesion. Chimpanzees offer the unique opportunity to study the outcome of this lesion under controlled experimental conditions. Therefore, experiments were conducted in chimpanzees to determine whether or not regression of hepatic fibrosis may occur after successful treatment.

Nineteen young chimpanzees from West Africa were used in this study. Of these 17 were exposed to 35-50 cercariae per kilogram of body weight and 2 were left as uninfected controls. The weight of each animal at the time of exposure and the total number of cercariae to which they were exposed are recorded in Table 8. Ten of the 17 infected animals were treated at various times after the onset of patency (Table 8).

Splenectomy was performed on most of the chimpanzees for use in an unrelated experiment with Plasmodium falciparum. These animals were utilized after termination of the malaria infection by therapy and were healthy when selected for this study. At autopsy accessory spleens were found in 5 splenectomized animals. Previous studies indicated that splenectomy had no demonstrable effect on the host response of chimpanzees to schistosome infections.

rcariae of the Japanese strain of S. japonicum were obtained from 50 to 100 infected Oncomelania hupensis nosophora snails that n exposed 3 to 4 months earlier to miracidia obtained by hatching rom livers of infected albino mice. The chimpanz re exposed as described previously. Mice were similarly exposed rcariae from the same suspension and used as infection control utopsy, 6 to 10 weeks after exposure, the recovery of adult wor m these controls varied from 60 to 75 percent.

Feces of each chimpanzee were examined weekly for schistosome eggs, using the same technique described before. Blood was collected from each animal at regular intervals for serological, biochemical and hematological studies. All infected chimpanzees except Nos. 364, 650, 651 and 759 were subjected to laparotomy and wedge biopsy of the liver and kidney two and one-half months after infection. All survivors were subjected again to open biopsy 5 months after infection and three of them were biopsied again 9 months after infection. Before autopsy the portal pressure of these animals was measured as described previously.

During autopsy the mesenteric and intrahepatic portal circulation were perfused separately. The number of eggs in various tissues was determined after digestion in 4% potassium hydroxide at 37°C for 12 to 18 hours. This method as well as methods for perfusion, pathological and histological studies have been described previously.

The methods employed for administration of the nitrovinylfuran derivative, the dosages and the attempts to promote better absorption of the drug will be described in a separate paper. From one to three

courses of treatment were given at various intervals after exposure to Schistosoma japonicum. The animals were observed frequently during and after treatment for weight loss, anorexia, lethargy or otherpossible side effects of the drug. Results of immunological studies conducted in these animals have been reported elsewhere. Complement fixation tests for the detection of Australia antibodies were performed on all chimpanzees. Four animals (Nos. 364, 370, 576 and 577) reacted at titers of 1 to 16 or greater. Of these, one (No. 364) was used as an uninfected control, one (No. 370) was infected but not treated and two (Nos. 576 and 577) were infected and treated.

Parasitological observations

No schistosome eggs were found in the stools of the chimpanzees before exposure to cercariae and for several weeks afterwards. The onset of patency as determined by the detection of eggs in the fexes varied from 6 to 9 weeks. The egg excretion pattern of the untreated animals followed a course similar to that observed in previous experiments. The passage of eggs in the feces of treated animals also increased initially, except in No. 759. Soon after treatment, egg passage was markedly suppressed and within 2 weeks after treatment eggs were no longer detected. However, a few eggs reappeared in the feces of 3 treated animals. The adult worms recovered from the untreated animals (Table 8) were active and well developed and both sexes were present in comparable numbers. Conversely, most of the worms recovered in the treated animals were stunted and male worms were predominate. Intravital staining revealed marked abnormalities in the reproductive organs of a high percentage of female worms recovered from the treated animals. Most of the eggs recovered from tissue digests were in the liver and large intestine (Table 9). The number of schistosome eggs recovered by digestion of various organs after necropsy was somewhat lower in all treated animals (Table 9). Most of the eggs in tissues of treated animals were dark in color and appeared to be dead.

Clinical observations

No obvious signs of illness were observed during the first 6 weeks after exposure and all chimpanzees gained weight early in the experiment. This trend was reversed after patency and blood was observed intermittently in the stools of some animals. Some chimpanzees lost their appetites, showed rapid weight loss, became lethargic and their mucosae became pale. Five of the 7 untreated animals died spontaneously, 2 of them shortly after laparotomy (Nos. 362, 587). A third chimpanzee (No. 359) died of intercurrent pneumococcal meningitis, pneumonia and septicemia confirmed by culture and autopsy. The fourth chimpanzee (No. 372) showed severe unresolved bacterial pneumonia. In addition, a fifth animal (No. 12) exhibited acute pyelonephritis at necropsy. Among the treated chimpanzees only one died spontaneously, five months after infection, presumably of septicemia. At autopsy many vessels contained fibrin thrombi and bacteria. Since all but one of these spontaneous deaths occurred in the untreated group, schistosomiasis probably played a role in fostering or aggravating complications.

Table 8

Worm recovery in treated and untreated chimpanzees infected with S. japonicum

Chimp no.	Rx	Weight (kg)	Number cercariae	Worm recovery				Duration of infection (months)
				M	F	Total	Percent recovery	
360	Yes	12	620	7	2	9	1.4	9
364	Yes	18	637	6	1	7	1.1	4
371*	Yes	13	654	41	48	89	13.6	5
576	Yes	15	763	4	1	5	0.6	9
577	Yes	15	757	14	5	19	2.5	16
650	Yes	19	674	5	0	5	0.7	4
651	Yes	17	633	0	0	0	0	4
759	Yes	13	320	0	0	0	0	4
955	Yes	11	553	0	0	0	0	16
956	Yes	10	750	5	2	7	0.9	16
12	No	24	1,750	201	229	430	24.6	3
359	No	13	658	ND†	ND	ND	ND	3
362	No	22	1,115	ND	ND	ND	ND	2
370	No	21	1,057	78	78	156	14.8	9
372	No	13	755	26	23	49	6.5	7
578	No	12	596	ND	ND	ND	ND	4
758	No	11	420	59	20	79	18.8	4

* This animal died 11 days after treatment.
† ND, not done.

Hematological observations

Anemia was noted in all infected chimpanzees. Whereas the hematocrit of the uninfected animals remained between 40 and 50 percent, that of the infected animals decreased rapidly and approximately 3 months after exposure reached a level of one-half of the original value. In the treated animals the hematocrit and hemoglobin levels rapidly returned to normal. Infection also resulted in marked leukocytosis. In the uninfected chimpanzees the total number of leukocytes varied between 20 and 30,000 per cubic millimeter. In the infected animals it increased rapidly to a maximum exceeding 100,000 in some cases. Three months after exposure to infection leukocytosis gradually subsided both in the treated and untreated animals, although more rapidly in the former group. While the percentage of neutrophils increased in the infected animals, the number and percentage of lymphocytes decreased markedly beginning with the second month after exposure to infection and remained depressed throughout the course of the infection. Thus, whereas in uninfected chimpanzees approximately two-thirds of the white blood cells were lymphocytes, in the infected animals the percentage of neutrophils far exceeded that of lymphocytes. Treatment reversed this abnormality so that toward the end of the experiment the treated animals had approximately the same percentage of neutrophils and lymphocytes as the uninfected controls.

Biochemical observations

An increase in blood urea nitrogen was observed in some chimpanzees toward the latter part of the study and low blood glucose values were obtained in some animals in the terminal stages of the disease. However, no concistent interpretations of these two parameters were possible in view of the large proportion of chimpanzees that died spontaneously. A decrease in prothrombin activity was observed in the infected animals beginning 2 months after exposure. Whereas in the untreated chimpanzees the prothrombin activity nevere exceeded 30% of the normal controls, in the treated animals it reached nearly normal values within 2 months after treatment. There was no significant elevation of transaminase levels in the infected animals over those of the treated chimpanzees or of the uninfected controls. Therefore, no significant trends were detected. Likewise no significant trneds were observed in the portal pressure measurements conducted at necropsy.

Table 9

Number and distribution of eggs in various organs in treated and untreated chimpanzees infected with S. japonicum

Chimp no.	Rx	No. worms recovered	Mean number of eggs/g tissue (1,000's)					Total eggs in tissue (1,000's)
			Liver	Lungs	Small int.	Proximal colon	Distal colon	
360	Yes	9	7.2	2.6	<0.1	6.6	3.1	5,592
364	Yes	7	1.0	<0.1	<0.1	0.9	0.8	1,058
371*	Yes	89	6.5	0.3	0.4	4.0	6.9	8,402
576	Yes	5	3.5	0.8	0.1	3.S	1.1	3,8[illegible]
577	Yes	19	6.0	0.3	0.4	8.4	11.6	10,785
759	Yes	0	0.1	0.1	0.2	0.7†		ND‡
650	Yes	5	1.8	0.1	0.2	1.8	0.8	1,863
651	Yes	0	1.2	0.1	0.1	1.1	0.5	1,187
955	Yes	0	6.2	5.1	1.8	11.6	21.8	14,292
956	Yes	7	3.7	1.0	0.3	14.0	6.0	6,127
12	No	430	10.3	4.1	0.2	13.2	18.9	14,177
359	No	ND	3.5	<0.1	0.2	7.3	1.9	4,353
362	No	ND	0.6	2.8	<0.1	5.7	2.9	4,800
370	No	156	4.0	3.8	0.4	6.9	7.0	8,717
372	No	49	9.5	5.2	1.0	13.6†		ND
578	No	ND	8.7	2.9	5.7	19.0	12.1	11,901
758	No	79	1.6	0.1	0.1	1.3	2.9	2,182

* This animal died 12 days after treatment.
† Entire colon.
‡ ND, not done.

Total serum protein concentration increased in most chimpanzees as the infection progressed. As previously recorded, the largest increases occurred in the chimpanzees with infections of long duration, particularly in those showing the greatest clinical deterioration. In contrast to the hematological observations which indicated a rapid return to preinfection levels shortly after treatment, the serum gamma globulin levels of the chimpanzees in the treated group remained elevated throughout the course of the experiment. This increase occurred

Table 10

Main pathological findings in S. japonicum-*infected, untreated chimpanzees*

Chimp no.	Months of infection D/K*	Colon	Liver	Lung	Lymph nodes	Kidney	Other
362	2.2 D	Active patches 30% estimated surface	Massive granulomatous involvement with *early* inflammatory portal lesions	Many active granulomas	Many eggs, active granulomas; perilymphadenitis	Minimal focal mesangial proliferation	Lymph nodes diffusely enlarged (Figs. 17, 18). Inflammatory focus of mitral valve. Adult female worm in liver
359	3.2 D	Active patches 15% estimated surface	Granulomatous involvement with early, *focal* pipestem lesions	Moderate no. active granulomas	Eggs and active granulomas focally	Minimal focal mesangial proliferation	None
12	3.8 K	Active patches 30–50% estimated	Established pipestem fibrosis, slender type active, *moderate*. Terminal triad fibrosis	Moderate no. active granulomas	Grossly enlarged. (Micro ND†)	Nephropathy Grade ++	Hoeppli phenomenon seen in liver granuloma (Fig. 13)
758	4 K	Active patches. Focal, maximal colon. Bilharziomas (2) rectosigmoid	Established pipestem fibrosis, broad (classic) active *moderate* early terminal triad fibrosis	Occasional active granulomas	Not studied	Nephropathy Grade +	Focal hyaline droplet arteritis, colon only
578	4.2 D	Active patches estimated 20% surface. Bilharziomas rectum, transverse colon	Pipestem fibrosis, broad (classic) pattern, active, *severe*. Terminal triad fibrosis	Many active granulomas	Focal involvement pelvis, mesocolon	Nephropathy Grade ++. Focal eggs and granulomas	Lymph nodes diffuse enlarged. Concomitant filariasis

* D/K, died or killed.
† ND, not done.

Table 10 (continued)

Chimp no	Months of infection: D/K*	Colon	Liver	Lung	Lymph nodes	Kidney	Other
372	7 D	Extensive, active patches. Bilharziomas, proximal colon	Pipestem fibrosis, *severe*, broad (classic), active. Terminal triad fibrosis	Many active granulomas	Grossly enlarged; eggs and granulomas	Nephropathy Grade +++ (Fig. 16). Focal eggs and granulomas	Focal arteritis, heart, kidney. Inflammatory focus of mitral valve; concomitant filariasis
370	9 K	Extensive, active patches (Figs. 14, 15). Bilharziomas rectum, splenic flexure	Pipestem fibrosis broad (classic) pattern, active, *severe* (Figs. 12, 21). Terminal triad fibrosis	Many active granulomas	Severely involved; bilharzioma, periaortic nodes	Nepropathy Grade +++	Necrotizing arteritis of gut, liver, kidney, urinary bladder and skeletal muscle (Figs. 19, 20)

Table 11

Main pathological findings in S. japonicum-*infected chimpanzees treated with SQ 18,506*

Chimp no.	Months of infection: D/K*	Colon	Liver	Lung	Lymph nodes	Kidney	Other
759	4 K	Grossly normal. Histology: sporadic egg shells	Grossly normal; no portal fibrosis seen. Sporadic degenerating and calcified eggs and involuting granulomas	Rare egg shells	Unremarkable	No significant glomerular lesion	Sporadic eggs, little residual pigment. Probably a mild infection initially
364	4	Grossly: no patches noted. Histology: focal subsiding and inactive lesions only	Grossly normal. Histology: Inactive and healed granulomas; *minimal* portal fibrosis, inactive, focal	Sporadic healed granulomas	Moderately hyperplastic	Mild focal mesangial expansion	Focal lymphoid aggregates of portal fields
650	4 K	Grossly: no patches noted. Histology. focal inactive lesions only	Grossly normal. Histology: healed granulomas, degenerate eggs; *minimal* portal fibrosis, inactive, focal; occasional recanalized portal veins	Sporadic healed granulomas	Unremarkable	Minimal focal mesangial expansion	None
651	4 K	Grossly: no patches noted. Histology: focal inactive lesions only	Grossly normal. Histology: inactive lesions only; *mild to minimal* portal fibrosis, focal; occasional recanalized portal veins	Unremarkable	Sporadic granulomas; mesenteric nodes focally enlarged	Mild focal mesangial expansion	None

* D/K, died or killed.

Table 11 (continued)

Chimp no.	Months of infection: D/K*	Colon	Liver	Lung	Lymph nodes	Kidney	Other
371	5 D	Active patches estimated 10% surface	Numerous granulomas. Established pipestem fibrosis, slender type, active, *moderate*. Terminal triad fibrosis	Moderate no. active granulomas	Many eggs and active granulomas; perilymphadenitis	Nephropathy Grade ++	Diffuse lymph node enlargement. Focal arteritis of esophagus, stomach, small intestine, colon; concomitant filariasis
576	9 K	10–15 small active patches throughout colon. Otherwise inactive	Numerous granulomas. Established pipestem fibrosis, slender type active, *moderate*; terminal triad fibrosis (Figs. 21, 25)	Moderate no. active granulomas	Few eggs and active granulomas	Nephropathy Grade + to ++	Diffuse lymph node enlargement. Lymph follicles in portal spaces
360	9 K	Two moderately active sites; other patches inactive	Few granulomas (Fig. 27). Delicate pipestem fibrosis, *mild* to *minimal* (Figs. 21, 26), lesions mostly inactive (Fig. 29). Dead worm lesions (Fig. 28). Terminal triad fibrosis, minimal	Many healed and healing granulomas, many calcified eggs	Few eggs; no active granulomas	Nepropathy Grade + to ++	Diffuse lymph node enlargement. Focal arteritis, liver only, minimal
956	16 K	20 small patches; some moderately active (Fig. 23)	Few granulomas. *Mild* to *minimal* pipestem fibrosis, slender type (L > R; lesions over 90% inactive (Fig. 30).	Sporadic healed granulomas, calcified eggs	Sporadic eggs, inactive	Minimal focal mesangial expansion (Figs. 31, 32)	Mild diffuse lymph node enlargement

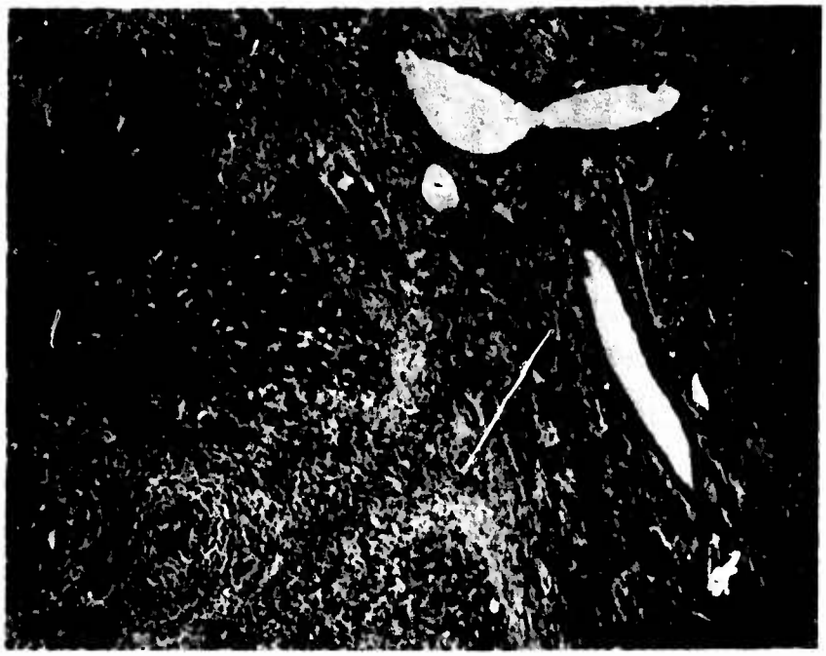


Fig 24 - Chimpanzee No. 360, liver biopsy at 2 1/2 months before treatment. Middle sized portal field showing diffuse inflammation, edema and early fibrosis. Note encroachment on liver end plates by inflammatory foci; large granulomas are seen on left, distal to the diffuse inflammation and there is slight arterial dilatation. Portal vein collaterals are present near the upper margin, but the main portal branch is not included in this field.

approximately 6 weeks after exposure to infection and was not reversed by treatment. At the end of the experiment the treated animals had approximately the same levels of gamma globulin as the untreated infected controls. Alpha 1, alpha 2 and beta globulin levels in the infected animals remained essentially unaltered regardless of treatment. A decrease in the albumin was observed in the infected chimpanzees as the infection progressed. Shortly after treatment the albumin levels rose somewhat in the treated animals but remained below those of the noninfected controls.

Pathological findings

a. Untreated chimpanzees. The principal gross and microscopic findings are summarized in Table 10. The stages, grades and histological appearances of the lesions showed the same spectrum as reported earlier. Moreover, there was similar correlation with the duration of infection. Approximately 2 1/2 months after exposure the liver involvement consisted largely of granulomatous changes with diffuse inflamma-

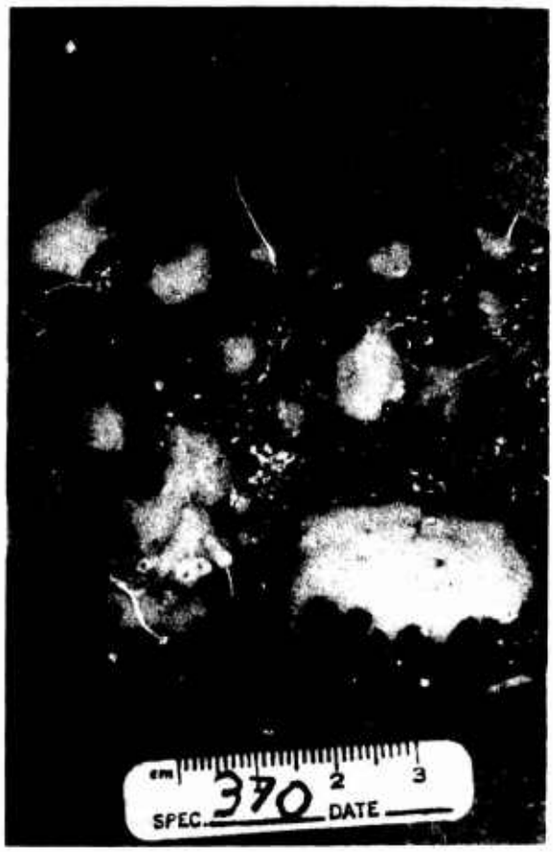


Fig. 25 - Chimpanzee No. 370. Cut liver surface at autopsy (9 months) - Untreated. Classical, broad banded pipestem fibrosis.

tion and mild fibrosis of the larger portal triads. Portal pipestem fibrosis, obliterative endophlebitis and arterial enlargement became well established during the third (Fig. 24) and fourth months, progressing steadily thereafter (Fig. 25). One animal (No. 12) showed both the stellate and the reverse form of the Hoeppli phenomenon as recently described in hamsters (Fig. 26). In the active colonic patches (Fig. 27), abnormalities of the mucosal pattern and focal proliferation of smooth muscle in the muscularis mucosae also seemed to increase with duration of infection (Fig. 28). The kidneys showed only mild focal mesangial cell proliferation of questionable significance before the 4th month of infection but thereafter the granular and mesangial lesion of schistosome nephropathy became progressively more evident (Fig. 29). Variable numbers of eggs and granulomas were observed in lymph nodes. Diffuse enlargement of abdominal and of some thoracic lymph nodes was present in most infected chimpanzees. These were characterized by follicular prominence with large, reactive germinal centers, expansion of sinuses

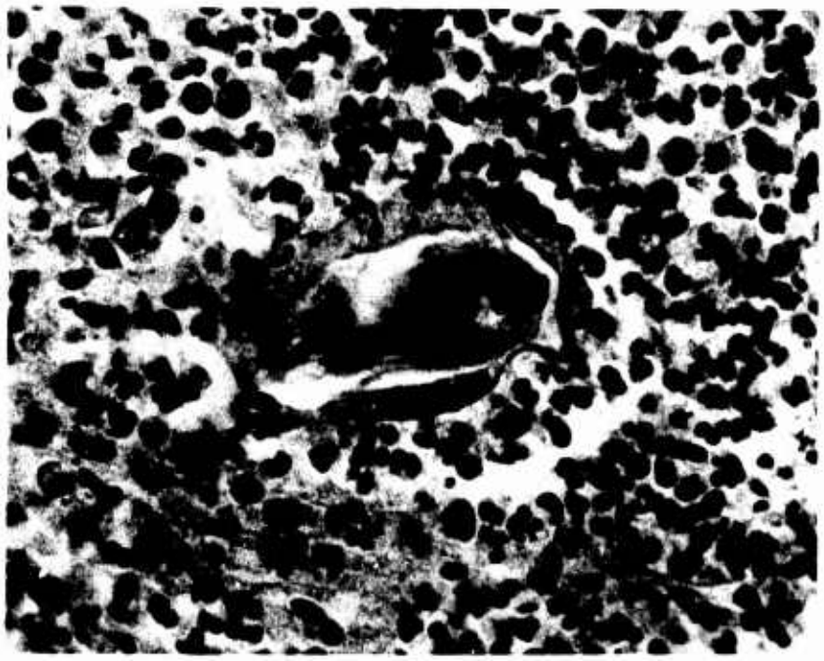


Fig. 26 - Chimpanzee No. 12, liver at autopsy (3.8 months). Untreated. A mature, secretory stage S. japonicum egg in the granuloma center is surrounded by degranulating neutrophils.

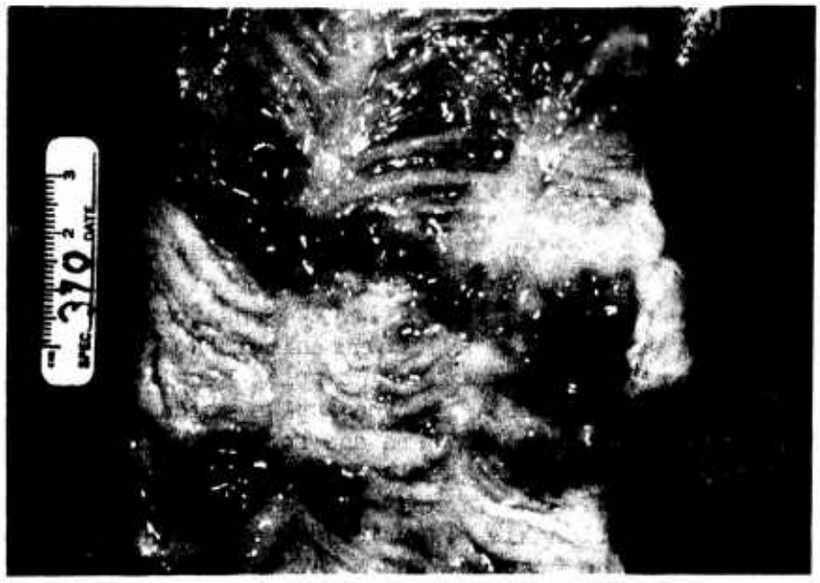


Fig. 27 - Chimpanzee No. 370. Colonic mucosa at autopsy (9 months) - Untreated.

and cords into the cortex with numerous plasma cells and eosinophiles and large numbers of vasculated macrophages which imparted a "starry sky" appearance to the cords, a pattern suggesting humoral antibody production (Figs. 30, 31). Some lymph nodes, which contained both eggs and granulomas, showed in addition perilymphadenitis and pericapsular fibrosis or bilharziomatous change. Two chimpanzees showed focal necrotizing inflammation of small arteries unrelated to sites of egg deposition (Fig. 32) with endothelial and smooth muscle proliferation and a well circumscribed largely mononuclear and eosinophilic infiltrate. Hyaline droplets similar to those described in schistosomal nephropathy were observed in some foci, lying free or within cells (Fig. 33). No significant parenchymal necrosis or hemoarrhage was associated with these lesions.

In addition to the complications listed elsewhere, other nonschistosome related lesions consisted primarily of ciliates in the colon of 4 animals and filarial worms in abdominal lymph node afferents.

b. Treated chimpanzees. The lesions in these animals are described in Table 11. They were similar to those of the untreated animals, but were less intense and frequently inactive or involuting (Fig. 34). The distinction between prevention and reversibility of lesions was sometimes not easily ascertainable. However, whenever possible, a comparison between treated and untreated chimpanzees and between pretreatment and post-treatment pathology was done to assess regression.

c. Comparative pathology of treated and untreated chimpanzees. One chimpanzee (No. 759) treated at 2 months, showed no gross lesions and only minimal, inactive microscopic pathology (Table 11). Two of the treated animals showed autopsy lesions indistinguishable in degree from those of the untreated group (Fig. 35). One of these (No. 371) died shortly after treatment. The other (No. 955) after approximately one year showed a larger proportion of inactive patches in the colon but severe pipestem fibrosis. The remaining 7 treated animals showed variable pathology.

In the colon of the treated animals the number and size of patches was reduced and the proportion of normal looking mucosa increased (Fig. 36). By gross examination the inactive patches lacked hyperemia and mucosal erosion. They were smoother, more brownish and less elevated than active patches, although a few ere polypoid in shape. Histologically, immature (basophilic) and mature (secretory) eggs were fewer in number, and in some instances, absent, as were the corresponding exudative granulomas. Conversely, clusters of egg shells, as well as degenerated and calcified eggs persisted in variable numbers and proportions, in the midst of declining or minimal tissue reaction (Fig. 37). However, in 4 of the 7 cases these inactive lesions coexisted with lesser numbers of active patches (Table 11) which were indistinguishable from those of untreated animals. Furthermore, alterations of the crypt pattern in the mucosa, mural fibrosis and focal smooth muscle hyperplasia persisted in larger patches.

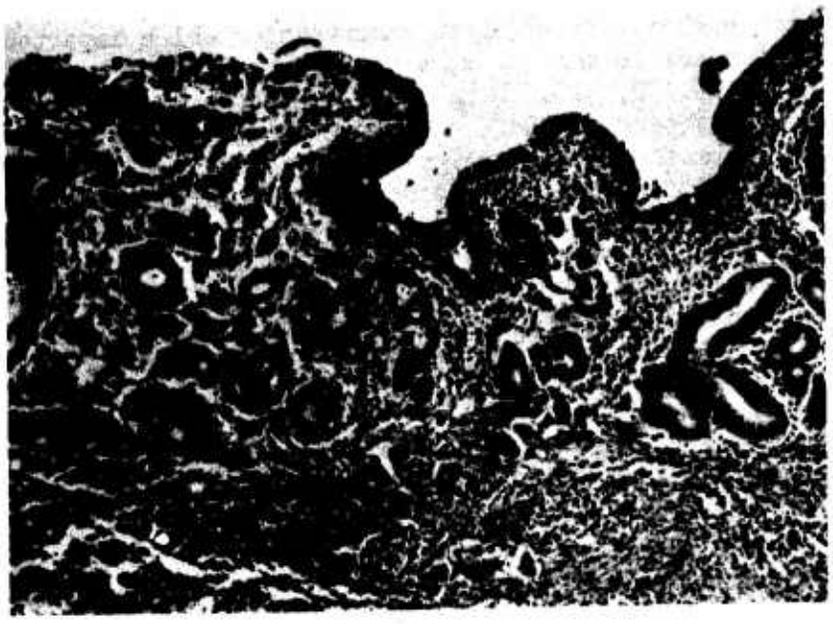


Fig. 28 - Chimpanzee No. 370. Section of sigmoid colon at autopsy (9 months) - Untreated.

Fig. 29 - Chimpanzee No. 372. Kidney at autopsy (7 Months) -

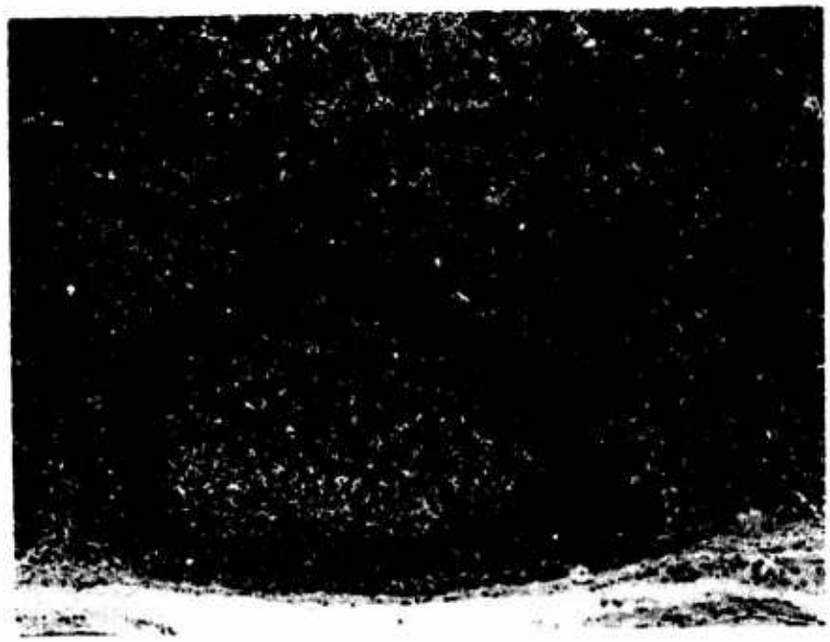


Fig. 30 - Chimpanzee No. 362. Mesenteric lymph node at autopsy (2.2 months). Untreated. X100.

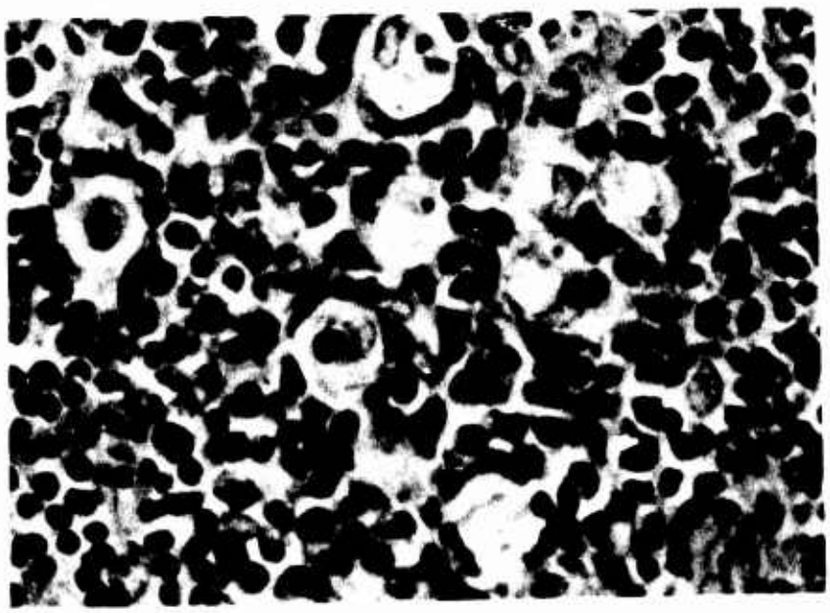


Fig. 31 - Same as in Figure 30, magnified X490.

In the liver, hard whitish nodules representing healed composite granulomas were seen in variable numbers (Fig. 38). None of the treated chimpanzees selected for comparison showed the classical broad banded pipestem pattern characteristic of the three untreated long-term controls. Slender, or minimal portal fibrosis was recorded, with the fibrous cuffs not surpassing the width of the vein diameter itself (i.e., 0.2 cm cuff around a 0.2 cm diameter vein) (Figs. 38,39). In addition, the pipestem lesions in the treated animals showed an angular or spidery configuration as opposed to the rounded, or redundant appearance on cross section in the untreated chimpanzees. This suggested consolidation and retraction of the fibrous structures. Similarly, the subcapsular fibrotic septa representing terminal triad fibrosis appeared thinner and sharper, especially at 16 months. Areas of grossly undisturbed parenchymal pattern were larger (Figs. 38, 39). Thus, gross evaluation of the liver fibrosis gave a good indication of the effects of treatment, and was predictive of the microscopic findings.

Histological examinations of the livers of treated chimpanzees revealed that the changes in egg characteristics in livers were like those in the colon. Variable but small numbers of mature eggs and exudative granulomas persisted focally in 4 of 7 animals. By contrast, groups of dead and calcified eggs and healed or involuting granulomas were numerous (Fig. 40), attesting to the greater infection intensity which had existed in the past. Two animals showed large, hyaline scars suggestive of dead worm lesions (Fig. 41). There was also a decline in the number of eosinophils and plasma cells and in the density of diffuse inflammatory infiltrates in the portal fields and septa. These changes resulted in a less crowded appearance of the fibrotic areas, and a sharper border along the hepatic parenchyma, with less encroachment (Fig. 42). However, lymphocyte aggregates persisted in moderate numbers (Fig. 40, 41). Portal collagen appeared compact with little waviness, neovascularization, or interstitial edema (Figs. 42, 43). Patent small and medium sized portal veins were more distinct in the treated group and portal endophlebitis was less active; however, some veins remained obliterated or narrowed by dense, fibrous intimal cushions. Others were recanalized, or totally disrupted so as to require trichrome staining for identification. Many arterial branches remained thick-walled and wide (Fig. 42).

In the lungs and lymph nodes viable eggs were fewer and involution of granulomas was observed. This was best seen in the long term survivors. Fibrosis around the lymph nodes was not affected by treatment.

In the kidneys of the selected 7 animals glomerular changes were significantly less than expected on the basis of the duration of infection. The only changes noted after 4 to 61 months were focal mesangial proliferation, or mild expansion of the matrix. In two long-term treatment survivors (Nos. 956, 577) mesangial expansion along the capillary stalks was minimal, with little cellular proliferation (Fig. 44), and with normal capillary basement membranes (Fig. 45). This change suggested regression of an earlier cellular proliferation, leaving the widened

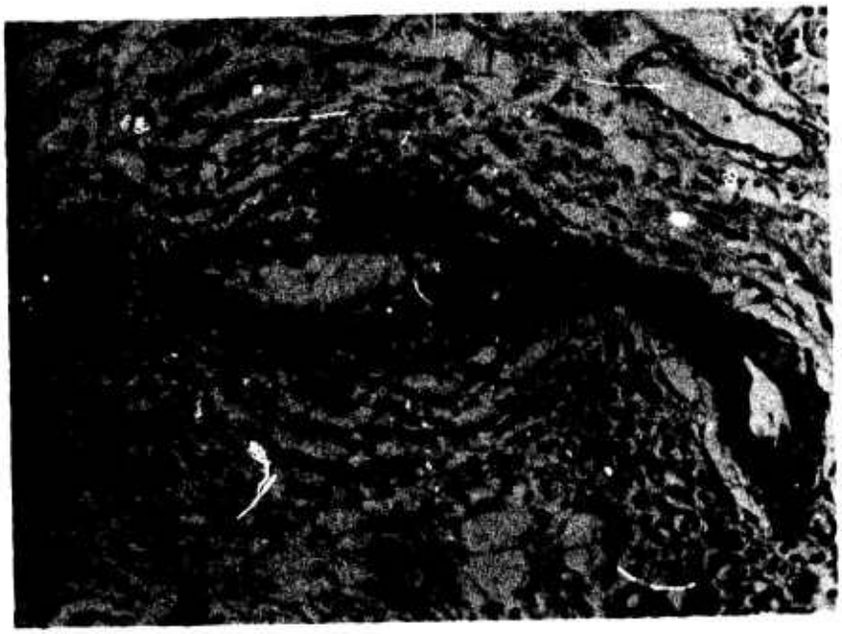


Fig. 32 - Chimpanzee No. 370. Colon at autopsy (9 months). Untreated.

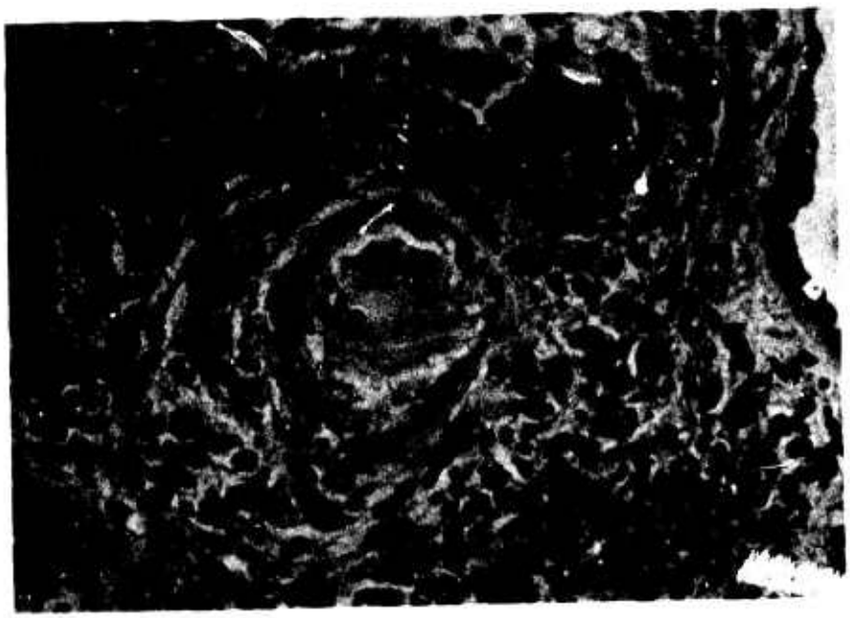


Fig. 33 - Chimpanzee No. 370. Kidney at autopsy (9 months). Untreated.

Fig. 34 - Left: Chimpanzee No. 370 (untreated, 9 months);
Middle: Chimpanzee No. 576 (treated, 8 months);
Right: Chimpanzee No. 360 (treated, 8 months).
Liver slices.

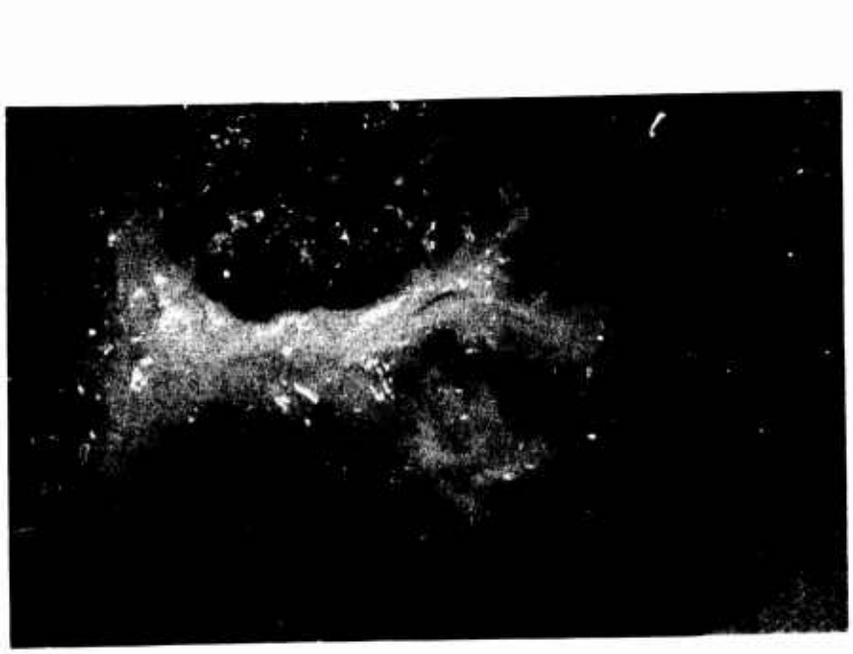


Fig. 35 - Chimpanzee No. 955. Cut liver surface (16 months);
treated at 4 1/2 months.

stalk as its sole stigma.

d. Sequential pathology in the liver of treated chimpanzees. Although an anatomical scoring method has been applied to autopsy livers, it was not discriminative enough for the biopsy samples of these uniformly heavy infections. Thus, the following evaluation is based on subjective observations.

Treatment within 2 1/2 months: Four of the 5 chimpanzees (Nos. 364, 650, 651, 759) in this category showed no grossly detectable pipestem fibrosis at autopsy 2 months to 2 months 6 days after treatment. Microscopically, all eggs were degenerate with subsiding or healed inflammatory reaction. Portal fibrosis was absent (No. 759) or minimal (Nos. 364, 650, 651). Biopsy had not been performed in these animals before treatment. The fifth animal (No. 956) which resumed egg excretion after the first course of treatment showed slight pipstem fibrosis at autopsy 2 months 12 days after the first treatment. Some improvement was noted in the first post-treatment biopsy of this chimpanzee with little change seen in the biopsy taken after the second course of treatment. However, at autopsy, 16 months post-infection, further decrease of inflammatory activity and egg viability was evident (Fig. 43).

Treatment at 3 months: In this initial biopsy, one animals was judged to have severe liver involvement (No. 577) and another somewhat less severe (No. 360), but both showed pipestem lesions (Fig. 24). Chimpanzee No. 360 responded to a single course of treatment with negative stools, and with dramatic reduction of inflammatory activity in the liver (Fig. 42) which persisted at the time of autopsy, 9 months after exposure (Fig. 39).

Chimpanzee No. 577 showed only moderate histological improvement after his first course of treatment. Two additional courses were followed by slight further reduction in histological inflammation. No further change was evident at autopsy, 7 months later. Overall, this animal benefited from treatment less than chimpanzee No. 360.

Treatment at 4 1/2 months: All three animals in this category (Nos. 371, 576 and 955) showed severe portal inflammation and early fibrosis on initial biopsy, and these lesions had progressed toward an established pipestem pattern in the second biopsy, prior to treatment. Animal No. 576 (treated twice) showed moderate reduction of inflammatory activity at autopsy, and fibrosis had progressed less than would have been expected in the absence of treatment (Fig. 48). Animal No. 371 which died shortly after a single treatment and animal No. 955 (treated twice) showed persisting inflammatory activity in all subsequent tissue samples and, at autopsy, hepatic lesions were of similar degree as in untreated chimpanzees (Fig. 45), as summarized in Table 11. Although in chimpanzee No. 955 there was less inflammation in the colon at autopsy, one year after treatment, the liver nevertheless showed severe, broad-banded fibrosis.

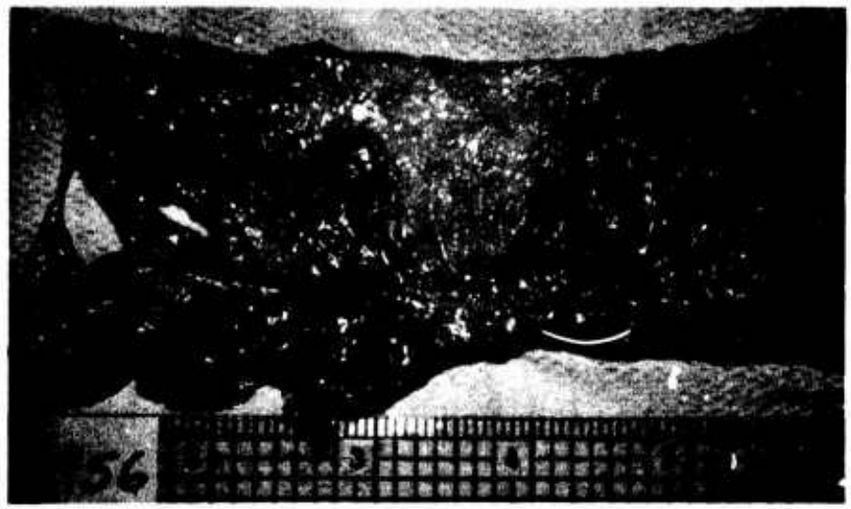


Fig. 36 - Chimpanzee No. 956. Colonic mucosa (16 months); treated at 2 1/2 months.

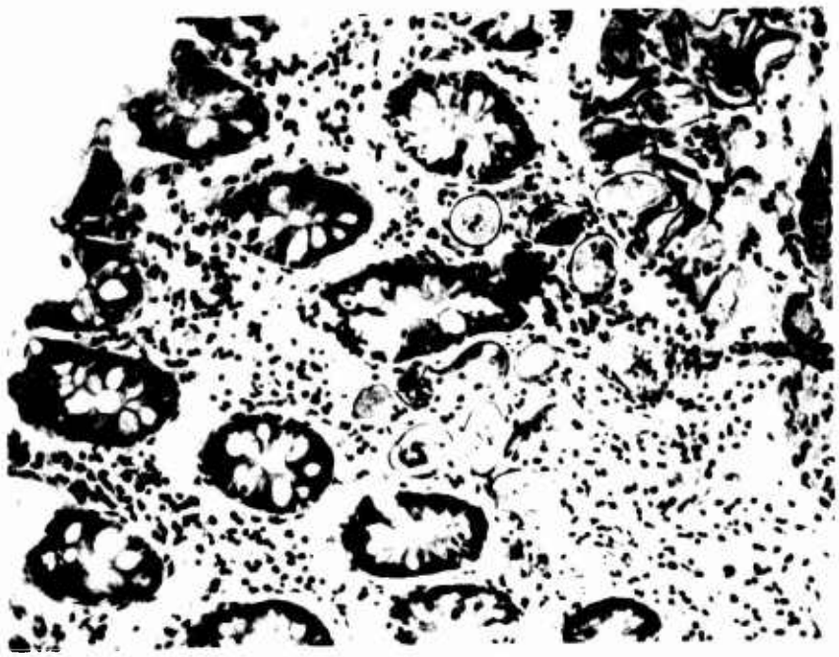


Fig. 37 - Chimpanzee No. 577 (treated, 16 months). Colonic patch.

Fig. 38 - Chimpanzee No. 576. Cut surface of liver (9 months); treated at 4 1/2 months.

Fig. 39 - Chimpanzee No. 360. Cut surface of liver (9 months); treated at 3 months.

Thus, the histopathological responses to treatment varied individually over a spectrum ranging from total success (No. 759) to failure (No. 955) with the timing of the initial course of treatment being an important determining factor. Some histological response to subsequent courses of treatment was also noted. Furthermore, the anatomical results were by far the best in those animals lacking evidence of residual oviposition after treatment (Nos. 759, 364, 650, 651): and correlated to some degree with the total number of eggs in organ digests (Table 9, last column) which in turn reflects the effect of treatment on the cumulative infection intensity (among other variables). It should also be noted that different drug formulations and routes of administration had been used in the course of this study which might account for some of the individual variations of the results.

The four chimpanzees which had reacted to Australia antigen had no pathological alterations attributable to this viral infection. Their livers and their hematological and biochemical findings were not demonstrably different from those of the nonreactors. No lesions attributable to nitrovinylfurane toxicity and no significant abnormalities of the CNS were noted in either the treated or the untreated group.

Although numerous investigators have compared bilharzial lesions before and after chemotherapy, this is the first time that such a study could be conducted in an experimental animal in which the major anatomical lesions resulting from infection are similar to those occurring in man.

Our findings contrasted with the proven frequent reversibility of serious lesions such as hydroureter and hydronephrosis following treatment of S. haematobium infection and agree with the traditional view that pipestem fibrosis due to either S. mansoni or S. japonicum once established is irreversible. On the other hand, the World Health Organiation's Scientific Group on Research in Bilharziasis (Chemotherapy) in its report to the Director General in 1964 concluded that there is some clinical evidence that chemotherapy can prevent the establishment of hepatic fibrosis if given early enough in the infection. The results of our studies with infected chimpanzees provide experimental evidence in support of this statement.

In our experience, the colonic, hepatic, and renal lesions of both series of untreated S. japonicum infected chimpanzees have been remarkably predictable in degree and time sequence. Typically, the onset of pipestem fibrosis can be placed at 2 1/2 to 3 months after severe infection. Terminal triad fibrosis, colonic fibrosis and bilharziomas begin at about the same time. After the fourth month, these lesions are well established and mesangial changes seen in the kidney progress to significant nephropathy.

The drug formulation used in this series was not completely parasiticidal. Nevertheless, when treatment was begun within 3 months, all the severe lesions of schistosomiasis japonicalisted above failed to

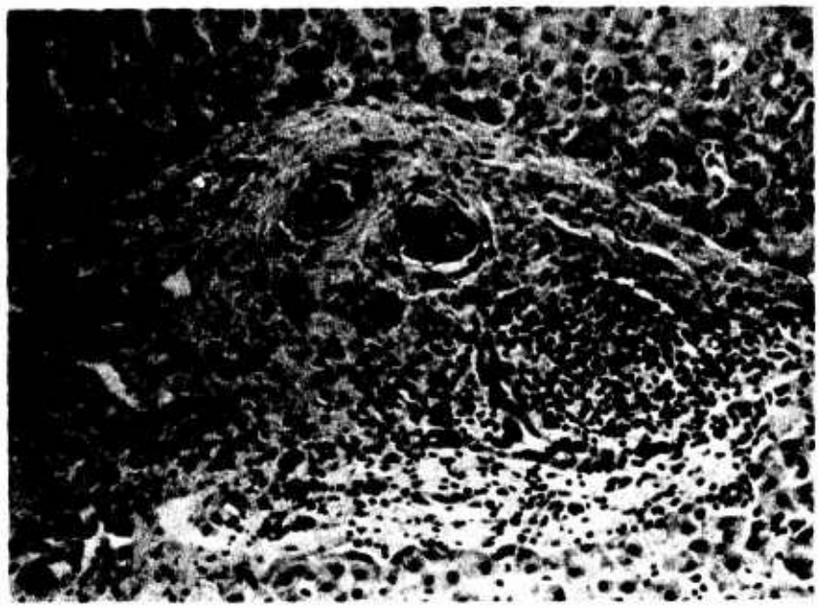


Fig. 40 - Chimpanzee No. 360. Liver (9 months) treated at 3 months.

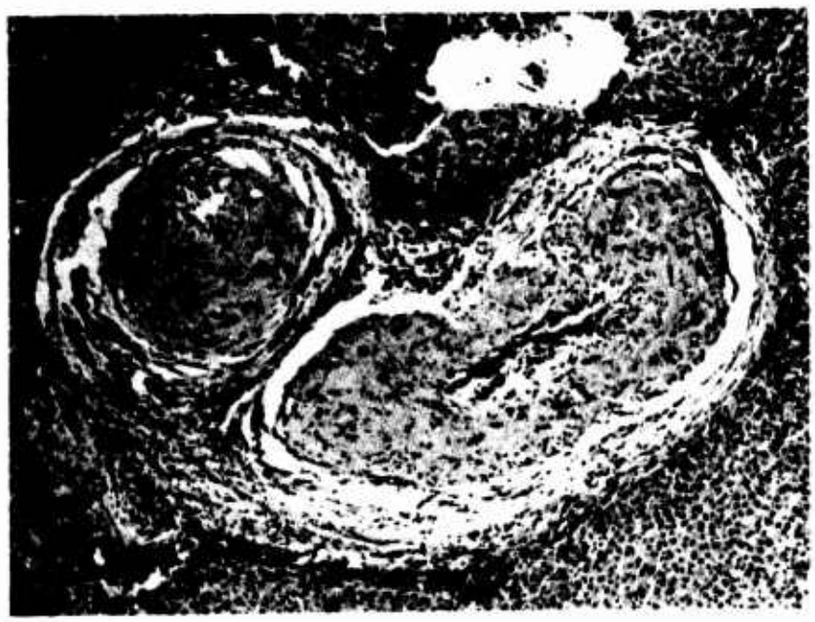


Fig. 41 - Chimpanzee No. 360. Large, convoluted, quasi hyaline scar.

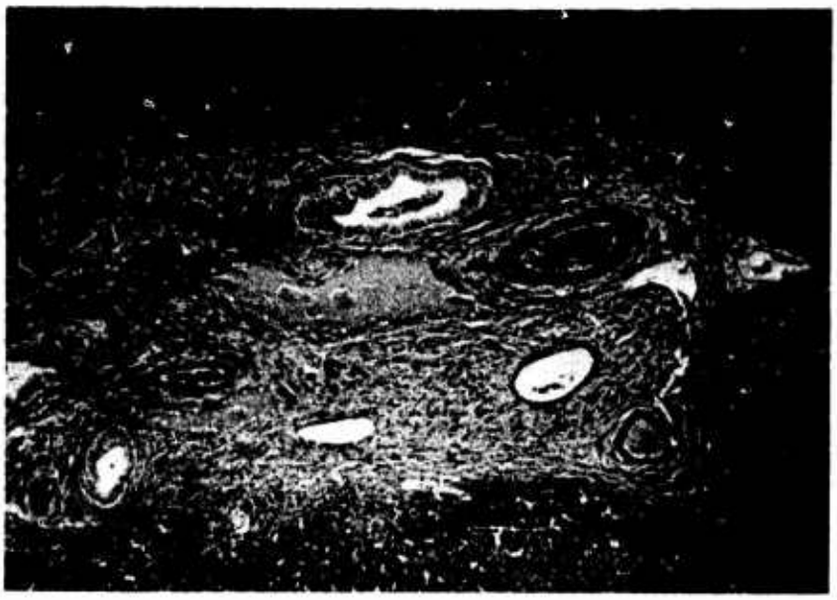


Fig. 42 - Chimpanzee No. 360, 2nd liver biopsy - after treatment.

Fig. 43 - Chimpanzee No. 966. Liver (16 months). Treated at 1/2 months.

appear. Conversely, when therapy was delayed, significant lesions persisted in the colon and liver. Whereas involuting patches and small fibrous lesions in the colon need not be regarded as important, persisting pipestem lesions in the liver cannot be similarly ignored. Despite the lesser degree of fibrosis seen in these livers and the static or quiescent appearance of most of the scars, evidence of obstructive portal lesions and of an arterial shift of circulation remained for over a year after initial treatment with some condensation and retraction of the fibrous tissue, but otherwise with little change. Thus, reduction of granulomatous and diffuse inflammatory activity in the liver was not accompanied by resorption of the fibrous tissue which had already been deposited before the treatment began. The same was true of mice treated 9 months after infection. If this irreversibility is a function of the host collagen metabolism, as seems likely, it could also be expected to occur with more effective drug formulations. On the other hand, we found no evidence that the liver pathology continued progressing after treatment as might be expected if fibrosis were caused by an autoimmune mechanism, as has been suggested. Rather, in all instances, persisting inflammatory activity in these livers was proportional to the degree of residual active infection. S. japonicum eggs present in the tissues of chimpanzees at the time of treatment were apparently destroyed only slowly. This is consistent with the increasing accumulation of eggs seen in untreated chimpanzees and in marked contrast to the rapid destruction of S. japonicum eggs in rhesus monkeys.

In the treated animals with improvement of the liver pathology, but with persisting portal fibrosis, there was a marked reduction in the degree of glomerular changes of the kidney suggesting at least a partial reversibility of these lesions. Indeed, the degree of nephropathy seemed more closely related to the degree of active oviposition and of diffuse inflammatory lesions, than of portal obstruction and fibrosis. This would indicate that parasite load is an important factor in the pathogenesis of schistosome nephropathy and, therefore, effective parasiticidal treatment might prevent or halt this particular complication even after irreversible fibrotic damage has already arisen in the liver. However, it must be noted that this evidence as yet is only suggestive. Further experimental and clinical studies may clarify this point.

Regardless of timing, treatment of our severely infected chimpanzees resulted in the rather prompt reversal of most clinical symptoms as well as of hematological and biochemical abnormalities with the notable exception of the elevated immunoglobulin levels and with only partial correction of hypoalbuminemia. These findings suggested a correlation of the hypochromic-microcytic anemia principally with the intestinal pathology, and of the serum protein abnormalities principally with the liver changes.

In contrast to the post-treatment crisis postulated by a number of authors, there was no indication of any immediate deleterious effects of treatment on the clinical or pathological conditions of these infected animals. It has long been known that in schistosomiasis severe

Fig. 44 - Chimpanzee No. 956. Kidney at autopsy (16 months). Treated at 2 1/2 months. Haematoxylin-eosin, X490. Compared with the full-blow nephropathy (Fig. 16), this glomerulus shows only minimal lesions; it is normal sized, with focal mesangial expansion; no basement membrane alterations or hyaline granules are seen.

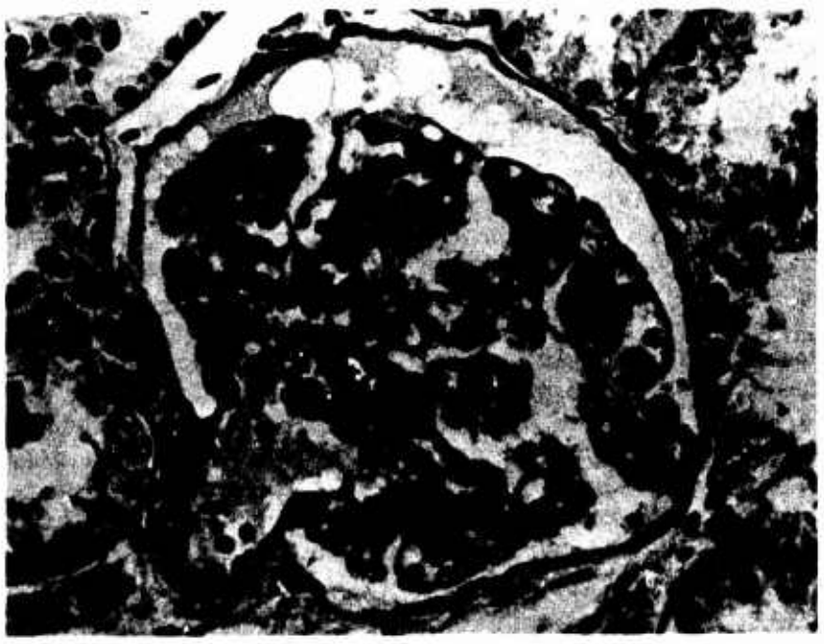


Fig. 45 - Chimpanzee No. 956. Kidney (16 months). Treated at less than 3 months.

anatomical changes may be accompanied by only mild clinical manifestations and that liver pathology may progress during periods of clinical latency. This was certainly the case in at least one of our chimpanzees treated late in the experiment (No. 955), in which progression of pipestem fibrosis occurred after clinical improvement and virtual absence of passage of eggs in the stool. Therefore, the efficacy of treatment must ultimately be judged by its effects on pathological lesions as well as by its clinical results. Since chimpanzees develop no appreciable portal hypertension in association with pipestem fibrosis, the effects of treatment on functional portal obstruction were not determined. However, the nearly uniform association of pipestem fibrosis with severe intrahepatic portal obstruction in man indicates that the presence of this anatomic lesion is a reliable indication of severe disease.

Under the conditions of this experiment, incomplete parasiticidal treatment given at any time up to 5 months after S. japonicum infection had prompt and favorable effects on the clinical and laboratory status of heavily infected chimpanzees, and no significant deleterious effect was noted in these animals. However, clinical or parasitological improvement was not a reliable indicator of the degree of the remaining anatomical pathology. Whereas egg excretion, colonic lesions, renal pathologies and anemia all improved markedly after treatment, portal fibrosis persisted at about the same stage which it had reached prior to treatment. Thus, in this experimental model, where progression to pipestem fibrosis occurs within the first 3-4 months of infection, it could be completely prevented only by treatment within 2 1/2 months; subsequent therapy succeeded in stabilizing the liver fibrosis, but not in reversing the damage which had already occurred.

The lack of effect of treatment on established portal fibrosis could not have been evaluated without wedge biopsy of the liver, although in general, serum gamma globulin levels did parallel the degree of pipestem fibrosis. Evaluation of the effects of treatment on pipestem fibrosis in man will presumably be equally difficult, although measurement of portal pressure should afford a quantitative index of portal obstruction.

Although there are significant differences between experimental schistosomiasis in chimpanzees and human endemic disease, our results indicate that parasiticidal treatment, even when only partially effective, cannot be considered as harmful at any stage of the disease. However, the greatest benefits will be obtained by treatment soon after the onset of patency in S. japonicum. The public health implications of the finding are obvious.

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable diseases and immunology

Work Unit 165 Parasitic diseases of military importance

Literature cited

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71Q	00	166
b. CONTRIBUTING				
c. XXXXXXXX	CARDS 114(f)			

11. TITLE (Precede with Security Classification Code)

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12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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e. KIND OF AWARD: f. CUM. AMT.

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FISCAL YEAR CURRENT 75	4.5	205

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS
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NAME: Brandt, Dr. Walter E.

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Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Viruses; (U) Immunology; (U) Ecology; (U) Human Volunteer; (U) Respiratory Tract Infections; (U) Arbovirus Infections

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To define etiology of acute infectious diseases of special hazard to military personnel, to determine and evaluate factors influencing occurrence, distribution, severity and medical result of human virus infections, and to develop means for reducing disability due to virus diseases.

24 (U) Contemporary virological and immunological methods are applied to disease problems occurring in troops or in susceptible civilian populations in strategically important areas. New conceptual approaches and methods are developed as needed for specific problems.

25 (U) 73 07 - 74 06 Two glycoproteins of the Group A arbovirus Sindbis were cleanly separated by isoelectric focusing; the one with isoelectric point of 6 has hemagglutinating activity and group antigenic determinants, while that with isoelectric point of 9 has type specific antigenic determinants. Soluble complement fixing (SCF) antigen of dengue types 1 and 2 purified by isoelectric focusing has produced specific anti-SCF antibody in mice. Effect of the temperature sensitive lesion of a naturally occurring small plaque variant of dengue-2 takes place between 8 and 12 hours after infection. The close temporal correlation between emergence of A. atlanticus and Keystone virus sentinel rabbit infections suggests that this vector transmits virus at its first blood meal, a finding consistent with transovarial transmission. During this year excellent control of adenovirus acute respiratory disease in basic trainees was achieved, due to potent and stable vaccines. Of the 6 BCT posts, only Fort Leonard Wood had sustained rates greater than 2/100 men/week and less than 20% of admissions at this post were associated with adenovirus. For technical report, see Walter Reed Army Institute of Research Annual Progress Report 1 July 1973 - 30 June 1974.

*Available to contractors upon originator's approval

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 166, Viral infections of man

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C. L. Parker; J. M. McCown; G. E. Holmes; C. M. Hampton
R. J. Jackson; G. P. Onley

Description

To define the etiology and ecology of human virus infections, particularly those of military medical significance; to devise and evaluate means for precise diagnosis, control and/or prevention of disease. Studies have applied virological, immunological, epidemiological and physiological approaches to understanding disease caused by respiratory, arthropod-borne and other viruses, the factors influencing transmission among men, other vertebrates and invertebrates, and their survival in nature.

Progress

I. The arthropod-borne viruses

A. Antigenic analysis of the structural proteins of Sindbis virus

The serological complexity of the alphaviruses (group A arboviruses) is not well understood in that the antigens responsible for group and type reactivity have not been defined. This group of viruses exhibits antigenic relationships similar to the flaviviruses currently posing sero-diagnostic problems. Studies of the physico-chemical separation of virus structural components for diagnostic antigens and immunogens of potential prophylactic value were initiated using Sindbis virus, an alphavirus, as a model. To date we have succeeded in isolating the nucleocapsid or "core" particle from three

alphaviruses, Sindbis, western (WEE) and eastern equine encephalitis (EEE) virus, and have shown them to contain common antigenic determinants, possibly relating all members of the alphavirus group (Dalrymple et al, 1973. In addition to the nucleocapsid protein, Sindbis virus contains two envelope glycoproteins which have heretofore been resolved only by techniques which at least partially denature these proteins, rendering them unsuitable for antigenic analysis (Schlesinger et al, 1972). We have described in previous annual reports attempts to separate these envelope glycoproteins in a biologically active form, using detergent disruption and isoelectric focusing. The present report describes the identification of the separated virus components and a description of their antigenic properties.

The observation (see last year's Annual Report) that the three separated proteins exhibited marked differences in isoelectric points (pI 3, 6, and 9) suggested that differences would be observed in the amino acid composition of these proteins. Purified Sindbis virus labeled with a commercial mixture of 3H-amino acids (New England Nuclear, Boston, MA) was analyzed as a control and compared to a similar analysis of each of the three proteins recovered from an isoelectric focusing experiment of the detergent disrupted virus. Differences were detected in the distribution of six amino acids in the 3 proteins (Table 1). The nucleocapsid protein (pI 3) appeared richer in threonine and glycine while the pI 6 envelope protein contained more methionine and tyrosine and the pI 9 protein more asparagine and histidine. Of the six amino acids in which differences occurred, only two (glycine and asparagine) were abundant in the virion preparations examined. These data are not meant to reflect the actual amino acid composition of the virus since differences in incorporation could well have resulted from differences in the concentration, specific activity, or rate of uptake of any of the amino acids in the mixture. Differences in distribution of any given amino acid in the three proteins, however, would suggest actual differences in primary structure.

To determine the correlation between the two envelope proteins pI 6 and pI 9 and the envelope proteins E_1 and E_2 described by Schlesinger et al, 1972), samples of each of the electrofocused proteins were analysed by discontinuous gel electrophoresis. Since this procedure is not routinely performed in this laboratory, these samples were sent to Dr. Sondra Schlesinger's laboratory for identification. The pI 3 protein was electrophoresed to the same point as the nucleocapsid protein and the migration of pI 6 and pI 9 proteins correlated with E_1 and E_2, respectively.

Hemagglutinating activity was assayed by dilution of each of the fractions from an isoelectric focusing gradient of detergent disrupted virus. Fig. 1 shows the distribution of hemagglutinin, using goose erythrocytes at pH 5.8. A clear peak of hemagglutinating

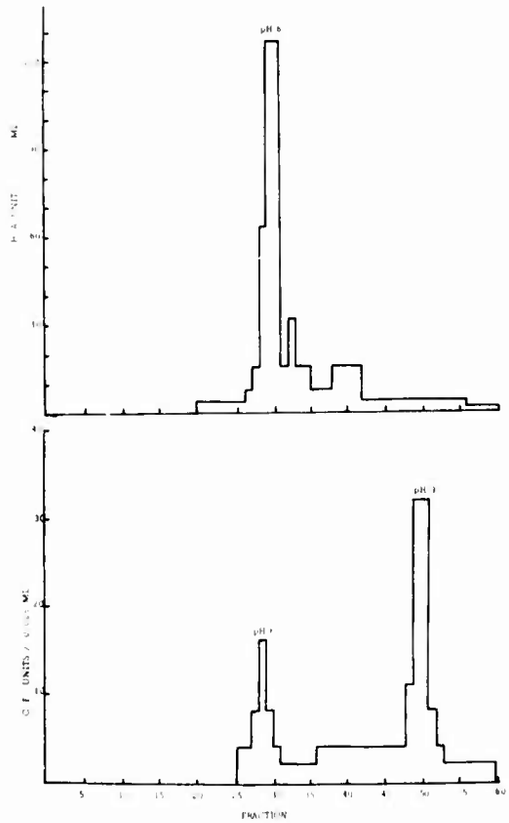

Figure 1. Hemagglutinating (top panel) and complement fixing (bottom panel) activity of electrofocus fractions from a pH 3-10 gradient of detergent disrupted Sindbis virus. Nonspecific lysis and anticomplementary effects exhibited in the acid region of the gradient have been deleted from the figure.

Table 1 - Incorporation of selected amino acids into Sindbis virion and individual protein components

Amino acid	Virion	Percentage distribution in virion proteins		
		pI 3	pI 6	pI 9
Threonine	1.63[a]	76.5[b]	12.0	11.6
Glycine	12.07	40.9	26.8	32.3
Methionine	0.13	13.9	45.9	40.4
Tyrosine	0.13	0	87.4	12.6
Asparagine	7.18	28.1	29.9	42.0
Histidine	0.91	27.3	27.3	45.5

[a] Percentage of total virion radioactivity found associated with the specific amino acid indicated

[b] Percentage of the combined total radioactivity (pI 3 + pI 6 + pI 9) for each amino acid

activity was detected coincident with the pI 6 protein. Similar hemagglutinating activity was not observed in the pH 9 region of the gradient. Lysis of the goose erythrocytes occurred in fractions below pH 3 and a 1:2 to 1:4 baseline of hemagglutinating activity was present in all fractions; however, these effects were attributed to the low pH and constant presence of detergent since control gradients containing no virus exhibited the same pattern of lysis and low titered agglutination. Further evidence that the pI 6 envelope protein reacts with erythrocyte receptors was obtained from experiments in which radioactive pI 6 and pI 9 antigens were incubated with goose erythrocytes at the pH optimum (pH 5.8) and at pH conditions that are not conducive to alphavirus hemagglutination (pH 7.0 and pH 8.6). Following incubation at room temperature for 20 minutes, the cells were pelleted by centrifugation and the radioactivity remaining in the supernatant was determined (Table 2). The pI 6 envelope protein attached to the erythrocytes at pH 5.8, but this interaction was markedly pH dependent and was greatly decreased at pH 7.1 and 8.6. The pI 9 protein exhibited very little interaction at any of the pH conditions examined.

Table 2 - Binding of pI 6 and pI 9 envelope proteins to goose erythrocytes

Envelope protein	% radioactivity bound		
	pH 5.8	pH 7.1	pH 8.6
pI 6	83.0*	22.5	8.6
pI 9	0.8	12.0	0

* Percentage radioactivity bound was calculated by measuring radioactivity of each reaction condition before and after incubation with erythrocytes

Fractions from the same column shown for hemagglutin analysis were assayed for complement fixing activity by reacting dilutions of each of the fractions with anti-Sindbis virus mouse hyperimmune ascitic fluid. The distribution of complement fixing activity is shown in the bottom panel of Fig. 1. Antigenic activity was demonstrated at both pH 6 and pH 9 and was somewhat higher titered at pH 9. Lysis and anti-complementary activity was associated with the acid region (< pH 3) of the gradient. Dialysis of these fractions resulted in the elimination of lysis but anti-complementary activity remained. Fractions from a control gradient exhibited the same anti-complementary activity.

Antigenic specificity of the three structural proteins was compared to the reactivity of the intact virion using the indirect radioimmune precipitation (RIP) test. Antiserum to Sindbis virus consisted of mouse hyperimmune ascitic fluids (obtained by immunizing adult mice with infected suckling mouse brain suspensions) known to contain antibody to both intact virions and detergent disrupted virus components (Dalrymple et al, 1973). Rabbit antisera to isolated viral antigens was prepared using electrofocus purified antigens. Virus antigens employed in the RIP test were either intact virions taken directly from sucrose gradients, isolated envelope proteins from isoelectricfocusing columns, or intact nucleocapsids and envelope preparations obtained by sucrose gradient centrifugation of detergent disrupted virus. The pI 3 protein antigen from electrofocus columns aggregated readily and yielded non-specific precipitation in the RIP test; however, the nucleocapsid particle antigen which was examined should exhibit similar specificity. The RIP reactions of intact virus and structural antigens with their respective antisera are compared in Table 3. Mouse antiserum to intact Sindbis virus exhibited a high titered homologous reaction and a significant cross-reaction with closely related WEE virions. Much lower titers were observed with the isolated structural protein

Table 3 - Radioimmune precipitation testing of Sindbis virus structural antigen

	Intact virions		Detergent solubilized virions		Electrofocused envelope fractions	
	SIN	WEE	Nucleocapsid	Envelope	pI 6	pI 9
Mouse anti-Sindbis virus	50,000[a]	10,000	1,000	1,000	200	100
Rabbit anti-pI 3 protein	<10[b]	<10	2,000	<10	<10	<10
Rabbit anti-pI 6 protein	20,000	500	10	1,000	20,000	(1,000)[c]
Rabbit anti-pI 9 protein	50,000	<10	<10	250	<10	1,260

[a] RIP titer = calculated endpoint dilution yielding 50% of the maximum precipitation observed. Unless otherwise indicated, all reactions precipitated greater than 70% of antigen containing 500 - 1,000 cpm in 50 microliters.

[b] <10 = no precipitation greater than 20% at any dilution tested.

[c] ( ) = questionable result, maximum precipitation was only 25% of total radioactivity added.

antigens. Positive RIP titers with 70-90% precipitation of the electrofocused membrane proteins were always obtained; however, titers were somewhat variable between different antigen preparations. The lower titers observed with detergent solubilized and separated antigens could be explained if detergent disruption of the virus results in a considerable increase in the exposed antigenic mass. Increased antigen concentration has been shown to reduce the apparent RIP titer of an antibody preparation. In these tests, all antigens were standardized using radioactivity (500-1,000 cpm) as the only basis for quantification and intact virions would be expected to contain more radioactivity per exposed antigenic determinant than a dissociated protein antigen.

Rabbit antiserum to the pI 3 protein reacted only with nucleocapsid antigen, suggesting that the immunogen from electrofocus fractions was not contaminated with either envelope protein. As previously demonstrated (Dalrymple et al, 1973), this antigen is not exposed on the surface of intact virions. The antiserum to the pI 6 envelope glycoprotein reacted with intact Sindbis virus and also exhibited some cross reaction with WEE virus. This antibody preparation had a high titer to the homologous pI 6 protein antigen and was free of any nucleocapsid reactivity but did, on occasion, react with the isolated pI 9 protein. This cross reaction was extremely variable and only 25% of the radioactivity was precipitated; the specificity of this antiserum must remain questionable pending further testing. Antibody to the pI 9 protein appeared specific in that it exhibited a high titer with Sindbis and no detectable cross reaction with WEE virus. No reactions were observed with either nucleocapsid or pI 6 protein antigen. Antibody to pI 9 protein gave precipitation with the homologous protein but at a titer low in comparison to Sindbis virus. Based on the high titered reaction with Sindbis virus and the difficulties encountered using the pI 9 protein in the RIP test in previous experiments, the low homologous pI 9 antibody titer is probably a function of the antigen preparation used rather than a real decrease in antibody avidity or content.

Rabbit antisera to isolated envelope glycoproteins were tested against purified Sindbis virions and envelope antigens by complement fixation (CF) tests and examined for neutralizing activity against infectious virus (Table 4). Mouse hyperimmune ascitic fluid to Sindbis virus served as a control. This control antiserum reacted strongly in CF tests with Sindbis virions and also reacted, although in somewhat lower titers, with both pI 6 and pI 9 protein antigens, indicating that the preparations were antigenically active. Antiserum to the pI 6 protein exhibited a homologous CF reaction, a low titer to the virion antigen, and did not cross-react with the pI 9 protein. These data contrast sharply with RIP results, indicating equal reactivity of this antiserum for both homologous pI 6 protein and virion. Antiserum to the pI 9 protein did not fix complement with homologous antigen, even though antisera to Sindbis virions reacted with that protein. In contrast to the high titer detected by RIP, pI 9 antiserum gave only a

low 1:10 titer against virion antigen. A low titer was also observed with the pI 6 protein.

Table 4 - Complement fixing and neutralizing antibody in antisera to Sindbis virus structural proteins

Antiserum	Complement fixation			Neutralization
	Virion	pI 6 protein	pI 9 protein	
Mouse anti-Sindbis virus	640[a]	160	160	5,120[b]
Rabbit anti-pI 6 protein	40	320	< 10	10
Rabbit anti-pI 9 protein	10	10	< 10	320

[a] - Reciprocal of highest dilution of antiserum exhibiting 3-4+ fixation of 5 units of complement. Antisera were tested against twofold dilutions of antigen in a grid titration.

[b] - Reciprocal of highest serum dilution that neutralized 50% of a 50-150 plaque-forming units dose of infectious Sindbis virus.

Antiserum to the pI 9 protein was shown to neutralize infectious Sindbis virus even though complement fixation with virion antigen had not been detected. The neutralizing antibody titer was not as high as antiserum to Sindbis virus but did differ markedly from pI 6 protein antiserum which only neutralized to a 1:10 dilution. Although there does not appear to be good agreement between the RIP and CF tests of these antisera, the neutralizing activity of antiserum to the pI 9 protein serves as a valuable marker for the presence of antibody.

Antiserum to the pI 3 protein was not compared in these studies because we have not yet been able to recover specific complement fixing antigens from this region of the electrofocus gradient; however, it was tested using virion and envelope antigens. Low titered CF reactions were detected with virion and both envelope antigens, suggesting possible immunogen contamination; however, no neutralizing activity was observed.

Three structural antigens of Sindbis virus have been separated by

detergent disruption and isoelectricfocusing. The pI 3 protein is not glycosylated and appears to represent the nucleocapsid protein of the virion. Isolated nucleocapsids, or "cores" have been previously demonstrated to broadly cross react with similar preparations of other alphaviruses. The two envelope glycoproteins E_1 and E_2 have been isolated at pH 6 and pH 9, respectively and differ in amino acid composition as well as biological activity. The pI 6 (E_1) glycoprotein appears to be the virus hemagglutinin and antigenically cross reacts with the closely related WEE virus antiserum. In contrast, the pI 9 (E_2) glycoprotein appears antigenically specific for Sindbis virus and antiserum prepared against this protein neutralizes infectious Sindbis virus. Studies are currently in progress to test these observations of antigenic specificity with other serologically related viruses.

B. Studies with the dengue non-structural soluble complement fixing (SCF) antigens

1. Search for antibody to dengue-2 SCF antigen in sera of children with dengue hemorrhagic fever

The nonstructural SCF antigen of the dengue virus serotypes was described in the 1969 and 1970 Annual Reports and subsequent publications (Brandt et al, 1970, Russell et al, 1970, Cardiff et al, 1971). Briefly, it was obtained by ammonium sulfate precipitation of protamine sulfate clarified suckling mouse brain suspensions. The ammonium sulfate precipitate was subjected to Sephadex G-100 chromatography and the peak of CF activity in the 39,000 dalton range was concentrated by nitrogen pressure filtration on Amicon PM-30 membranes which restrict the flow of material larger than 30,000 daltons. Since the antigen was found to circulate in relatively high titers in the blood of infected suckling mice (Brandt et al, 1970 b), the question was raised as to whether patients with dengue or dengue hemorrhagic fever (DHF) had SCF antigenemia or developed antibody to dengue SCF. We examined acute and convalescent sera of primary dengue cases and found no SCF antibody. The SEATO laboratory examined sera from secondary cases of dengue (associated with hemorrhagic fever) and found no antibody; however, convalescent sera represented specimens obtained on hospital discharge which generally occurred before 8 days after onset of illness.

Falkler et al (1973), using convalescent sera obtained more than 2 weeks after onset from secondary infections and hemorrhagic fever cases in Burma and the South Pacific, found SCF antibody in all those that he tested. Dengue hemorrhagic fever sera collected in 1970 at the SEATO laboratory and selected to include only patients with convalescent sera obtained weeks after appearance of symptoms, were examined for the presence of SCF antibodies using the CF test standardized according to Kent and Fife (1961). Table 5 lists the day of disease and the CF antibody titer to both crude mouse brain D-2 antigen and Sephadex

Table 5 - Complement-fixing antibodies in dengue hemorrhagic fever sera reacted with either crude dengue-2 infected mouse brain suspensions or the Sephadex purified soluble complement fixing (SCF) antigen

Grade disease	Acute				Convalescent			
	SEATO No.	Day of disease	Antigen		SEATO No.	Day of disease	Antigen	
			Crude	SCF			Crude	SCF
III[a]	48616	8	1024[b]	0	48685	20	1024	16
II	49115	7	128	0	49250	18	1024	8
III	49174	7	2048	0	49355	21	2048	32
IV	49190	5	512	0	49362	19	2048	0
III	48943	4	256	0	49033	18	1024	0
III	48619	9	1024	0	48707	21	1024	0
II	51138	5	256	0	51355	16	1024	0
III	51153	5	1024	0	51287	13	2048	0
II	51122	4	128	0	51288	15	2048	0
II	51196	3	64	0	51372	14	1024	0
III	51232	4	256	0	51447	18	2048	0
II	51276	7	2048	0	51432	15	1024	0
II	51374	3	1024	0	51567	19	2048	8
I	51376	2	32	0	51548	16	512	4
III	51377	4	128	0	51521	12	512	0
II	51408	3	1024	0	51568	17	512	4
III	51405	6	1024	0	51537	15	1024	8
III	51142	9	512	0	41145	12	1024	4
IV			QNS[c]		49249	18	2048	8
III			QNS[c]		48981	19	1024	4

[a] Severity of disease (Nimmannitya et al)

[b] Reciprocal of titer; 0 = < 1:4

[c] QNS = quantity not sufficient

purified D-2 SCF. The high CF titers to crude antigen in the sera collected relatively early are compatible with secondary dengue infection; these same sera (collected up to the ninth day of disease) had no detectable antibody to the SCF antigen. However, ten of the 20 convalescent sera, collected 12 days later after onset of illness, had SCF antibody titers ranging from 1:4 to 1:32. Most of those sera having titers of 1:8 to 1:32 were collected 18 to 21 days after onset. Dengue-2 SCF antibody was not detected in 10 convalescent sera. During this study, dengue-1 and 3 strains were isolated in addition to D-2; the lack of reactivity may reflect, among other possibilities, infection of these patients by serotypes other than dengue-2. The cross reactions with 2 or more serotype SCF antigens reported by Falkler et al (1973) may have been due to virion surface proteins likely contaminating their antigen preparations. Further definition of SCF antibody in DHF will require highly purified SCF antigens to the 4 dengue serotypes, as well as a more sensitive assay than the CF test. We are currently pursuing these goals, first, by including isoelectricfocusing as an additional and powerful step for purification (see following section) and second, by developing a radioimmune assay procedure.

2. Further purification of the dengue SCF antigen

The nonstructural soluble complement fixing (SCF) antigens of each of the dengue viruses have been investigated in a variety of analytical test systems. All of the experiments prior to those described in this report were performed using antigen preparations that were known to be contaminated with many mouse brain proteins. Methods for the purification of SCF were recently pursued as a prerequisite to extrinsic radiolabeling experiments which would require either pure SCF or a separation method for the removal of radiolabeled contaminants.

The preparation of SCF was described in the previous section. Continuous SDS polyacrylamide gel electrophoresis (PAGE) of the Sephadex G-100 purified SCF antigens has shown them to contain greater than twenty separate proteins as evidenced by stained bands on these gels. The antigenic activity of SCF has been shown to be associated with only one of these bands migrating to an estimated molecular weight of 39,000 (Cardiff et al).

Attempts to purify dengue SCF consisted of isoelectricfocusing the standard SCF preparation on a sucrose column (pH 3-10 gradient); procedures for this technique have been described in previous annual reports. Although considerable precipitation occurred at various points in the column, these bands of precipitate appeared to dissolve upon collection and did not interfere with either the linearity of the pH gradient or the distribution of markers with known isoelectric points (Sindbis virus proteins). It was soon discovered that concentration by pressure dialysis was an essential step prior to electrofocusing, since the large volume fractions from the G-100 Sephadex column contained sufficient salt to cause excessive current flow.

Initial attempts to purify dengue SCF used dengue type 2 preparations. A typical electrofocus profile of dengue-2 SCF is shown in Fig. 2. All fractions were examined by complement fixation using a constant dilution of dengue-2 mouse hyperimmune ascitic fluid as well as normal ascitic fluid as a control for anti-complementary effects of the fractions. Lysis of the indicator cells and anti-complementary effects (A/C) were prominen: in the first ten fractions (anode region) and a trace of A/C activity was often associated with the higher fractions (cathode region). The remainder of the gradient appeared free of such non-specific effects and could be assayed for dengue specific effects and could be assayed for dengue specific antigens without dialysis, etc. A reasonably sharp peak of dengue virus antigenic activity was observed at pH 5.2. Activity was detected in neighboring fractions but was principally confined to the pH range 4.8 to 5.5. Antigen harvested from peak fractions appeared free of mouse brain contamination by PAGE; however, the SCF band was not sharp and covered approximately 1/8 of the gel length.

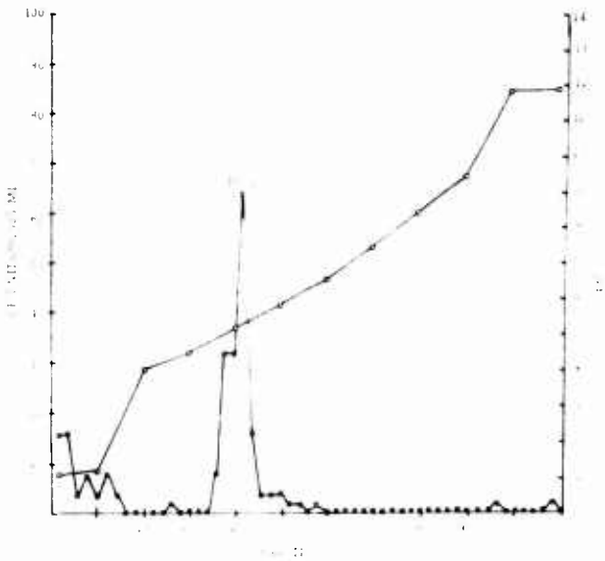

Figure 2. Distribution of dengue-2 soluble complement fixing antigen on an isoelectric focusing gradient pH 3-10. Fractions were diluted and tested by complement fixation using dengue-2 hyperimmune mouse ascitic fluid.

Repeated isoelectric focusing experiments over a period of six months using different dengue-2 SCF preparations have shown the procedure to be reproducible, averaging about pH 5.15 (range 5.0 to 5.2). In each instance, a peak of complement fixing activity was observed at

the indicated pH, even though the CF titer varied from 1:8 to 1:256.

Antiserum preparation was attempted in a single rabbit using an electrofocus purified dengue-2 SCF preparation that titered 1:64. The animal was given four injections of antigen mixed with Freund's complete adjuvant over a six-month period. This extensive immunization scheme was utilized because of previous difficulty encountered in mouse immunization experiments requiring substantial antigenic mass. Antisera obtained from three successive bleedings had high titered antibody to dengue-2 SCF (Table 6) but did not fix complement with 1:2 dilutions of normal mouse brain. These sera did not inhibit D-2 hemagglutination nor neutralize D-2 virus in plaque reduction neutralization tests at a 1:10 dilution. They gave precipitin lines by double diffusion in agar with either purified dengue-2 SCF or crude infected mouse brain suspensions.

Table 6 - Complement fixing antibody titers of rabbit antisera prepared to purified dengue-2 SCF

Antiserum		Antigen	
		Crude DEN-2 CF antigen	Purified DEN-2 SCF
#1	3/26/74	64/128*	64/64
#2	3/28/74	N.T.	64/64
#3	3/24/74	N.T.	64/128

* Reciprocal of highest antibody dilution exhibiting 3-4+ complement fixation over the reciprocal dilution of highest antigen dilution yielding maximum antibody titer.

Attempts to obtain purified SCF preparation from each of the other three dengue serotypes have not been as easily accomplished primarily because the lower antigen titers obtained in mouse brain necessitates larger quantities of infected mouse brain material than dengue-2 virus. Each of these antigens have been repeatedly electrofocused and the isoelectric point of each is presented in Table 7. Each isoelectric point represents at least two successful experiments in which only a single peak of antigen was observed and titers of 1:8 or greater were obtained. The dengue-4 virus serotype presented the greatest difficulty with exceptionally low titers and antigen peaks at somewhat

higher isoelectric points (pH 6.8 - 7.6); however, upon refocusing, a single peak at pH 5.5 was obtained.

Table 7 - Isoelectric point determinations for each of the dengue virus serotypes compared with the relative electrophoretic mobility

Dengue serotype SCF	Isoelectric point (pH)	Relative free solution electrophoretic mobility*
D-2	5.0-5.2	1.122
D-4	5.5	1.001
D-3	5.8	0.866
D-1	6.2-6.5	0.793

* Cardiff et al, 1970

It can be seen in Table 7 that the isoelectric points of the four dengue SCF antigens in ascending order of pH (D-2, D-4, D-3 and D-1) are in the same order in terms of descending relative free solution electrophoretic mobility. The latter was described in the 1970 Annual Report and elsewhere (Cardiff et al, 1970); briefly, the free solution electrophoretic mobilities of the SCF antigens were obtained by extrapolating to zero gel concentrations the plots of relative mobilities in various concentrations of polyacrylamide gel. These lines were parallel to each other, and since the molecular weight of each dengue serotype SCF antigen was the same, they were proposed to be "charge isomers". The isoelectric point data presented here correlates with the relative free solution electrophoretic mobility since the more alkaline the isoelectric point of an SCF antigen, the less charge migration should be observed in acrylamide gel electrophoresis carried out at pH 8.5.

Rabbits are currently being immunized with purified SCF preparations of each of the dengue serotypes. Sera have been obtained from the dengue types 1 and 2 animals, and although CF titers to purified antigens range from 1:512 to 1:1024, they do appear to contain some antibody to normal mouse brain antigens (1:20-1:40) and anti-complementary titers approach 1: 28. Comparison of the antigenic relation-

ships of the SCF from the four dengue viruses and the proposed radiolabeling experiments will be continued following the satisfactory completion of the rabbit immunization experiments.

C. Characterization of a small plaque clone of dengue-2 virus.

A naturally occurring small plaque variant of the PR-159 (Puerto Rico) strain of dengue-2 virus was plaque purified in certified primary African green monkey kidney cell monolayers as described in the previous Annual Report. When the small plaque clone was compared to the parent virus from which it was derived, it was relatively avirulent for mice, it did not produce detectable viremia in monkeys - yet produced a good antibody response, and it could not replicate in cell cultures at as high a temperature as the parent virus could replicate. Temperature sensitive mutants of animal viruses are of considerable interest because of their usefulness in genetic studies as well as defining the events that occur during viral replication. From a more practical viewpoint, a lower permissive temperature of replication than that of the wild virus has generally been associated with those viruses which have been attenuated for use as live virus vaccines.

The effect of temperature on the small plaque clone in cell culture is shown in Fig. 3. Raising the temperature from 36 to 37°C

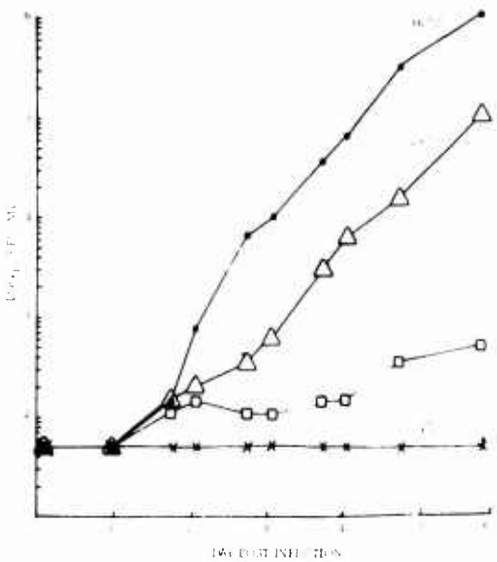

Figure 3, Replication of dengue-2 small plaque clone S-i in LLC-MK_2 cells.

significantly decreased the titer of infectious virus found released into the culture medium (approximately 1 $\log_{10}$ unit). An additional one degree increment from 37°C to 38°C caused a dramatic drop in infectious virus titer (about 2.5 $\log_{10}$ units); 39°C was totally non-permissive for replication of the virus in this experiment. In order to determine if the nonpermissive temperature was inactivating any virions that were released into the culture medium, a heat stability curve with the small plaque clone as compared to the parent virus and the mouse brain seed of the New Guinea C strain was carried out at 39°C and 35°C (Table 8). It can be seen that the small plaque clone appears even more heat stable than the parent virus from which it was derived at both 35°C and 39°C. Inactivation of the New Guinea C mouse brain seed at 35°C parallels that of the small plaque clone but appears somewhat greater at 39°C.

Table 8 - Stability of the infectious virions at 35°C and 39°C

	Small plaque 19A		Parent-GMK-6		New Guinea C sm35	
	35°C	39°C	35°C	39°C	35°C	39°C
	33*		68		4900	
1 hr	31	58	55	60	3700	4200
2	21	20	51	10	2700	2300
3	21	6	36	0	2700	880
4	24	7	29		2600	150
5	19	5	0		1600	< 100
6	13	1			1500	
7	12	0			1150	
8	5				970	
24 hr	0				< 100	

* pfu/ml

The temperature sensitive step during viral replication is being determined by temperature shift experiments, in LLC-MK_2 cells here, and in primary African green monkey kidney cells in the Hazard Laboratory at Forest Glen. The multiplicity of infection that could be obtained with the cell culture seeds during routine passage of the virus was

insufficient (0.001) to yield detectable virus from LLC-MK_2 cells that had been placed at the nonpermissive temperature for various periods of time. However, when the infected cells were frozen and thawed several times for determination of intracellular virus content after several days of incubation at the permissive temperature, small quantities of infectious virus were found under the following conditions. Four times as much virus was obtained when the temperature was shifted up to 39°C at 12 hours than when it was shifted up at 8 hours post infection. In temperature shift-down experiments, virus was found when the temperature was shifted to 35°C at 8 hours, but no virus was found when the temperature was shifted down at 12 hours. The tentative conclusion is that the temperature sensitive lestion is probably somewhere between 8 and 12 hours post infection, but better data is required using higher multiplicities of infection. This is being accomplished by using virus seeds concentrated 100-fold by centrifugation on smaller numbers of cells in small culture vessels. Good yields were obtained in this system, but slightly higher temperatures are required to adequately differentiate the effects of temperature shifts during the replication cycle.

Concentrated small-plaque-clone seed virus (at the 20th cell culture passage) was also required to obtain a sufficiently high multiplicity of infection for the production of radioactive virions for structural analysis by polyacrylamide gel electrophoresis (PAGE). LLC-MK_2 cells were infected at a multiplicity of 0.1 with the GMK-6 parent, and 1.0 with the New Guinea C mouse brain seed (prototype - control). Radioactive amino acids were added to the amino-acid deficient medium 12 hours later. The culture fluids were harvested at 68 hours post infection and the radioactive virions obtained by successive series of centrifugations as described in the previous annual reports. PAGE was performed on virions boiled in SDS as described previously. Three radioactive peaks typical of the group B arboviruses (1971 Annual Report) were observed with the small plaque clone virions and the prototype New Guinea virions (Figs. 4-A, B). In the GMK-6 parent virion, very little radioactivity was present in the V-2 (core or nucleo-protein) region of the gel shown coelectrophoresed with the New Guinea C prototype in Fig. 4-C; the 10-fold lower multiplicity of infection may have been responsible. Radioactivity in the V-2 region was also depressed when the small plaque clone and the New Guinea C virions were boiled again after -20°C storage for coelectrophoresis (Fig. 4-D). Regardless of the problems with incorporation of radioisotopes into V-2, coelectrophoresis indicates that the only differences between the prototype New Guinea C virus and the cell culture passaged Puerto Rican strain (both parent and the small plaque clone) is the slightly smaller V-1 (minor envelope protein) of the New Guinea C virus.

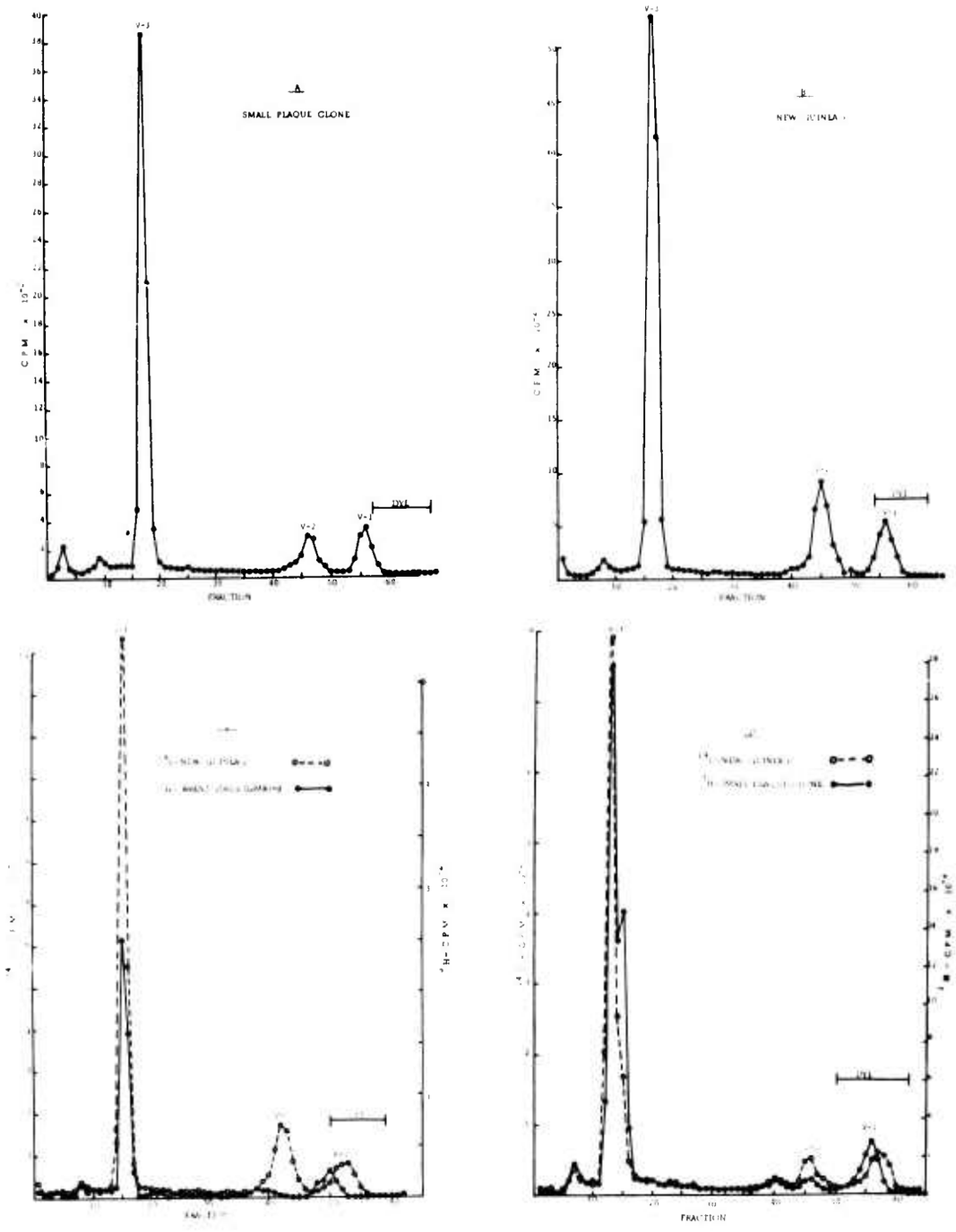

Figure 4. Polyacrylamide gel electrophoresis of detergent and heat-disrupted dengue-2 virions. A. PR-159 small plaque clone (S-1) passage 21 (^{3}H-amino acid label). B. Prototype New Guinea C (^{14}C-amino acid label). C. Coelectrophoresis of the PR-159 parent virus (^{3}H-amino acid label) and the New Guinea C strain. D. Coelectrophoresis of A and B after storage, mixing the samples together, and reboiling them.

D. Differential ability of various dengue type-2 strains having different passage histories to replicate in several cell culture systems

Replication of dengue virus in lymphoid cells was considered essential in elucidating or attempting to duplicate the antigens produced in dengue infections that participated in activating the complement system. The ability of dengue-2 virus to infect several lines of human lymphoblastoid cells was determined in collaboration with the Department of Experimental Pathology, Scripps Clinic and Research Foundation. We also examined replication of several dengue-2 strains in Aedes albopictus (Schneider) cells in initial attempts to determine the effects of various types of cell culture passage on dengue viruses.

In the first experiments, the New Guinea C strain of dengue-2 virus that had been passaged 35 times through suckling mouse brain, was used to infect the Raji, Wil-2, and 8866 human lymphoblastoid cell lines. No newly replicated virus was found in the supernatant culture fluids or the cells up to and including 5 days after attempted infection. However, when these cells were infected with the SEATO 16681 strain of dengue-2 (isolated in cell culture, passed in a monkey, 3 passages in Aedes albopictus mosquitoes, and then in Raji cells) the virus replicated as indicated by the titers listed at daily intervals in Table 9. Generally, 3 days were required before a significant

Table 9 - Replication of dengue-2 virus (strain 16681, cell culture seed) in human lymphoblastoid cell lines

Time (hrs)	Raji		Wil-2		8866	
	Supe	Cells	Supe	Cells	Supe	Cells
0	0	1.6×10^{1}*	0	3.3×10^{1}	0	6.5×10^{1}
24	8.0×10^{1}	1.1×10^{1}	1.0×10^{2}	1.6×10^{2}	1.4×10^{2}	2.8×10^{2}
48	4.0×10^{1}	9.5×10^{1}	4.0×10^{1}	9.6×10^{1}	8.5×10^{1}	1.6×10^{3}
72	2.0×10^{1}	3.5×10^{3}	2.3×10^{3}	8.6×10^{2}	9.5×10^{1}	5.2×10^{3}
96	2.5×10^{1}	5.8×10^{3}	1.3×10^{3}	3.2×10^{4}	8.5×10^{1}	1.9×10^{3}
120	1st	8.8×10^{3}	9.9×10^{2}	2.5×10^{4}	5.5×10^{1}	6.6×10^{2}

* Titers corrected for the 10-fold concentration of the cells relative to the culture medium prior to frozen storage

increase in virus yield could be detected, and by the fourth day post infection, there appears to be more intracellular than extracellular virus. Since the Wil-2 cells released more dengue virus into the culture fluids, we examined the ability of a cell culture seed of another strain of dengue-2 virus (PR-159) and a naturally occurring small plaque clone isolated from it (neither of which had been passed in suckling mouse brain) to replicate in these cells. Virus replication was detected only in those cells infected with the 16681 strain, used as a positive control for replication. Finally, the New Guinea C strain in mouse brain was passed through LLC-MK_2 cells and the infected culture medium was used in an attempt to infect Raji cells. The mouse brain seed and the 16681 strain of dengue-2 were used as controls, all at an MOI of 0.02. There was some replication of the New Guinea C strain in mouse brain this time, no replication with the New Guinea C smb35/LLC-MK2-1 seed, and the 16681 strain replicated as usual (Table 10). It was found that chronic infections of the human lymphoblastoid cell lines could be established with the 16681 strain of dengue-2 virus (Table 11).

Table 10 - Ability of two dengue-2 strains to infect the Raji line of human lymphoblastoid cells[a]

Hour post infection	New Guinea C mouse brain seed		New Guinea C Smb/LLC-MK_2 seed		16681 strain Raji-LLC-MK_2 seed	
	Supe	Cells	Supe	Cells	Supe	Cells
0	0	0	0	0	2.5×10^1	1.3×10^2
24	0	1.5×10^1	0	0	0	1.0×10^1
48	5	9.5×10^1	0	0	7.0×10^1	1.1×10^3
72	1.0×10^1	1.0×10^2	0	0	4.4×10^2	7.6×10^3
96	2.5×10^1	1.0×10^2	0	0	4.6×10^3	5.7×10^4
120	2.5×10^1	1.1×10^2	0	0	4.9×10^3	Contamin.
144	2.0×10^1	9.0×10^1	0	0	5.0×10^3	1.2×10^5

[a] Raji cells were infected at the same multiplicity of infection

[b] 0 = < 5 pfu/ml

Table 11 - Chronic dengue-2 infections established in several lines of human lymphoblastoid cells (pfu/ml)

Passage	Raji		Wil		8866	
	Supe	Cells	Supe	Cells	Supe	Cells
5th	2.5×10^4	$9.0 \times 10^{4*}$	1.5×10^2	1.0×10^5	4.5×10^3	6.4×10^4
6th	9.8×10^3	Contamin.	4.0×10^2	6.5×10^4	6.9×10^3	4.4×10^4
7th	5.5×10^4	9.8×10^4	5.0×10^2	1.8×10^4	7.0×10^2	7.2×10^3
8th	7.0×10^3	4.8×10^4	1.9×10^3	1.0×10^5	3.5×10^2	2.4×10^4
9th	7.5×10^3	5.6×10^4	4.1×10^4	6.1×10^4	5.0×10^2	1.7×10^4
10th	2.2×10^4	1.5×10^5	4.5×10^4	2.5×10^5	1.7×10^3	5.5×10^4
20th	3.2×10^4	3.1×10^5	1.3×10^3	2.7×10^4	5.3×10^3	1.3×10^4
27th	2.9×10^4	6.7×10^4	No samples		1.7×10^3	Contamin.

* Not corrected for volume

Next, Aedes albopictus cells were infected with the mouse brain seed of New Guinea C, the small plaque clone of the PR-159 strain of dengue-2, and the 16681 Raji/LLC-MK2-1 seed. Several passages were attempted to determine if Raji cells could be infected with Aedes passage virus (see preceding section). All 3 strains appeared to replicate in the albopictus cells (Table 12), but when the virus-containing day 4 culture fluids were used to infect another lot of albopictus cells, we only obtained successful passage (measured by release of infectious virus) with the 16681 strain. We are examining the multiplicity of infection, defective particles, strain variation, and the effect of the host cell during passage.

Table 12 - Passage of dengue-2 strains in Aedes albopictus cell cultures

Strain	Time (day)	Passage no. 1	Passage no. 2
New Guinea C smb seed	1	7.0×10^2*	< 50
	2	8.0×10^2	< 50
	3	1.0×10^4	< 500
	4	1.6×10^4	< 500
PR-159 SP-1 cell culture seed . .	1	< 50	< 50
	2	< 50	< 50
	3	1.5×10^3	< 500
	4	1.5×10^3	< 500
16681 Raji-LLC-MK_2 seed	1	3.5×10^2	< 50
	2	4.0×10^3	1.1×10^3
	3	2.2×10^5	3.2×10^4
	4	4.2×10^5	1.4×10^5

* Plaque-forming units per ml in LLC-MK_2 monolayers

E. Growth of Togaviruses in enucleated cells

When cytochalasin B, a metabolite of the fungus Helminthosporium dematoideum, is used at relatively high concentrations (10 µg/ml) it has been shown to cause some vertebrate cells in culture to extrude their nuclei (Carter, 1967). By subjecting cells exposed to this concentration of the drug to centrifugation one can obtain mass enucleation (> 90%) of populations of cultured vertebrate cells with no obvious cytoplasmic damage. In collaboration with Dr. Richard A. Goldsby of the Dept of Biochemistry, University of Maryland, we took advantage of these observations to ask if the nucleus is necessary for the replication of alphaviruses and/or flaviviruses.

1. Production of infectious virus

Secondary cultures of chick embryo cells were grown on plastic discs 12 mm in diameter. Enucleation was accomplished by placing the discs cell side down in the bottom of a 50 ml Sorvall centrifuge tube containing 8-10 ml of Dulbecco's Modified Eagle's (DME) medium with 10% fetal bovine serum (FBS) and 10 µg/ml of cytochalasin B. The tubes containing the coverslips were then centrifuged in a Sorvall RC-2B centrifuge prewarmed to 37°C for 30 min at 12,000 rpm in the SS-34 rotor. After centrifugation the cells were placed in medium lacking cytochalasin and after 60-90 min at 37°C the cells regained their normal morphology with > 90% lacking nuclei.

Enucleated cells, cells treated with cytochalasin but not centrifuged and untreated cells were infected with either Sindbis or Japanese encephalitis virus at an MOI of 100. After incubation at 37°C for 90 min for viral adsorption, they were washed through three beakers containing about 100 ml of either normal saline or Hank's balanced salt solution. They were then placed in two ml of 10% FBS DME and incubated at 37°C. The culture fluids were harvested at the appropriate times and frozen in three aliquots at -70°C.

Two experiments were performed: in the first, culture fluids were harvested at 2 1/2, 14 1/2, and 24 hours post infection; in the second, 2 1/2, 18 and 26 hours post infection were the times of harvest. The culture fluids were assayed for infectious virus by plaque assay on LLC-MK_2 cells and the results are shown in Figures 5 and 6. In the first experiment the final yield of Sindbis virus was reduced by 93% from the controls; in the second, the yield was reduced by 94%. In the case of JE the yield of infectious virus was reduced 99.5% in the first experiment and 99.4% in the second.

In the first experiment, there was no increase in the titre of the JE-infected enucleated cultures, whereas in the second, there was an increase of one log in the enucleated cultures with time. However, since a separate coverslip was used for each time point, the variation of one log from the 2 1/2 hour point to the 24 hour point may be due to different efficiencies of washing, as can be seen in the variation of the 2 1/2 hour points in the first Sindbis experiment. We therefore concluded that JE did not replicate in the enucleated cultures.

In contrast, the Sindbis-infected enucleated cultures showed a rise of 1 1/2 logs in the first experiment and 2 logs in the second, indicative of replication in the enucleates. However, as was noted above, the final yield in both experiments was reduced by > 90%. This reduction could possibly be explained by: 1) loss of cells in the enucleated cultures during centrifugation; 2) a non-specific cytotoxic effect of the drug. The second explanation seems unlikely since the cytochalasin treated unenucleated cells showed no such effect.

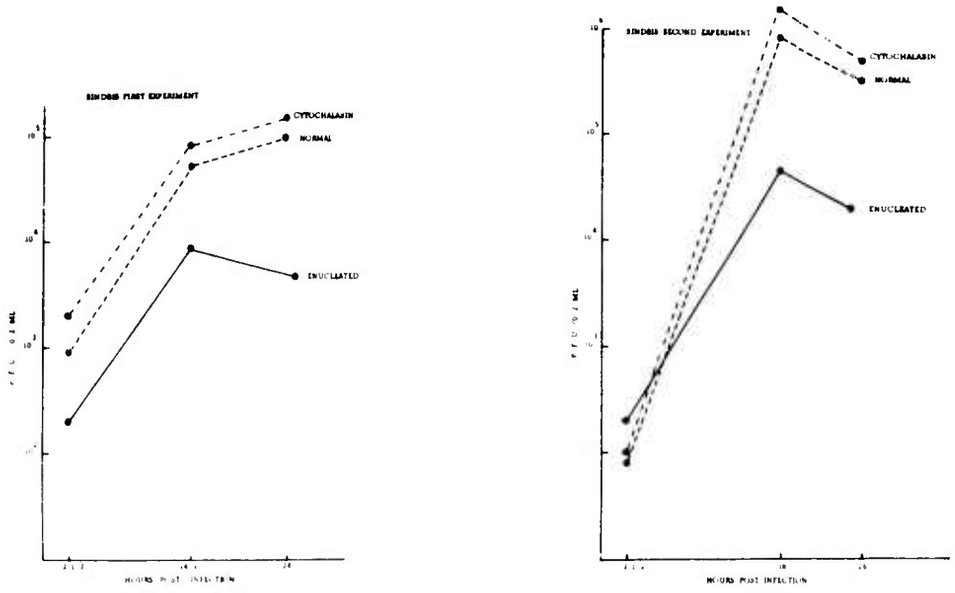

Figure 5. Growth curves of Sindbis virus in enucleated, cytochalasin-treated and normal chick embryo cells.

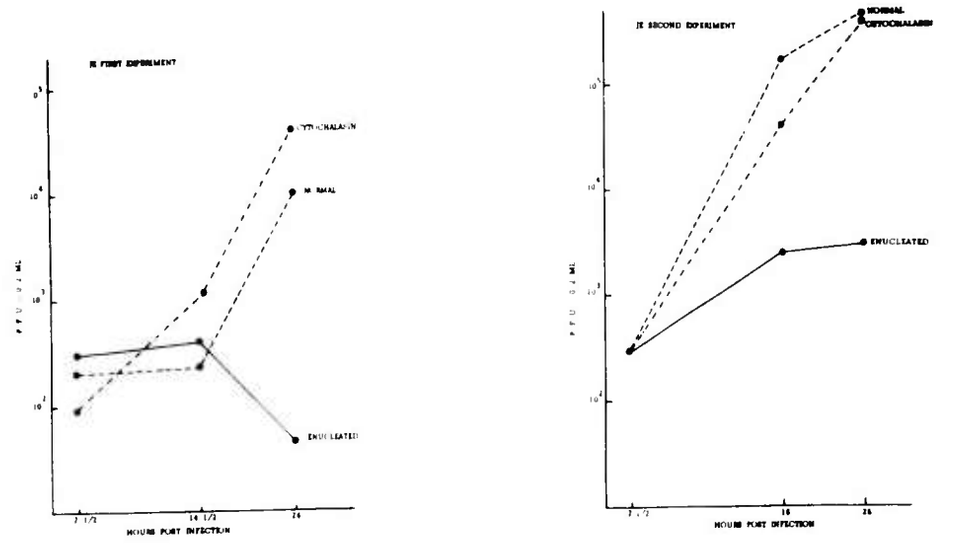

Figure 6. Growth curves of Japanese encephalitis virus in enucleated, cytochalasin-treated and normal chick embryo cells.

2. Detection of viral antigen by the indirect fluorescent antibody technique

To investigate the involvement of the nucleus more directly, we decided to use the indirect fluorescent antibody technique, whereby cells that showed signs of replication could be examined directly for the presence of a nucleus, thus eliminating the problem of cell loss during centrifugation.

Cells were enucleated and infected as before. They were then subjected to extensive washing to remove excess inoculum. The cells were removed from the coverslips by trypsin and plated on glass slides. At 18 hours post infection the cells were washed with phosphate buffered saline (PBS), fixed in methanol for 5 min, rewashed in PBS and frozen at -70°C until the fluorescent antibody test was performed.

The methods used for fluorescent staining were essentially those described by Cardiff (1973). Infected enucleated, cytochalasin treated but not enucleated, and normal chick embryo cells were observed for fluorescence, and the percentage of fluorescent cells was determined (Table 13).

Table 13 - Replication of Sindbis and Japanese encephalitis viruses in normal vs enucleated chick embryo cells, as judged by the indirect fluorescent antibody technique

Experimental conditions	No. of fluorescent cells	Total no. of cells counted**	Percentage of fluorescent cells
SIN - normal	93	100	93
SIN - cytochalasin treated	87	100	87
SIN - enucleated*	15	20	75
JE - normal	76	100	76
JE - cytochalasin treated	65	100	65
JE - enucleated*	3	36	8

* Percentage of enucleated cells was > 90% and only enucleated cells were counted in the enucleated cultures.

** In the case of the normal and cytochalasin treated cultures, 100 cells were counted. Since the cell density in the enucleated cultures was much lower, fewer cells were counted.

From these results it seems that Sindbis replicates almost as efficiently in enucleated cells as it does in normal cells (93% vs 75%). In contrast, replication of JE in enucleated cells is very inefficient. Although 3 JE-infected cells lacking nuclei were seen that did fluoresce the percentage of fluorescing cells was only about one-tenth that of the normal cultures. We therefore suggest that the nucleus is important in the replication of JE in chick embryo cells. However, our studies do not exclude the possibility that rather than the nucleus itself, some structure located perinuclearly is essential for flavivirus replication. The presence of the three JE-infected enucleated cells that fluoresced could be explained if, in most cases, the perinuclear area was removed along with the nucleus during centrifugation and that it remained intact in only a small percentage of the enucleates. Some credence is given to this hypothesis by the observation that flavivirus-infected cells show intense perinuclear fluorescence early in infection (Cardiff, 1973).

F. Studies on the replication of Japanese encephalitis virus in Culex tritaeniorhynchus cells.

Japanese encephalitis virus (JE) grows to high titer (10^8 pfu/ml) in Culex tritaeniorhynchus cells, without any observable cytopathic effect (Annual Report, FY 1973). Because of these observations it was thought that adequate amounts of radioactive virus could be obtained to permit characterization of purified virions from mosquito cells.

1. Attempts to obtain radiolabeled JE from Culex tritaeniorhynchus cells

Attempts to obtain radioactive virus from mosquito cells using the same timetable for addition of label as was used for mammalian cell propagated virus (Annual Report FY 1971, 1973) were unsuccessful. In an attempt to get more radioactivity into the cells, starvation in a minimal media along with prelabeling prior to infection were tried. Bottles of Culex cells were incubated in a minimal media prepared from salts of Hsu's media, tricine, sodium bicarbonate, vitamins, phenol red and penicillin and streptomycin. Twenty-four hours later 1 uCi/ml of ^{14}C-amino acids were added to half of the bottles and 20 uCi/ml of ^{3}H-amino acids to the rest. After 24 hours of prelabeling, the ^{3}H-amino acid labeled bottles were infected with JE(M1/311) according to previously described methods (Annual Report FY 1973). The infection was allowed to continue in the presence of the labeled amino acids for 4 days. At the time of harvest, the ^{14}C-labeled uninfected culture fluids were mixed with the ^{3}H-labeled infected fluids and processed together through the following purification scheme. The fluids were first clarified at 800 x g for 10 min and then at 3,500 x g for 30 min. This supernatant was then centrifuged at 136,000 x g for 140 min. This supernatant was discarded and the pellet resuspended in 0.5 ml of 0.02 M tris (hydroxymethyl) amino methane, 0.15 M NaCl, 0.001 M EDTA, pH 8.2 (TNE 8.2).

The resuspended pellet was sonicated for 1 min in a 10 Kc-Raytheon Sonic Oscillator, clarified at 800 x g for 10 min and then layered onto a 4.8 ml linear 5-30% sucrose gradient with a 0.2 ml pad of 70% sucrose (all sucrose solutions are w/v and made in TNE 8.2). The gradient was centrifuged at 50,000 rpm for 40 min in the Beckman SW 50L rotor. Fractions of 0.2 ml were collected from the bottom and 20 μl of each fraction was assayed for radioactivity. Hemagglutinating activity of each fraction was determined, using 25 μl. The results are shown in Fig. 7-A. No peaks of radioactivity over the background of ^{14}C labeled uninfected control were found in the ^{3}H labeled infected material; however, hemagglutinin peaks were found at fractions 2, 10, and 21. These peaks were diluted and layered on to 5.0 ml linear 20-70% sucrose-D_2O gradients and centrifuged in the SW 50L rotor for 15 hrs at 50,000 rpm (Figs. 7-B and 8). Once again, the radioactive profiles of infected and uninfected cells closely paralleled each other. Polyacrylamide gel electrophoresis (PAGE) was performed on the hemagglutinin peaks from all of the gradients. The gel profiles of infected versus uninfected preparations were similar; no significant increase in radioactivity of the infected material could be observed. Although we could get good yields of hemagglutinin and recover infectivity from the gradients associated with hemagglutinating peak (data not shown) the degree of purity was low and the efficiency of labeling very poor. More information on the labeling properties of these cells has to be obtained before meaningful experiments can be designed.

2. Comparison of uptake and incorporation of radioactive amino acids by LLC-MK_2 and Culex tritaeniorhynchus cells

Because of the problems encountered in the preparation of radiolabeled virus from mosquito cells, their ability to take up and incorporate radioactive precursors was compared with that of LLC-MK_2 cells. A minimal media containing no serum or unlabeled amino acids but containing 1 μCi/ml of ^{3}H-amino acids was added to each of 7 flasks of LLC-MK_2 cells, and 5 flasks of Culex cells. At specified times after isotope addition, a sample of the labeled culture fluid was removed. The cell sheets were washed with normal saline and 1.0 ml of 1% sodium lauryl sulfate in 0.01 M phosphate buffer was added. Both the culture fluid and the cell sheets were frozen until the time of analysis, when aliquots of the culture fluid were assayed for radioactivity. The cell sheets were boiled for 5 min to decrease viscosity and then aliquots were assayed for both total and trichloroacetic acid (TCA) precipitable radioactivity. The protein content of all samples was determined by the method of Lowry (1951).

By dividing the TCA precipitable radioactivity by the radioactivity of the culture fluids, incorporation could be measured. Uptake was determined by division of total counts in the extract by total counts in the culture fluids. Samples were corrected to a constant amount of protein and the data is presented in Fig. 9. Total uptake by Culex cells over a 2 hr period was only 20% of that of LLC-MK_2 cells.

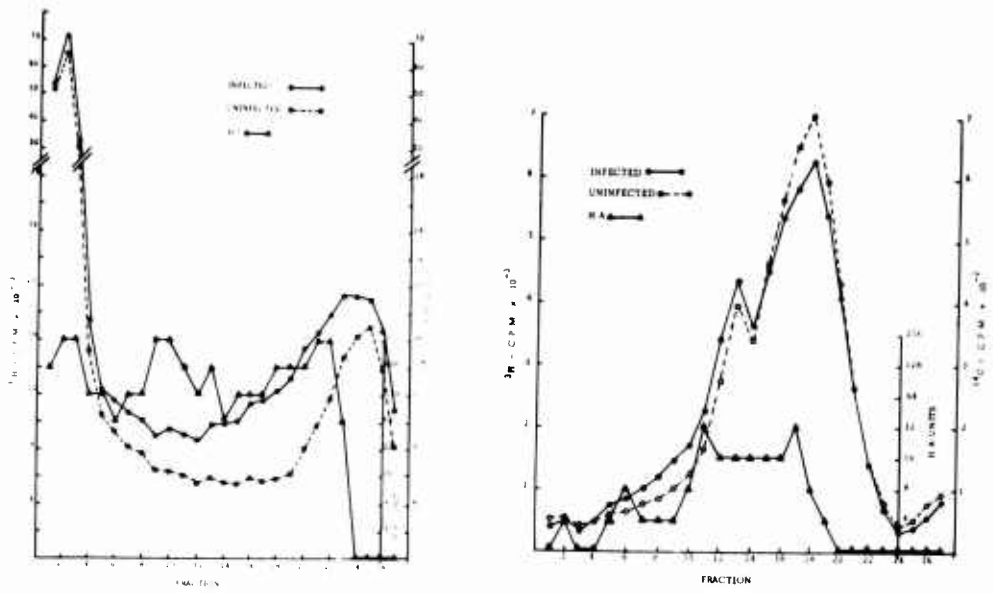

Figure 7. A. Rate zonal sucrose gradient centrifugation of culture fluids from infected and uninfected Culex tritaeniorhynchus cells. B. Isopycnic sucrose gradient centrifugation of fraction 2 from the original rate zonal gradient.

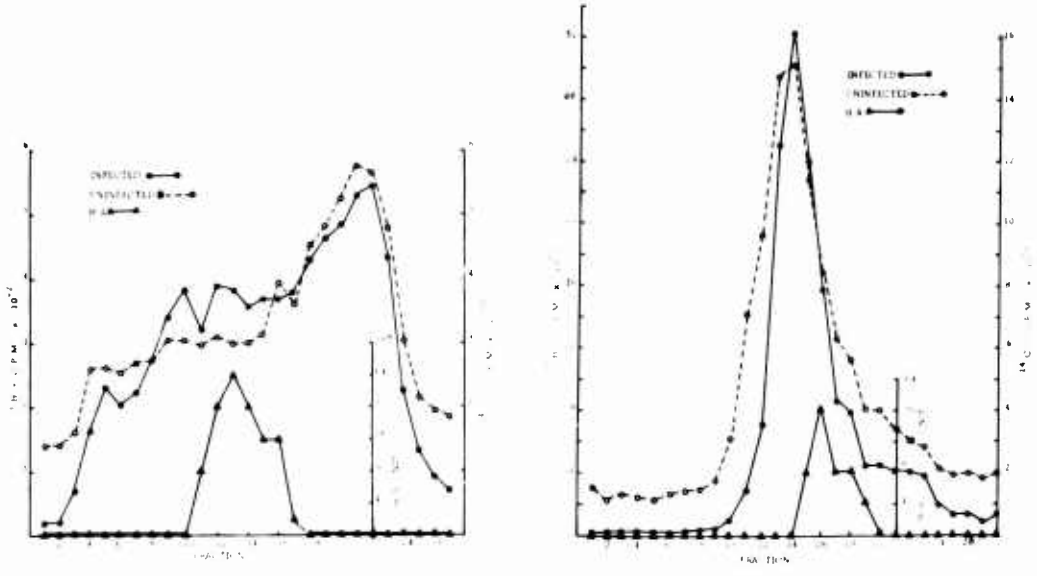

Figure 8. A. Isopycnic sucrose gradient centrifugation of fraction 10 from the original rate zonal gradient. B. Isopycnic sucrose gradient centrifugation of fraction 21 from the original rate zonal gradient.

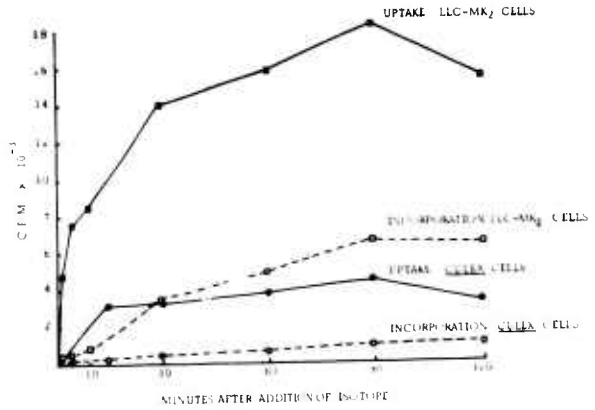

Figure 9. Comparison of the ability of LLC-MK_2 and Culex tritaeniorhynchus cells to take up and incorporate radioactive amino acids.

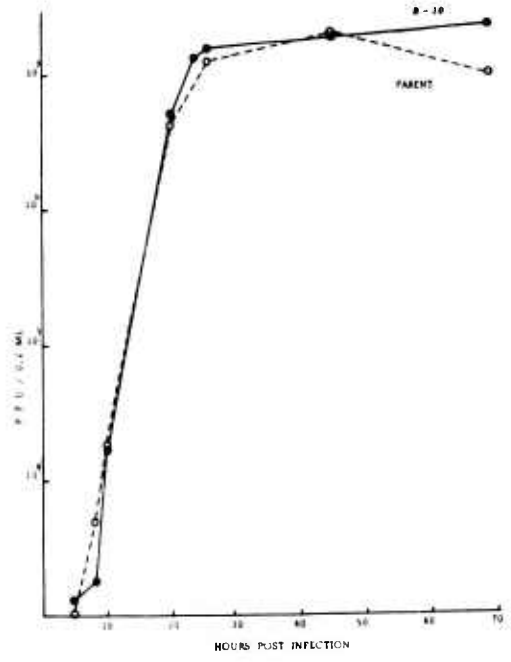

Figure 10. Comparative growth curves of Japanese encephalitis virus in the parent line of Culex tritaeniorhynchus cells and the clone, B-10.

Furthermore, LLC-MK_2 cells incorporated 40% of what they took up, whereas Culex incorporated only 30%. Overall, it appears that poor uptake is the major reason for inefficient labeling of mosquito cells. This is suggestive of either the presence of a very large intracellular pool or requirements for specific amino acids, or both. Studies to investigate these possibilities are currently underway.

3. Detection of infected mosquito cells by the fluorescent antibody technique

The indirect fluorescent antibody (FA) technique was used to determine the percentage of infected cells in a culture of Culex tritaeniorhynchus that had been infected with a high MOI of JE (M1/311). Cells were grown on glass coverslips and infected as previously described (Annual Report FY 1973). The infection proceeded for 30 hours at which time the cells were fixed and stained for FA as previously described (Cardiff, 1973)

By this method 3-10% of the cells fluoresced. This low percentage of cells showing signs of virus replication could represent another factor in the difficulty of obtaining purified radiolabeled virions from mosquito cells. It may be possible to overcome this problem through the use of metabolic inhibitors such as actinomycin-D or cycloheximide. Studies of this nature have not yet been performed. If the susceptibility of the cells to infection is genetically based, the problem could be approached by isolating a genetically pure subpopulation of the cells with a higher susceptibility to infection. Such cloning experiments are described in the next section.

4. Cloning of Culex tritaeniorhynchus cells

Cloning of Culex tritaeniorhynchus cells was accomplished by plating about 5,000 cells in a 60 mm petri plate. To promote growth at such low cell densities, 50% Hsu's media containing 10% FBS and 50% conditioned Hsu's media with 10% FBS was used. Cells observed microscopically daily, were allowed to grow for several days until distinct clones of about 100 cells of the same morphology were formed. At this time the clones were withdrawn using fine glass capillaries and replated into small microtiter wells (96 hole plates) in about 0.1 ml of 50% normal, 50% conditioned media. These cells were allowed to grow for several weeks at which time those that survived were plated into larger wells (24 hole plates). These were then transferred to tubes and finally flasks. Initially 21 clones were picked. Only one, B-10 (so designated by its position in the original microtiter plate), survived and is now in its 14 passage.

A growth curve of JE(M1/311) in B-10 versus the parent line of Culex is shown in Fig. 10 (previous page). Although B-10 is morphologically homogeneous and exhibits some cultural characteristics different from the parent line, i.e., better adherence to the growth

surface, growth of JE in the two cell populations appears identical. Further studies to test for susceptibility to infection and labeling characteristics of the cells are now in progress.

G. Further studies on the effects of metabolic inhibitors on cells infected with Flaviviruses

Fractionation of Japanese encephalitis (JE) virus-infected chick embryo cells into smooth and rough membranes revealed a quantitative difference in the virus-specified polypeptides present in these fractions, the most significant difference being the increased amount of NV-5 in the smooth membrane fraction (Annual Report 1971). These studies were performed with cells inhibited with both actinomycin-D and cycloheximide, and which were labeled during the late logarithmic phase of the replication cycle, using previously described methods (Shapiro, 1971). In order to obtain a sufficiently radioactive preparation to permit analysis, a four-hour labeling period was used, thus the observations we have made pertain to a static system. To be able to trace the flow of polypeptides through the cell by pulse chase experiments, it is necessary to develop drug treatments which will effectively inhibit cellular protein synthesis while at the same time allow better incorporation of radioactive precursors into viral-specified polypeptides. Preliminary experiments of this type are described below.

1. Effect of actinomycin-D on protein synthesis in chick embryo cells

In the following experiment the effect of a two-hour pulse of actinomycin-D at a concentration of 1 μg/ml was measured over a forty hour period. Monolayers of primary chick embryo cells were grown in 25 cm^2 plastic flasks. One set of flasks received a 2-hour pulse of actinomycin-D (1 μg/ml). After the pulse, they were washed with five 2 ml aliquots of normal saline and 5 ml of medium 199 with 1/10 the normal concentration of amino acids and 2% FBS were added to each flask. One set of flasks received no actinomycin-D but otherwise were treated identically, while another set received no drug and were not washed. At specified times after the removal of the drug one flask of each set received 2 μCi/ml of ^3H-amino acids for a period of 1 hr, at which time the flask was washed twice with 5 ml of normal saline. The cell sheets were then dissolved with 1.0 ml of 1% sodium lauryl sulfate in 0.01 M phosphate buffer and frozen until the time of analysis. When the experiment was completed the cell sheets were boiled for 5 min. Acid-precipitable radioactivity was assayed, using two 50 μl aliquots of each extract. Protein determinations were made for each sample, using the method of Lowry (1951). Results were expressed as acid-precipitable counts per minute per μg of protein (Fig. 11). Washing stimulated protein synthesis in chick embryo cells, with the washed controls showing about 4 times the specific activity of their unwashed counterparts, forty hours after washing. The actinomycin-D treated cells showed a

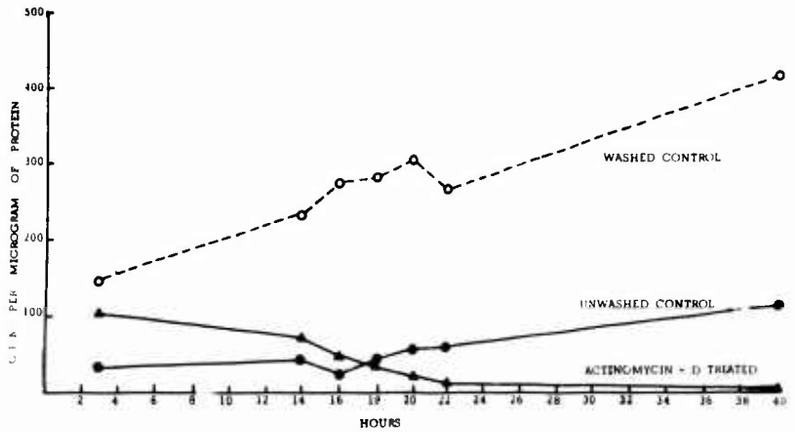

Figure 11. Effect of a two hour pulse of actinomycin-D on protein synthesis in chick embryo cells.

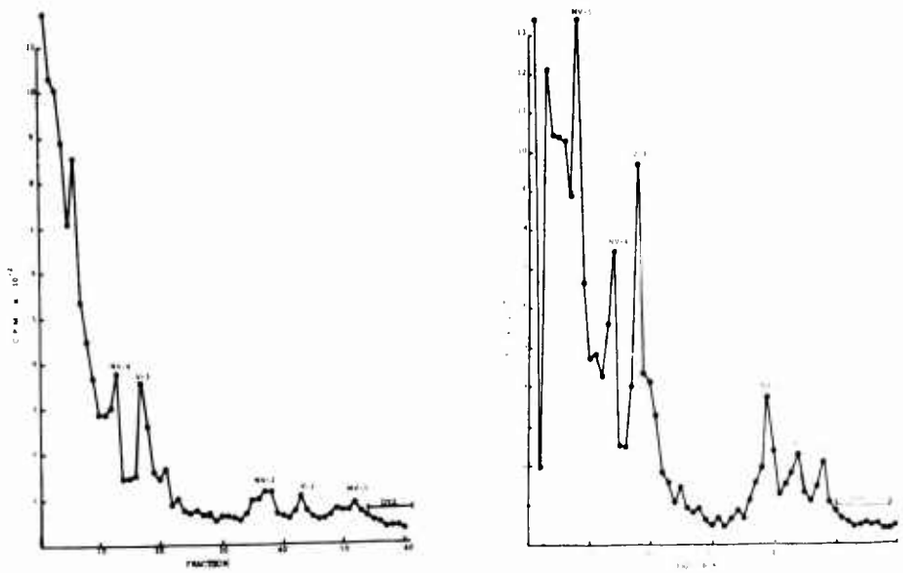

Figure 12. PAGE of Japanese encephalitis virus-infected chick embryo cell extracts. A. Pretreated with actinomycin-D for two hours prior to infection. B. Treated with actinomycin-D from 4 to 6 hours after infection.

steady decline in specific activity. By 22 hours after drug treatment, the drug treated cultures showed about 20% of the specific activity of the unwashed controls and less than 5% of that of the washed controls. By 40 hours, the specific activity of the actinomycin-D treated cells was < 5% of the unwashed controls and < 1% of the washed controls. These results imply that a 2-hour pulse of actinomycin-D (1 μg/ml) is sufficient to cause severe inhibition of protein systhesis in chick embryo cells.

2. Effect of time of addition of actinomycin-D on the synthesis of proteins in JE-infected chick embryo cells

Monolayers of chick embryo cells were grown in 25 cm^2 plastic flasks. They were infected by previously described methods (Shapiro, 1971). At specified times cells received a 2-hour pulse of 1 ug/ml of actinomycin-D which was removed by washing with five 2 ml aliquots of normal saline. All flasks were labeled with a 2-hour pulse of 20 uCi/ml from 24-26 hours after infection. At the end of the labeling period the cell sheets were washed, dissolved in 1% sodium lauryl sulfate in 0.01 M phosphate buffer, boiled in 1% 2-mercapto ethanol and subjected to PAGE as previously described (Shapiro, 1971).

Results are shown for cells treated with actinomycin-D for 2 hours before infection, or from 4-6 hours, 6-8 hours, or 16-18 hours post infection (Figs. 12 and 13). In the pretreated flask, viral proteins are present but levels of radioactivity are low, and the high molecular weight region of the gel is obscured. Somewhat better incorporation is seen in cells receiving the 4-6 hour post infection actinomycin-D treatment, but the high background in the high molecular weight region is still present. Gel patterns of the cells treated with actinomycin-D from 6-8 hours after infection are similar but with slightly better incorporation.

When cells treated with actinomycin-D from 16-18 hours post infection were co-electrophoresed with intracellular proteins prepared by treatment with both actinomycin-D and cycloheximide (Shapiro, 1971) virtually identical gel patterns were obtained. The level of radioactivity incorporated by the cell receiving the 16-18 hour drug treatment was > 5-fold higher than the cells that had been treated with actinomycin-D from 6-8 hours after infection. These results suggest that early treatment with actinomycin-D may permit the study of viral polypeptides synthesized during the latent period of the viral replication cycle, which could not be examined with previous drug treatments. Secondly, the ability to get high levels of radioactivity incorporated, using the 2-hour pulse of actinomycin-D from 16 to 18 hours post infection, may make possible pulse chase experiments. However, it still remains to be shown that the background of host cell proteins in uninfected controls is sufficiently low to permit this.

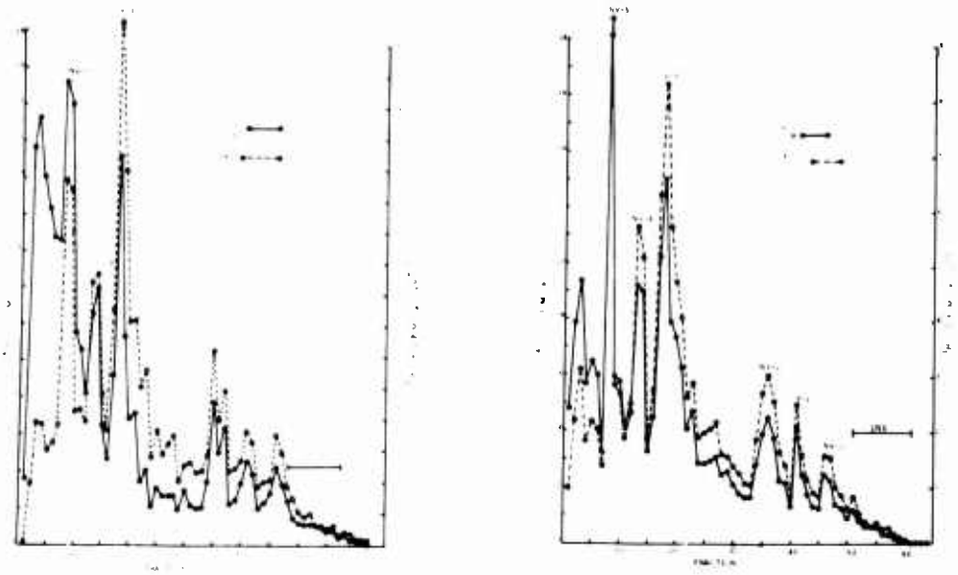

Figure 13. Co-electrophoresis of ^{14}C-labeled Japanese encephalitis virus-specified marker polypeptides with ^{3}H-Japanese encephalitis virus-infected chick embryo cell extracts. A. Treated with actinomycin-D from 6-8 hours after infection. B. Treated with actinomycin-D from 16-18 hours after infection.

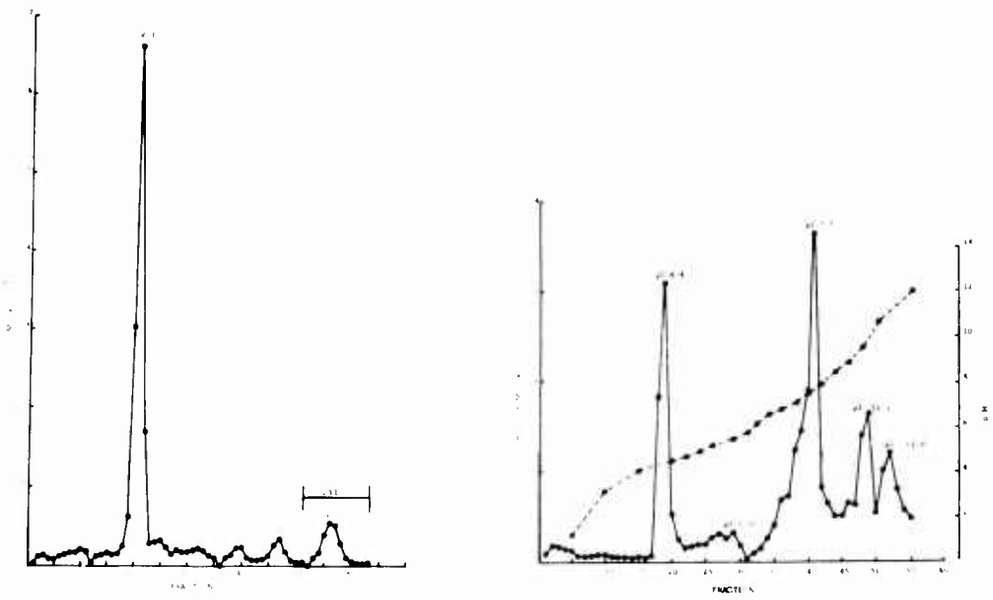

Figure 14. A. PAGE of purified Japanese encephalitis virus. B. Isoelectric focusing column of purified Japanese encephalitis virus.

H. Isoelectric focusing of Japanese encephalitis virus proteins

1. Virion proteins

Complete separation of Sindbis virion proteins by isoelectric focusing permitted the absolute identification of two glycoproteins previously detected only by methods of discontinuous PAGE. These studies suggested that this method might be useful to confirm or extend the number of structural proteins in group B arboviruses, using Japanese encephalitis virus (JEV) as a model. Initial experiments were performed using purified JEV from 5-25 percent sucrose gradients as described in the 1971 Annual Report, and then using isoelectric focusing procedures identical to those described for Sindbis virus (1972 Annual Report). At the time of these experiments, purified preparations of JEV exhibited a slightly altered polypeptide pattern by polyacrylamide gel electrophoresis (PAGE); the nucleocapsid protein V-2 was greatly reduced in radioactivity (Fig. 14-A, previous page). The ratio of radioactivity and migration of V-3 (the major coat protein) and V-1 (the small envelope protein) was typical of JEV and other group B arboviruses (shown in the 1971 and 1972 Annual Reports). Since other virus characteristics appeared unaltered (sedimentation rate, hemagglutinin, infectivity), initial experiments utilized these V-2 deficient virion preparations as starting material.

Disruption of ^{3}H-amino acid labelled virion by gentle mixing in 1 percent Triton X-100 was followed by electrofocusing at 850 volts for 48 hours in a sucrose gradient containing 0.1 percent Triton X-100 and ampholytes to yield a gradient from pH 3 to pH 10. The distribution of radioactivity from a typical experiment is shown in Fig. 14-B, previous page. The pH gradient was essentially linear over the range pH 3 to pH 10 and four separate radioactive peaks could be readily identified. The four peaks were tentatively assigned isoelectric points of pH 4.4, pH 7.7, pH 10.2, and pH 11 (approximately) from left to right, or anode to cathode. Peak fractions were heated in sodium dodecyl sulfate and 2-mercaptoethanol and analyzed by PAGE for identification. The pI 4.4 fraction (Fig. 15-A) contained both V-3 and V-1 as well as some radioactivity that did not enter the gel and a small peak of large molecular weight protein that was possibly an aggregate of one of the structural proteins. The V-3/V-1 ratio appeared less than one as opposed to the value of 10 normally observed in JEV purified virions. These data suggest an intimate association of these two peptides that is not disrupted by gentle treatment with non-ionic detergent and isoelectric focusing. The gel of the pI 7.7 peak showed only the V-3 polypeptide (Fig. 15-B). Repeated experiments have always yielded a peak near pH 7.7 and PAGE has consistently identified only V-3 without contamination. The pI 10.2 fraction was shown by PAGE to contain a trace of V-3, but principally a broad irregular peak that covered the range of both V-2 and V-1, making interpretation difficult (Fig. 15-C). Since radioactivity was low in

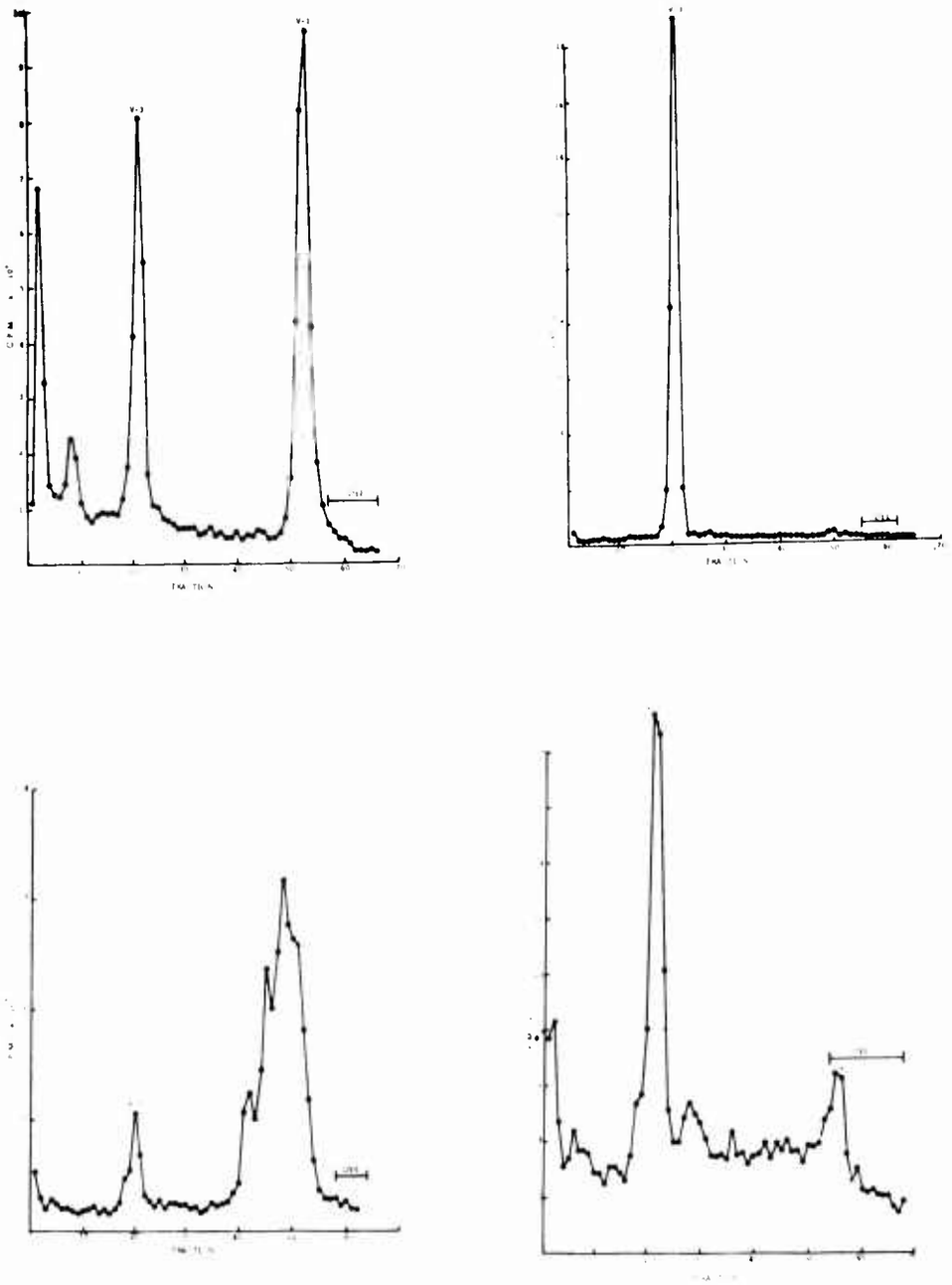

Figure 15. PAGE of peaks from the isoelectric focusing column. A. pI 4.4 peak. B. pI 7.7 peak. C. pI 10.2 peak. D. pI 11 peak.

these preparations, it is possible that this fraction represents the small amount of V-2 that was present in the starting material. The peak at approximately pH 11 (Fig. 15-D, previous page), consisted of V-3 and a small amount of V-1 resembling the profile of the starting material. In a single experiment we have tentatively identified the isoelectric point of undegraded JEV at pH 5.2 to pH 5.5, making an interpretation of undegraded virion for the pH 11 peak untenable.

Subsequent to the initial studies using V-2 deficient preparations of JEV, modification of the concentration and purification procedures have resulted in preparations of JEV with the "normal" polypeptide composition (Fig. 16-A). A single preparation of JEV that had been purified by pelleting the virions from the radioactive culture medium at 78,000 x g (average) for 3 hours (instead of 136,000 x g for 2 hrs) and rate zonal centrifugation in 5-35% sucrose gradients was subjected to isoelectric focusing. In an attempt to resolve the question of the association of V-3 and V-1 in the pH 4.4 peak previously shown for other JEV preparations, the detergent disruption procedures were changed to include 5% Triton X-100 for one hour at room temperature and vigorous vortex mixing at 10 minute intervals prior to electrofocusing. The distribution of radioactivity in this electrofocusing experiment is shown in Fig. 16-B. The pH gradient was not linear over the range pH 3 to pH 10 as intended; however, protein precipitation occurred and a protein "overload" could account for the distortion of the pH gradient. (Similar pH gradients have been observed in experiments with dengue SCF antigens containing high concentrations of mouse brain proteins and visible precipitation in the column). A sharp peak of radioactivity was observed at pH 4.06. A peak in fraction 39 with a "shoulder" at fraction 43 was also observed; however, isoelectric point determinations could not be made because of the irregular pH gradient. The pI 4.06 peak was analyzed by PAGE and the gel profile illustrated in Fig. 17-A. Although some radioactivity again failed to enter the gel and a trace of large molecular weight protein was observed, the V-3 and V-1 association was no longer present. These samples have not been co-electrophoresed with marker polypeptides, but this polypeptide has been tentatively identified as V-1. PAGE of the fraction 43 material from the pH 7 region of the gradient is shown in Fig. 17-B. As previously demonstrated, the V-3 glycopeptide was observed with little or no contamination with other polypeptides. Other fractions from the electrofocus gradient could not be resolved by PAGE.

On the basis of these preliminary observations, we feel that at least the V-3 and V-1 polypeptides of JEV can be separated by isoelectric focusing. The isoelectric points of these proteins can be tentatively identified as pH 4-5 for V-1 and pH 7-8 for V-3. It would also appear from these experiments that isoelectric focusing does not result in the separation of two envelope glycoproteins as described for Sindbis virus, a critical question in terms of location of group and type specific antigenic determinants of the virion. The antigenic characterization of V-3 and V-1, as well as the resolution of the V-2 popypeptide, are under study.

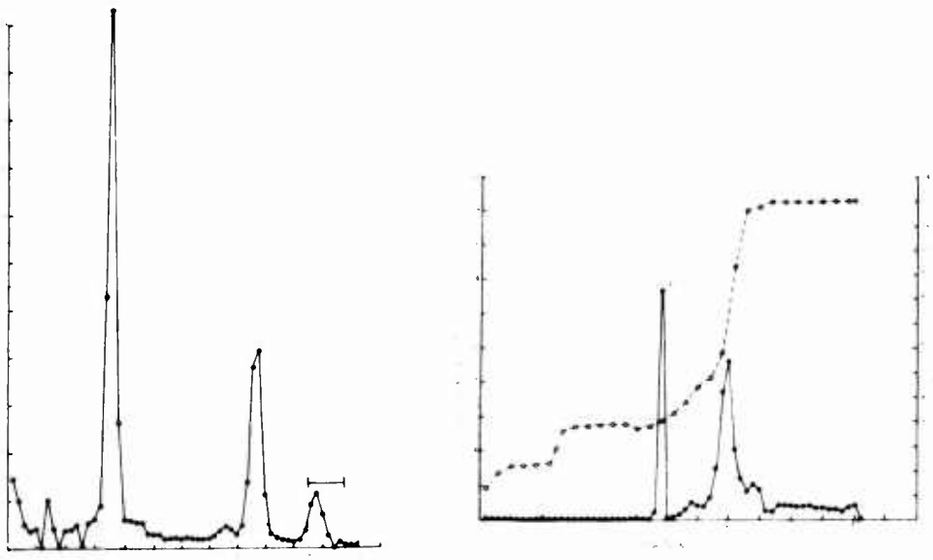

Figure 16. A. PAGE of purified Japanese encephalitis virus. B. Isoelectric focusing column of purified Japanese encephalitis virus.

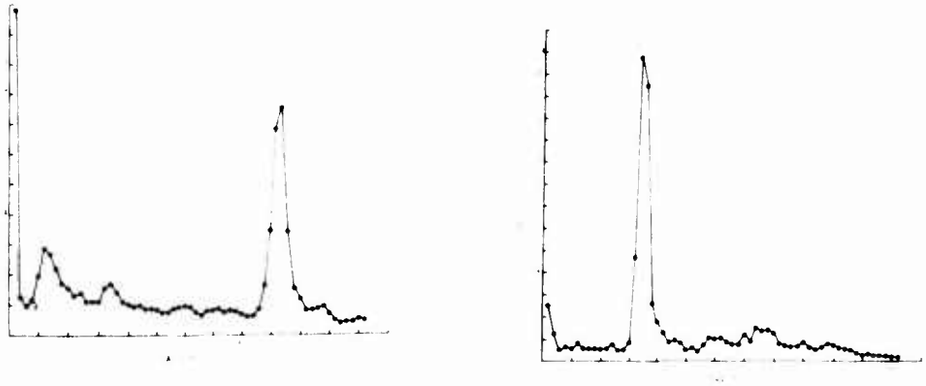

Figure 17. PAGE of peaks from the isoelectric focusing column. A. pI 4.06 peak. B. pI 7-8 peak.

2. Isoelectric focusing of Japanese encephalitis virus-specified polypeptides from chick embryo cell extracts

Two roller bottles of chick embryo cells were infected with JEV(M1/311) at an MOI of 10. They were adsorbed for 90 min at 37°C. At 9 hours post infection, actinomycin-D was added to each to a final concentration of 1 μg/ml. At 18 hours post infection cycloheximide was added to a final concentration of 300 μg/ml. Thirty minutes later the cells were washed extensively, and 33 μCi/ml of ^{3}H-amino acids were added to each bottle; actinomycin-D was also readded. At 24 hours post infection the cells were scraped off the bottles with a rubber policeman, into 10 ml of 1% Triton-X 100 in Dulbecco's phosphate buffered saline. They were then homogenized in a Sorvall omnimixer for 30 seconds at full speed. The homogenate was spun at 800 x g for 10 min to remove nuclei. The supernatant was dialysed overnight against 2 liters of distilled water. After dialysis, the homogenate was clarified at 800 x g for 15 min and 7 ml were applied to the electrofocusing column containing a pH 3-10 gradient in 0.1% Triton-X 100. An aliquot of 0.2 ml was saved for PAGE.

Results of the focusing column are shown in Fig. 18-B. Prominent peaks occur at the following pH's: 5.60, 6.89, 8.30, 9.33, and 11.39; with minor peaks at: 4.03, 4.34, 7.48, 8.02, 11.22, and 11.37. All of the peaks were boiled for 10 min in 1% sodium lauryl sulfate, 1% 2-mercapto-ethanol and co-electrophoresed with a ^{14}C marker of JE-intracellular polypeptides on 8% polyacrylamide gels, as previously described (Shapiro, 1971).

PAGE of the starting material (10% gel) co-electrophoresed with ^{14}C JE intracellular marker, showed an aberrant pattern (Fig. 18-A). The two largest non-structural polypeptides, NV-5 and NV-4 are absent, V-3 is reduced, and prominent peaks in the area of NV-3, NV-X are present. A large amount of small molecular weight material obscures the region of the gel where NV-2 1/2, NV-2, V-2, and NV-1 are usually found. Such a result could have been caused by enzymatic digestion in the presence of Triton-X 100 during processing, but attempts to reproduce this pattern have failed.

Most of the peaks from the electrofocusing column gave multiple protein peaks on gels. Peaks at pH-s 4.03, 4.34, 8.30 and 11.39 gave a series of unresolvable protein peaks on gels. Still other peaks at pH's: 7.48, 9.33, and 11.22, qualitatively resembled the ^{14}C marker, suggesting to us that these were pieces of virus modified cellular membranes that had not dissociated under the conditions we used. The peak at pH 5.60 yielded three protein peaks upon electrophoresis, tentatively identified as NV-3, NV-X, and another migrating at about the same place as V-1 usually does (Fig. 19-A). The peak at pH 5.60 was refocused to a single peak at pH 5.8 (PAGE) of the refocus peak confirmed its composition (Figs. 19-B & C). Since the two peaks of "NV-X" and "V-1" usually absent in infected cell extracts were highly

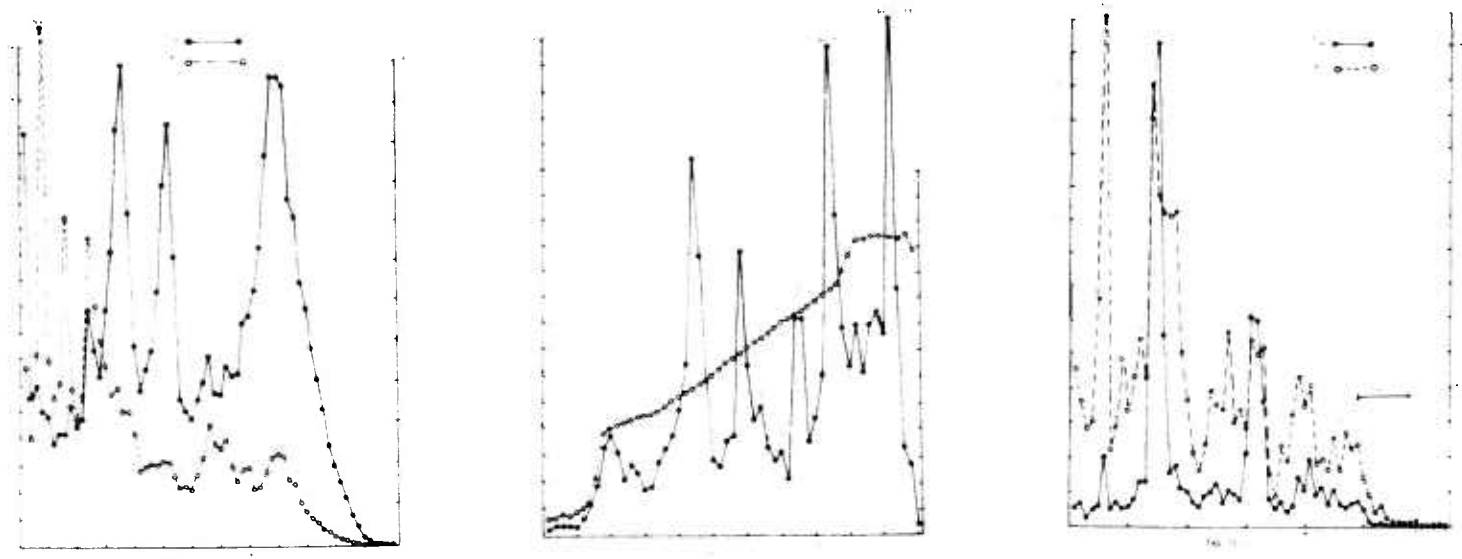

Figure 18. A. Co-electrophoresis of ^{14}C-labeled Japanese encephalitis virus-specified marker polypeptides with ^{3}H Japanese encephalitis virus-infected chick embryo cells that had been dissociated with Triton-X 100. B. Isoelectric focusing column of Japanese encephalitis virus-infected chick embryo cells dissociated with Triton-X 100. C. pI 8.02 peak from the isoelectric focusing column.

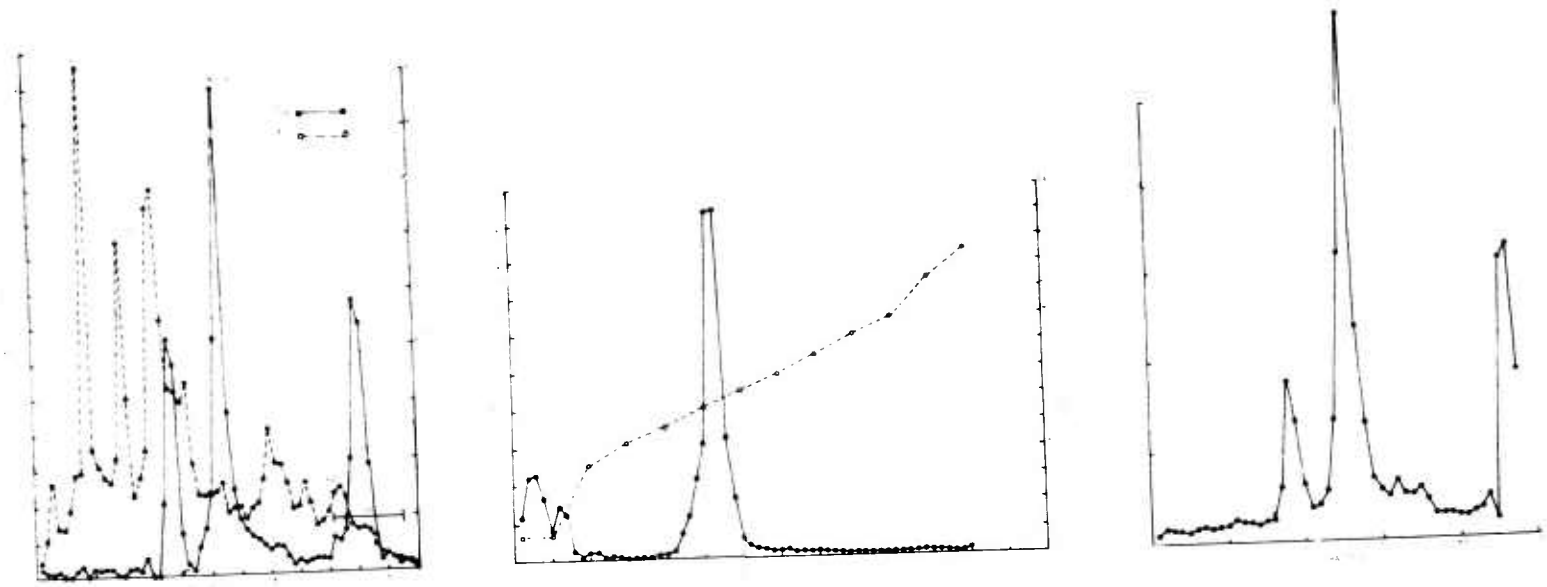

Figure 19. A. Co-electrophoresis of ^{14}C-labeled Japanese encephalitis virus-specified marker polypeptides with pI 5.6 peak from the isoelectric focusing column. B. Refocus of the pI 5.6 peak. C. PAGE of the refocused peak.

prominent in our starting material, it is possible that these peaks may be artifacts.

PAGE of the material at pH 6.89 in the original column yielded a single peak which co-migrated with NV-3 (Fig. 20-A). The original focusing peak could be refocused to pH 7.2 and PAGE of the refocused peak again yielded NV-3 (Figs. 20-B & C). PAGE of the peak at pH 8.02 on the original column revealed V-3 and NV-2 (Fig. 18-C), but there were not sufficient counts to permit refocusing.

These preliminary attempts to isolate intracellular polypeptides from cellular extracts by isoelectric focusing point out several problems: 1) For unknown reasons an aberrant gel pattern was obtained from the starting material, making interpretation difficult. Subsequent experiments have shown that it is possible to obtain normal distribution of the virus specified polypeptides following disruption with Triton-X 100; 2) Some other methods must be worked out to permit more adequate disruption of virus modified cellular membranes if single polypeptides are to be obtained.

I. Characterization of mouse peritoneal lymphocytes for studies of virus-induced cell mediated immunity

1. General properties of normal peritoneal lymphocytes

It has been well established that the uninflamed or "normal" peritoneal cavity of most rodents (Shelton et al., 1970) and man (Dixon et al., 1934) contains a large number of cells with the morphologic characteristics of lymphocytes. In contrast to lymphocytes from other sources, relatively little is known about lymphocytes from the normal peritoneal cavity (hereinafter designated normal peritoneal lymphocytes, or NPLs). Some of the characteristics which have been attributed to NPLs, however, suggest that they represent a unique subpopulation of lymphocytes.

The following are examples of several unusual properties of NPLs: a) Freshly isolated suspensions of NPLs (in all the studies to be described in this section, peritoneal lymphocytes have been "purified" from peritoneal macrophages by some variant of selective adherence techniques) bind much more antigen in the antigen binding reaction than any other population of lymphocytes studied (Ada, 1970); b) When compared with suspensions of splenic lymphocytes, NPLs took up larger amounts of soluble protein by pinocytosis (Mandel et al., 1970; Catanzaro and Graham, 1970); c) Mouse NPLs, when placed in cell impermeable chambers in the peritoneal cavity of a rat, with time gave rise to cells with morphologic characteristics of macrophages, plasma cells and fibroblasts (Shelton et al., 1959); d) If NPLs were cultured in vitro, they acquired the ability, with time, to phagocytose carbon and latex (Vernon-Roberts, 1969; Catanzaro and Graham, 1974).

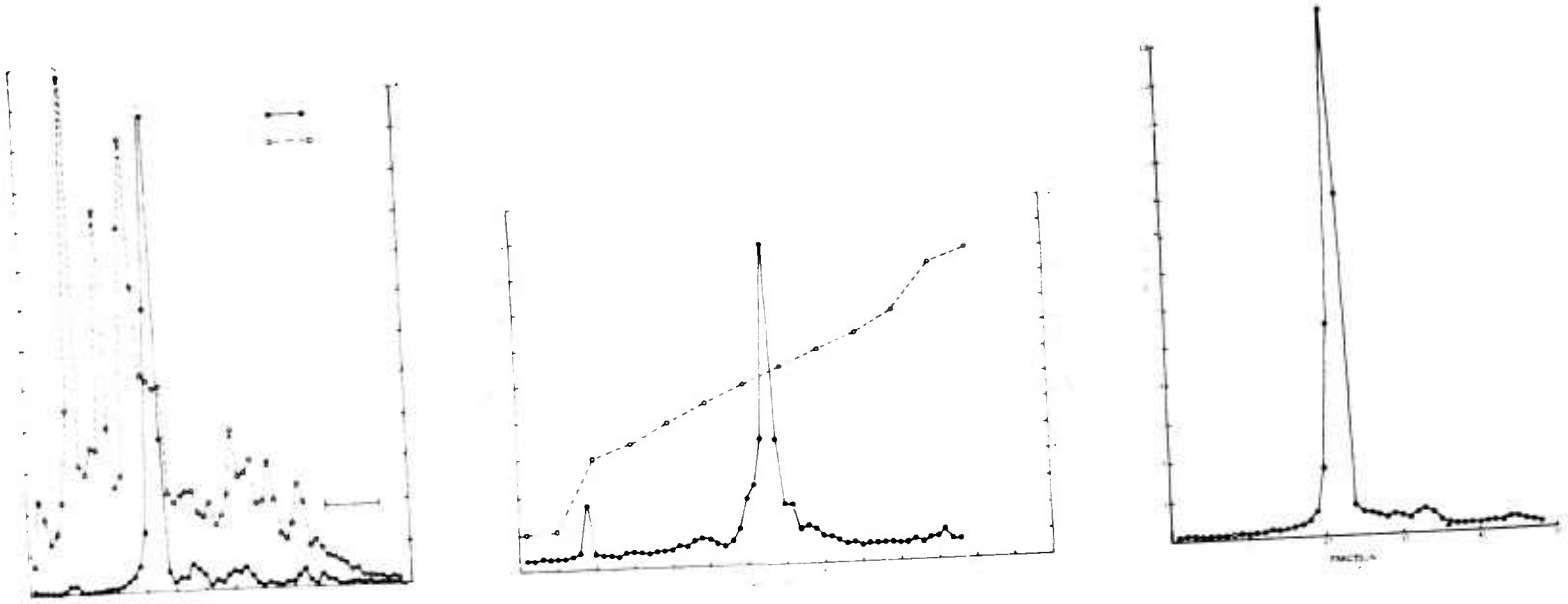

Figure 20. A. Co-electrophoresis of ^{14}C-labeled Japanese encephalitis virus-specified marker polypeptides with pI 6.89 peak from the isoelectric focusing column. B. Refocus of the pI 6.89 peak. C. PAGE of the refocused peak.

When such "transformed" cells, containing phagocytized carbon particles, were injected intraperitoneally into syngeneic recipients, they could be detected subsequently at distant sites of experimentally induced inflammation (Vernon Roberts, 1969); e) Among NPLs were cells capable of synthesizing a 19S hemolysin in response to sheep erythrocytes in vitro without an initial proliferative phase, i.e., antibody was synthesized even though colchicine was present in the culture (Bussard et al., 1967, 1970); f) Finally, NPLs have a unique traffic pattern when injected intravenously, homing only to spleen and not to lymph nodes; in contrast, similarly injected spleen and lymph node cells migrate to both sites (Gillette and Lance, 1971, 1972; Gillette et al., 1973).

Thus, it seemed to us useful to explore in more detail the immunologic and general properties of NPLs. In this regard, we have compared such data with results obtained with more familiar and better characterized lymphoid populations, splenic lymphocytes (SpLs), and where appropriate, with peritoneal exudate lymphocytes (PELs). PELs were obtained from an exudate induced by the intraperitoneal injection of an irritant, and differ at least in that respect from NPLs harvested from the undisturbed or normal peritoneal cavity. In the studies reported here, we have investigated the presence of cell surface immunoglobulin with fluorescent antibody; the response to the T-cell mitogen, concanavalin A (CON A); the proliferative response to allogeneic histocompatibility antigens in mixed lymphocyte cultures; and the cellular metabolic activity as measured by glucose metabolism.

Materials and methods

Conventionally maintained female BALB/c and or (BALB/c x A)F1 mice (Jackson Memorial Laboratories, Bar Harbor, Maine) 12 to 20 weeks old were used according to the experimental situation.

Cells were harvested with RPMI 1640 (GIBCO, Grand Island, NY) containing hepes buffer (0.006M; Calbiochem, San Diego, CA) and heparin (10 units/ml). In the case of spleen cells, bovine plasma albumin (Calbiochem), 2% (w/v) was added to the harvest medium. Post-harvest washing procedures employed the harvest medium without heparin or added protein. Medium for cell culture contained heat inactivated (56°C for 45 minutes) fetal calf serum (GIBCO), 10%-20% (v/v) and a commercially prepared antibiotic-antimycotic mixture (100X; GIBCO) at a concentration of 1% (v/v).

Peritoneal exudate cells were harvested 3 days after the intraperitoneal injection of 2 ml of sterile light mineral oil, and normal peritoneal cells were obtained from the previously undisturbed peritoneal cavity. Mice were anesthetized with ether and exsanguinated by incision of the right axillary artery. Following reflection of the peritoneal skin, 3 ml of warm harvesting medium were injected intraperitoneally. The abdomen was gently massaged for 1 minute, the peritoneum incised and the cell rich fluid aspirated under sterile conditions

with a Pasteur pipette. Spleen cell suspensions were prepared by teasing minced spleen fragments, obtained from spleens removed aseptically from anesthetized and exanguinated mice, through 60 mesh stainless steel screens into the harvesting medium. The cells were filtered through sterile gauze to remove large clumps and slowly drawn through a 25 gauge needle to produce a suspension of single cells. The suspensions from several animals were pooled in 50 ml plastic centrifuge tubes (Falcon Plastics, Los Angeles, CA) and centrifuged for 10 minutes at 180 x g. The cells were washed twice and resuspended in a medium appropriate for the particular experiment. Viability was determined by exclusion of trypan blue.

To accomplish separation into adherent and nonadherent cells, cells were resuspended in culture medium and pipetted into plastic T flasks, (75 cm^2; Falcon Plastics). The flasks were maintained tightly capped and stationary at 37°C for 3 hours. At the end of this period, the flasks were tilted and the cell rich fluid which drained to the bottom was collected. The monolayer was gently rinsed twice with RPMI 1640 and the wash pooled with the above. Those cells which had not attached to the plastic surface at the end of this period were designated non-adherent cells. The adherent cells were usually discarded. However, if needed, these were gently scraped from the flask with a rubber policeman.

Fluorescent microscopy: Nonadherent lymphocytes were washed 3 times in ice cold Hank's balanced salt solution (HBSS, Microbiological Associates, Bethesda, MD). A suspension of 1.5 x 10^6 cells in 0.2 ml of HBSS was mixed with an equal volume of a 1:5 dilution of fluorescein-conjugated rabbit anti-mouse-γ-globulin (Cappel Laboratories, Downingtown, PA) or goat anti-mouse-γ-globulin (Antibodies, Inc., Davis, CA). The specificity of the antisera was established by immunoelectrophoresis against whole BALB/c serum (Jackson Memorial Laboratories). The suspension was maintained in an ice bath for 30 minutes and then washed 3 times with ice cold HBSS. A drop of the suspension was mixed with phosphate buffered glycerine and mounted on a glass slide with a cover slip. The suspensions were examined with a Leitz fluorescence microscope equipped with an FITC and BG23 excitor filter and a barrier filter 50. Controls included incubation with fluorescein-conjugated rabbit anti-mouse albumin (Cappel Laboratories) and specific inhibition of fluorescence with unconjugated rabbit or goat anti-mouse - γ-globulin (Meloy Laboratories, Springfield, VA).

Cells were incubated with 1 uCi of glucose-1-^{14}C (New England Nuclear, Boston, MA) for determination of glucose utilization (Stjernholm, 1967). Incubations were carried out in siliconized 50 ml center well Erlenmeyer flasks containing 28-40 x 10^6 leukocytes in 5 ml of routing culture medium (with 20% v/v autologous serum). The flasks were flushed with 5% CO_2/95% O_2 for 3 minutes and placed in a metabolic shaker bath (70 strokes per minute) at 37°C. After 4 hours, the cells were disrupted with 35% perchloric acid. Sodium hydroxide

(carbon dioxide free) was introduced into the center well to absorb the respiratory carbon dioxide. Labelled carbon dioxide in the sodium hydroxide was determined after conversion to barium carbonate. The latter was dispersed in a thixotropic gel powder-toluene scintillation fluid mixture for counting. Radioactivity of all products was determined with a Packard 3320 Scintillation Spectrometer. Results were expressed as μ moles of glucose utilized/hour/10^8 cells.

Routinely, 1.5 million NPLs or SpLs were cultured in 1 ml of TCM containing 5% (v/v) fetal calf serum (heated 56°C for 40 minutes; Grand Island Biological Co.) as described by Stobo et al (1972). To the cultures were added either Concanavalin A (CON A) (prepared by a modification of the method of Agrawal and Goldstein (1965), and kindly supplied by Dr. A. E. Powell), or phosphate buffered normal saline (pH 7.4) only. The cultures were maintained upright in a humified atmosphere of 5% CO_2/95% air at 37°C. Two microcuries of titrated thymidine (2.6 Ci/mM; New England Nuclear, Boston, MA) were added for the final 7 hours of culture. At the end of the culture period, the cells were collected by vacuum filtration through glass fiber filters (Whatman GF/C; WR Balston Ltd., England). The filters were washed twice with ice cold normal saline followed by 2 washes with ice cold 5% trichloracetic acid and finally 2 washes with ice cold methanol. The filters were then dried with radiant heat, dissolved in a toluene based scintillant fluid and the radioactivity measured in a liquid scintillation spectrometer. Results were expressed as the arithmetric mean of quadruplicate samples in counts/minute (cpm) or in terms of a stimulation ratio, i.e., cpm stimulated/cpm control. Additional cultures at 72 hours were centrifuged gently and the resulting pellet resuspended in 0.2 ml of heat-inactivated fetal calf serum. Smears were prepared on glass coverslips, air-dried and stained with Wright's stain. The percentage of blasts was determined using morphologic criteria (Chessin et al., 1966).

Mixed lymphocyte culture: Standard culture tubes contained either 5.5 x 10^6 or 2 x 10^6 nucleated cells in 1 ml of RPMI 1640 with 5% v/v fetal calf serum (heated 56°C for 30 min; Grand Island Biological Co.). In control tubes, BALB/C NPLs or SpLs and (BALB/c x A)F_1 SpLs were cultured alone. Experimental tubes contained (BALB/c x A)F_1 SpLs, the stimulating cell, and either SpLs or NPLs from BALB/c mice in a 1:1 ratio. The cultures were incubated upright in a humidified atmosphere of 5% CO_2 and 95% air at 37°C for up to 5 days. One microcurie of triated thymidine (^{3}H-T dr; 2.6 Ci) mM; New England Nuclear Corp., Boston, MA) was added for the last 8 hrs of culture. The cells were collected with vacuum filtration through glass fiber filters (Whatman GFC; WR Balston LTD., England). The filters were washed twice with ice cold normal saline followed by two washes with ice cold 5% trichloracetic acid and finally with two washes of ice cold methanol. The filters then were dried with radiant heat, dissolved in a toluene based scintillant fluid and the radioactivity measured in a liquid scintillation spectrophotometer. Each experiment was conducted twice in either triplicate or quadruplicate and the results expressed in counts per minute (cpm). Stimulation ratios were calculated as cpm

experimental/cpm control. The value of cpm control was arbitrarily arrived at by summing the control values for each of the cell types in the experimental tube and dividing by two. In come experiments, the tubes were centrifuged, the supernatant discarded and the pellet resuspended in heated fetal calf serum. An aliquot of this suspension was smeared on cover slips, air dried, and stained with Wright's stain. Blasts were scored according to the morphologic criteria of Chessin et al (1966).

Results

The amount of glucose utilized by NPLs, PELs, and SpLs is summarized in Table 14. Each population was contaminated with varying numbers of macrophages (see Table 14). Since Stjernholm et al have shown that cells of the monocyte/macrophage series metabolized glucose at much higher rates than did lymphocytes (1970) the values listed in Table 14 for all three populations are somewhat higher than would be the case in "pure" populations. Further, it has also been shown that the metabolic activity of activated macrophages such as are found in induced exudates was many times higher than non-activated macrophages such as are found in the normal peritoneal cavity (Stjernholm, 1970). Thus macrophage contamination of PELs probably contributes more to the total metabolic activity than a comparable number of macrophages among NPLs. With this in mind, it is not certain that PELs are metabolically more active than NPLs, as would be indicated by Table 14. Both peritoneal populations, however, appear to be almost two-fold more metabolically active than SpLs.

To obtain direct measurements of the glucose utilization of normal macrophages, these would first have to be isolated from a cell mixture by adherence to a plastic surface and subsequently detached for the incubations with isotope. It was our experience that this procedure resulted in a significant amount of cell damage or death and was for this reason unacceptable. Instead, the glucose utilization of a freshly isolated normal peritoneal population containing both NPLs and macrophages was determined. Since the differential count was known (see Table 14), the contribution of NPLs (obtained in other experiments) to the total glucose utilization of the unfractionated peritoneal population could be calculated. Subtracting the glucose utilization of NPLs from the unfractionated population gives the glucose utilization of normal peritoneal macrophages, i.e., 4.35 u moles glucose/10^8 cells/m.

In these experiments, specificity controls included preincubation with non-fluorescein conjugated anti-mouse globulin (control I; see Table 15) and incubation with fluorescein conjugated anti-mouse albumin alone (control II; see Table 15). Where positive, fluorescence was usually distributed circumferentially along the plasma membrane and exhibited a continuous, interrupted or granular pattern. Clumps and contaminating macrophages usually exhibited strong fluorescence and

Table 14 - Glucose utilization

Exp #	NPL		NPL (Unfractionated)[b]		SpL		PEL	
	glucose utiliz.[a]	% macro-phages	glucose utiliz.[a]	% macro-phages	glucose utiliz.[a]	% macro-phages	glucose utiliz.[a]	% macro-phages
1	1.41	5.0	2.25	25	1.03	1.0	1.77	2.0
2	1.61	4.2			0.78	1.0	1.63	2.0
3	1.45	6.0			0.89	2.0	2.25	8.2
4					0.96	2.0	2.10	8.2
mean	1.55	5.4	2.25	25	0.92	1.5	1.94	5.1

[a] All values expressed as μ moles glucose/10^3 cells/hr. All experiments done in quadruplicate. Values in replicate samples varied less than 5 percent.

[b] The unfractionated population contains both adherent and nonadherent cells and represents those cells freshly isolated from the normal peritoneal cavity.

Table 15 - Surface immunoglobulin

	% Positive-exp.	% Positive-control I[c,b]	% Positive-control II
NPL	46 - 52[a]	3% - 5%	0%
SpL	38 - 41	3% - 5%	0%
PEL	2 - 5	0%	0%

[a] Range of values in 3 separate experiments obtained by counting 400-500 cells

[b] Control I-cells were preincubated with non-fluorescein conjugated antibody to specifically inhibit fluorescence

[c] Control II-cells were incubated with fluorescein conjugated anti-mouse albumin

were not included in the differential count. Indeed, the fluorescence of the clumps was not inhibited by preincubation with non-conjugated anti-mouse γ globulin and exhibited fluorescence with anti-mouse albumin, suggesting a non-specific nature for this binding. The data from three separate experiments are summarized in Table 15. It can be seen that NPLs and SpLs are B cell rich populations with NPLs containing a slightly higher percentage of surface immunoglobulin (Ig) bearing lymphocytes (Ig + lymphocytes). In contrast, PELs from mineral oil induced exudates never contained more than 5% Ig+ cells, indicating that this is a B-cell-poor population. To determine whether the mineral oil interfered in some way with the binding of the fluorescein conjugate, SpLs were mixed with oil, washed thoroughly and processed as usual for the presence of surface Ig. Preincubation with oil did not change the results described above.

Mitogenic stimulation: In this series of experiments, the ability of NPLs to respond to the T cell mitogen CON A (Stobo et al., 1972) was studied and compared with the responses of SpLs. In preliminary experiments we varied the cell density, serum source, and time of maximal response.

At cell densities above 3 x 10^6 cells/ml, tritiated thymidine

(3Tdr) incorporation averaged 12.5 x 10^3 cpm in control cultures of both NPLs and SpLs at 72 hours, resulting in a lower stimulation ratio than could be obtained at lower cell densities. We found that cell concentrations between 1 x 10^6 and 1.5 x 10^6 nucleated cells/ml gave the best stimulation ratios for NPLs and SpLs. If we used 5% or 10% heat inactivated autologous serum as a protein source, blastogenesis of NPLs was markedly reduced while that of SpLs was abolished. It appeared that autologous serum, even when heat inactivated, was toxic to SpLs. Finally, although there was stimulation at 48 and 96 hours, this was far less than at 72 hours.

Thus maximal stimulation was achieved at 72 hours of culture with a cell density of 1.5 x 10^6 cells in 1 ml in a medium containing 5% heat inactivated fetal calf serum. These conditions were identical to those described by Stobo et al (1972) for mouse SpLs.

The results of two typical experiments are summarized in Table III. The peak blastogenic response of NPLs, as measured either by the morphologic appearance blasts or by tritiated thymidine incorporation, is two-fold less than SpLs. While the maximal stimulatory concentration of CON A in NPL cultures was always 0.3 ug/ml (1.5 x 10^6 cells/ml), this varied from 0.3 ug/ml to 1.0 ug/ml in SpLs cultures. This variation was probably due in large part to the presence of a variable number of erythrocytes which bound CON A and thus lowered its effective concentration. Cultures of NPLs contained few contaminating erythrocytes.

Response to allogeneic histocompatibility antigens: In this series of experiments, we have compared the response of BALB/C NPLs and SpLs ($H\text{-}2^d$) to A strain histocompatibility antigens ($H\text{-}2^a$) (Adler, et al, 1970). To this end, we examined the response of BALB/c NPLs and SpLs in the one way mixed lymphocyte culture (MLC) to (BALB/c x A)F_1 SpLs. The results are summarized in Table 17.

In preliminary experiments, a variety of cell densities and ratios were tested. Maximal incorporation of ^{3}HTdr was obtained with a responding cell/stimulating cell ratio of one and at a cell density of 2.0 x 10^6 with both NPL and SpL cultures. Higher cell densities resulted in increased incorporation of ^{3}HTdr in control tubes and consequently lower stimulation ratios. Maximal incorporation occurred on day 5 (Table 17) with significant incorporation occurring on day 4 also (Table 17). Much lower stimulation was observed on day 3 (Table 17) and day 6 (not shown in Table 17).

With conditions for maximal stimulation, SpLs were at least two times more active than NPLs with respect to ^{3}HTdr incorporation and at least three times more active with respect to percent blasts present in the culture. This was the only (and minor) exception to the usual pattern of percent blasts paralleling the ^{3}HTdr incorporation.

Table 16 - Con A stimulation of SpLs[a] and NPLs[b]

Con A[c] (ug/ml)	SpLs						NPLs					
	Mean CPM[e]		S -- R[d]		% Blasts		Mean CPM[e]		S -- R[d]		Blasts	
	I	II	I	II	I	II	I	II	I	II	I	II
None	2.2×10^3	2.6×10^3	1	1	3	5	4.7×10^3	3.2×10^3	1	1	6	6
0.3	316.0×10^3	48.4×10^3	143	18	62	-	125×10^3	171×10^3	27	53	36	32
0.6	-	298×10^3	-	114	-	65	-	45×10^3	-	14	-	-
1.0	20.4×10^3	-	9.3	-	-	-	21×10^3	-	4.5	-	-	-
3.0	0.2×10^3	-	0.09	-	-	-	0.4×10^3	-	0.08	-	-	-
10.0	0.2×10^3	-	0.09	-	-	-	0.2×10^3	-	0.05	-	-	-

[a] Splenic lymphocytes

[b] The table lists results from two representative experiments (I and II)

[c] The value for each concentration is the arithmetic mean for quadruplicate

[d] Stimulation ratio

[e] Counts per minute

Table 17 - Mixed Lymphocyte interaction[a]

Cell composition[b]	Day 3	Day 4[d]	Day 5[d]	% Blasts day 5
BALB/c SpL	43,492	19,974	29,503	14%
BALB/c NPL	7,441	15,260	23,282	11%
(BALB/c x A)F_1	36,848	19,958	22,309	13%
BALB/c SpL vs F_1 Hybrid SpL	74,895 1.85[c]	45,262 2.26[c]	85,040 3.46[c]	30%
BALB/c NPL vs F_1 Hybrid NPL	16,834 0.76[c]	21,011 1.19[c]	37,789 1.61[c]	9%

[a] Each experiment was conducted twice either in triplicate or quadruplicate. Counts in replicate tubes rarely varied more than 5%. Data is expressed in counts per minute (cpm)

[b] Each tube contained approximately 5.5×10^6 nucleated cells in 1 ml of RPMI containing 5% heated fetal calf serum. All cultures were pulsed with one microcurie of ^{3}H-Tdr before harvest

[c] Cpm experimental/cpm control. CPM experimental is the counts in mixed culture. CPM control is arbitrarily calculated as follows:

$$\text{CPM (BALB/c; NPL or SPL)} + \text{CPM (hybrid)}/2$$

[d] Each tube contained approximately 2.0×10^6 nucleated cells. All cultures received were pulsed with one microcurie of ^{3}H-Tdr before harvest

Discussion

It has been suggested that many NPLs actually represent macrophage precursors (Shelton et al, 1959; Vernon-Roberts, 1969). In studies reported elsewhere we investigated this possibility in detail (Catanzaro and Graham, 1974). We have shown that NPLs, in addition to being nonadherent to glass or plastic surfaces, are peroxidase-negative and do not phagocytose latex particles when freshly isolated. Monocytic cells are peroxidase positive (Van Furth, 1970) and actively phagocytic (Fedorko et al, 1970). Further, NPLs resembled lymphocytes by ultrastructural morphology (see following section). The glucose utilization of leukocytes affords yet another means of distinguishing between cells of the monocytic series and lymphocytes; monocytic cells metabolizing glucose at a far greater rate than lymphocytes (Stjernholm et al, 1970). It was clearly shown that nonadherent normal peritoneal cells utilized at least three times less glucose than corresponding adherent normal peritoneal cells. These data support our previous conclusion that NPLs were indeed members of the lymphoid series (Catanzaro and Graham, 1974).

These studies, however, also indicated that lymphocytes, although metabolically much less active than monocytes, exhibited a wide spectrum of metabolic activity. Thus, both the normal and exudate populations were much more active than SpLs. Perhaps this higher metabolic activity of NPLs and PELs resulted from existence in the suspension-like environment of the peritoneal cavity compared to the closely packed configuration of the cells of the spleen, many of which are sessile. Lymphoid cells bearing surface immunoglobulins (Ig^+) easily detectable by conventional methods have been shown to be bone marrow derived or B cells; T cells generally lack surface immunoglobulins detectable by such methods (Ig^-) (Raff, 1971).

It was not surprising that NPLs contained B cells, since others have demonstrated their ability to synthesize a hemolysin in vitro to sheep red blood cells (Bussard et al, 1967, 1970). However, this hemolysin response was unusual in several respects. First, despite the fact that NPLs were harvested from unimmunized mice, the kinetics of the hemolysin production resembled a secondary rather than a primary response. Second, the response proceeded with colchicine present in the medium; i.e., it was not dependent on mitosis. These findings suggest that the B cells among NPLs may differ in some way from B cells of the spleen. However, it has been shown recently (Shands et al, 1974) that NPLs respond to endotoxin with blastogenesis in the same way as SpLs.

PELs contained less than 5% Ig^+ cells. Therefore, NPLs represented a B cell rich population and PELs a B cell poor population. In the following section, we showed that uropod-bearing lymphocytes, which are thymus derived (Rosenstreich et al, 1972) were found almost exclusively in PELs and only rarely in NPLs. It thus appears that

NPLs and PELs comprise functionally distinct populations. The functional differences between NPLs and PELs are important since it is not uncommon that both NPLs and PELs are referred to collectively as "peritoneal exudate lymphocytes", a lack of discrimination which could certainly lead to conflicting results. The proliferative responses to the mitogen CON A (Stobo et al, 1972) and to allogeneic cells (Adler et al, 1970) have been shown to be thymic dependent functions. These responses can be expected to give a reasonable approximation of the T cell functional capacity of a given population.

The results indicated that NPLs were twofold less responsive than SpLs in the proliferative response to CON A. It should be pointed out that CON A stimulates a much broader spectrum of T-cells than phytohemagglutinin (PHA), stimulating both unselected thymocytes (Janossy and Greaves, 1972) and peripheral theta (θ) poor T cells (Stobo et al, 1972), which are both unresponsive to PHA. Thus most T cells should be responsive to CON A. The same relationship holds with respect to the response to allogeneic lymphocytes. The data indicate that there are twice as many histocompatibility antigen reactive cells among SpLs than NPLs, and taken together with the above, there were at least twofold as many T cells in SpLs than NPLs.

Since NPLs contained only 10% more Ig^+ cells than SpLs, it was unexpected that SpLs would contain twice as many cells capable of responding to mitogen and allogeneic cells, i.e., twice as many T cells. Thus, there must be a large number of Ig^- NPLs which are not classic T cells. Stobo et al (1973) have recently described a population of lymphoid-like cells in mouse spleen which are both θ^- and Ig^- (null cells). They offer strong evidence that these are not T-cells and consider among other possibilities that these null cells represent B cells with subliminal amounts of surface Ig. They have also described the ultrastructural morphology of such cells as being essentially inactive lymphocytes with occasional large mitochondria and large segregated nucleoli. In the subsequent section we have demonstrated that such morphologic types are quite common in NPLs.

It seems probable that in addition to classic T and B cells, NPLs contain many of these null cells. Studies are now in progress with anti θ and anti-mouse-kappa chain antiserum to define further the nature of these cells.

These studies have raised the following questions: (1) Is there a subpopulation of lymphocytes in the peritoneal cavity which belongs to the recirculating pool? (2) As a corollary to number one, what factors determine whether a particular cell enters, leaves, or remains in the peritoneal cavity? (3) What is the immune capacity of NPLs?

2. A morphologic comparison of normal and exudate peritoneal lymphocytes

The lymphoid system is heterogeneous in several respects. One of the most familiar is the functional distinction between thymus derived lymphocytes (T-cells), responsible for cell mediated immunity and bone marrow derived lymphocytes (B-cells), responsible for antibody production (Meuwissen et al, 1969). Another component of lymphoid heterogeneity involves the differential concentration of functional lymphoid classes in separate anatomic locations (Meuwissen et al, 1969). Thus, for example, lymph nodes contain a higher percentage of T cells (Raff, 1970) and a greater proportion of its cells belong to the re-circulating pool of lymphocytes (Fors, 1966; Ford and Gowans, 1969) than does the spleen. In this regard, we have noted with particular interest the reports of Koster et al (1970, 1971a, 1971b) and McGregor et al (1971) which have shown that short-lived cellular immune effector cells preferentially emigrated into experimentally induced peritoneal exudates.

In the preceding section, we described the properties of the resident lymphocytes of the uninflamed or "normal" peritoneal cavity. These normal peritoneal lymphocytes (NPLs) were found, when compared to splenic lymphocytes, to be B cell rich and T cell poor. In some of those experiments we also studied lymphocytes harvested from mineral oil induced peritoneal exudates. In contrast to NPLs, of which approximately 50% contained surface immunoglobulin (Ig^+), less than 5% of peritoneal exudate lymphocytes (PELs) contained surface Ig^+. Thus the resident lymphoid population of the peritoneal cavity is normally B cell rich and is converted to a B cell poor, T cell rich (Berke, et al., 1972; Brunner et al., 1971) population following an induced inflammatory response.

These results indicated that the functional immune capability of the lymphocytes of the peritoneal cavity varied with the presence or absence of inflammation. Since the ability to mobilize lymphocytes with a particular functional capability to or away from target tissues is potentially useful, this phenomenon seemed worthy of further study. It is hoped that an understanding of the underlying mechanisms whereby lymphocytes accumulate in the normal and inflamed peritoneal cavity will give useful information about such events in other tissues. A first step in the elucidation of these relevant factors would be to compare the resident populations in each state. This section compares NPLs and PELs with respect to ultrastructural features.

Materials and methods

Conventionally maintained female Balb/c mice (Jackson Memorial Laboratories, Bay Harbor, ME) 12 to 20 weeks old, were used throughout. The procedures for cell preparation and separation into adherent and

nonadherent cells were described in detail in the previous section. For studies of morphology, equal volumes of RPMI 1640 and 1.4M phosphate buffer (pH 7.35) containing 0.25M sucrose were mixed and designated buffered TCM. Nonadherent cells (from T flasks) were added to an equal volume of 0.1% glutaraldehyde in buffered TCM at 37°C. The mixture was immediately centrifuged for 5 minutes at 200 xg. The supernate was decanted and the pellet resuspended in 2.5% glutaraldehyde in buffered TCM at room temperature for 15 minutes. Following centrifugation, the pellet was resuspended in ice cold 2.5% glutaraldehyde in buffered TCM for 18-22 hours and then post-fixed in 2% OsO_4 in phosphate buffer (0.05M, pH 7.35). After 2 saline washes, the pellets were suspended in hot (60°C) 2% (w/v) Noble Agar (Difco Labs., Detroit, MI), quickly centrifuged and placed in an ice bath. The cell rich portion of the solidified agar was cut into small blocks, sequentially dehydrated in absolute ethanol and propylene oxide and embedded in Epon 812 (Luft, 1961). Thin sections (silver to gray) were cut on a Porter-Blum MT-1 ultramicrotome with a diamond knife, mounted on collodion-coated copper grids and stained with uranyl acetate and lead tartrate (Millonig, 1961). The sections were examined in an Hitachi (HUII) electron microscope. "Thick" (one micron) sections were useful for precise differential counts. These were mounted on glass slides, stained with toluidine blue and examined by light microscopy.

Results

Differential cell counts were made on the freshly isolated suspensions and on the cells which did and did not adhere to the surface of a plastic T flask after two hours exposure under standard culture conditions. The cells were fixed and processed as usual for electron microscopy and toluidine blue stained one-micron sections were utilized for the counts. Based on a frequency of occurrence, we have arbitrarily divided the cells into a major population (macrophages, lymphocytes) and a minor population (polymorphonuclear leukocytes, eosinophils, basophils and plasma cells).

Cells were considered to be macrophages which varied in diameter from 10 to 30 microns and had an eccentrically placed uniform nucleus with a low nuclear/cytoplasmic (N/C) ratio. Macrophages possessed prominent cytoplasmic vacuoles. We have designated those cells with a diameter less than or equal to 8 microns with a centrally placed nucleus and a high N/C ratio as small lymphocytes. Small lymphocytes had rare recognizable cytoplasmic granules. Those cells with a diameter of greater than 8 microns with a centrally placed round to oval nucleus and an N/C ratio between the small lymphocyte and macrophage, were scored as large lymphocytes. Cytoplasmic granules were uncommon and nucleoli variably prominent in these cells. The criteria for the minor cell population have been sufficiently well defined elsewhere (Bloom and Fawcett, 1968) and were utilized in the present study.

The data concerning the major population are summarized in Table 18. The cells scored as lymphocytes were never found in the population of cells adherent to the bottom of the flask. Hereinafter the non-adherent cells of each population and the terms NPLs (normal peritoneal lymphocytes), PELs (peritoneal exudate lymphocytes) and SpLs (splenic lymphocytes) will be used interchangeably. It can be seen that there were about twice as many small lymphocytes in SpLs than in either peritoneal population and that this relationship was reversed with larger lymphocytes. Table 18 also indicates that "purification" of lymphocytes by adherence was never complete; a small percentage of macrophages was always present in the nonadherent population. This was especially true in PELs where lipid laden macrophages appeared to adhere poorly.

The minor population was asymmetrically distributed among the three populations and is summarized in Table 19. Polymorphonuclear leukocytes (PMNs) represented approximately 3% and 1.0% of the normal peritoneal and spleen populations, respectively, but were still quite common in the exudate population three days after the intraperitoneal injection of mineral oil. These exudate PMNs were poorly adherent to plastic and frequently contaminated the nonadherent population. Plasma cells were seen only in spleen suspensions and never in normal or exudate peritoneal populations. While mast cells (Fig. 21A) were commonly seen among normal peritoneal cells, they were rarely seen in splenic or the peritoneal exudate populations. Eosinophils (Fig. 21B), on the other hand, were only seen commonly among peritoneal exudate cells and only very rarely among normal peritoneal cells or spleen cell suspensions.

Using ultrastructural criteria suggesting increased capacity for protein synthesis, e.g., content of round surfaced endoplasmic reticulum (RER), polyribosomes, nucleolar prominence, etc., Zucker-Franklin (1969) and Waldo et al (1972) have classified lymphocytes as inactive, intermediate and blast cells. These are not mutually exclusive criteria and intermediate forms between the classes are identifiable. The term activity in this paper will refer exclusively to morphologic evidence of capacity for protein synthesis. Our observations of mouse splenic lymphocytes are similar to the recently reported observations of Waldo and Zucker-Franklin (1972) and thus will not be described in detail here.

The inactive lymphocytes from PELs (Fig. 21-C and NPLs (Fig. 21-D) were identical in morphology to their counterparts in spleen, lymph node, thymus, etc. These were usually cells less than 10 microns in greatest diameter with a bland cytoplasm, i.e., one devoid of RER and polyribosomes, and with infrequent mitochondria. Consistent with this cytoplasmic appearance, the nucleolus was usually inconspicuous. While inactive lymphocytes constituted the vast majority of SpLs, less than half of the NPLs and PELs could be so classified. Approximately 20% of NPLs which would be ordinarily classified as inactive were unusual in that they contained a prominent segregated macronucleolus

Table 18 - Proportion of cell types (major population) in NPL, PEL[a] and SpL[b]

	Macrophages		Small lymphocytes		Large lymphocytes	
	Pre(%)	Post (%)	Pre(%)	Post (%)	Pre(%)	Post(%)
NPL	34	5	27	45	33	50
SpL	10	3	77	80	13	17
PEL	60-65%	8	8	38	12	54

The major population includes only lymphocytes and macrophages. In these experiments, the cellular composition of the unfractionated, freshly isolated suspension (PRE) is compared to that of the cells remaining nonadherent after two hours exposure to plastic (POST). The NPL and PEL are pooled from approximately 20 mice, the SpL from 6 to 8 mice. All determinations are made by light microscopy on one-micron sections of suspensions embedded for electron microscopy and stained with toluidine blue. The values are expressed as percentage of total major population cell count and represent the means of two separate experiments. Three hundred cells were counted in each determination.

[a] PEL = peritoneal exu[illegible]mphocytes; harvested 3 days after injection of sterile light mineral [illegible]

[b] SpL = spleen lymphocytes

Table 19 - Proportion of cells of minor populations in NPL, PEL[a] and SpL[b]

	Plasma cells	Polymorphonuclear leukocytes	Bosinophils	Mast cells
NPL	0	3%	0	4%
SpL	3%	1%	1%	1%
PEL	0	5%-10%	5%	1%

The determinations were made on the same experiments and in the same manner as detailed in Table 18. They represent observations on the freshly isolated, unfractionated population. The values are expressed as percentage of the total population and represent the means of two separate experiments. Three hundred cells were counted in each determination.

[a] PEL = peritoneal exudate lymphocytes harvested 3 days after the intraperitoneal injection of sterile light mineral oil

[b] SpL = spleen lymphocytes

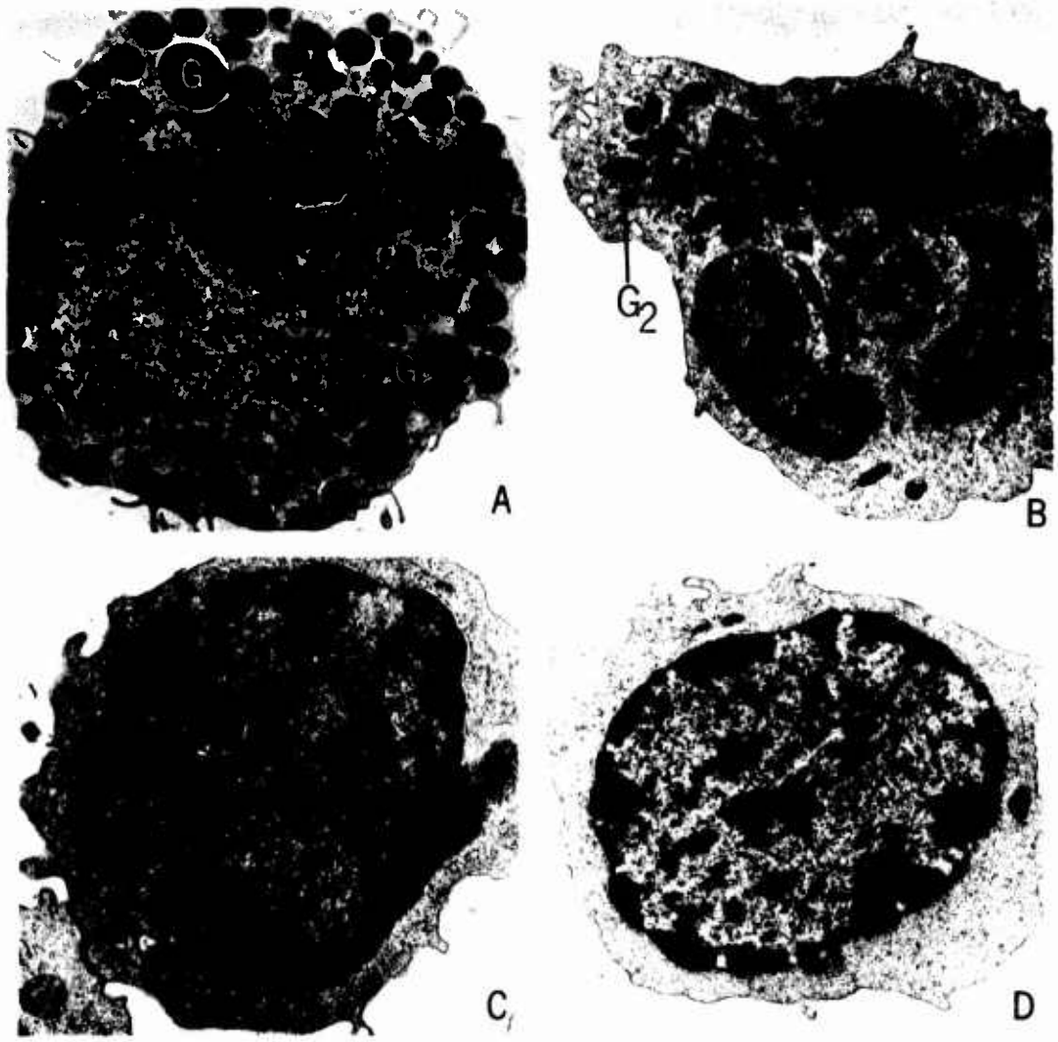

Figure 21. A. Mast cell from normal peritoneal cavity. Prominent ovoid granules (G) of varying electron density are seen. N indicates nucleus. All sections in this report were stained with uranyl acetate and lead tartrate. 8,350X. B. Eosinophil found only in mineral oil induced peritoneal exudate. Two types of granules are seen: G_1 - homogeneously electron dense and round to elliptical, and G_2 - elliptical, less electron dense with a dark central bar. Stacks of Golgi (Go) and a centriole (C) are in the "hof" of the cell. N denotes the trilobed nucleus. 16,000X. C. and D. Inactive lymphocyte from peritoneal exudates (C) and normal peritoneal lymphocyte (D). Note the high nucleus/cytoplasm ratio, the abundant peripheral heterochromatin and inconspicuous nucleolus. No mitochondria are in this section. Ribosomes are distributed singly and profiles of rough endoplasmic reticulum are not seen. N indicates nucleus; C. 20,000X; D. 16,000X

(Figs. 22-A, B). Such nucleolar prominence is usually associated with a much more active cytoplasm, i.e., increased profiles of RER, etc. These cells were found in much smaller numbers among splenic lymphocytes and uncommonly in PELs.

The remaining NPLs and PELs were of a more active variety and were classified as intermediate cells. Such intermediate cells from NPLs and PELs were morphologically similar (Figs. 23, 24). These lymphocytes were usually larger than inactive cells, possessed a lower N/C ratio and a much more active cytoplasm containing frequent profiles of RER (Figs. 23-A - D) and in some cases numerous polyribosomes (Figs. 24-A, B). Intermediate lymphocytes containing numerous polyribosomes were much more prominent among PELs. Lymphoblasts were never seen among NPLs but comprised approximately 10% of PELs (Fig. 25-A).

Of most interest was the uropod bearing lymphocyte (Figs. 25-B, C). The morphology of the uropod bearing lymphocyte from mouse PELs is similar to that described in guinea pig PELs (Rosensteich et al, 1972) and human peripheral blood lymphocytes (Biberfeld, 1971; McFarland et al, 1970). In our preparations, these represented approximately 10% of PELs, were rarely seen in NPLs, but never observed among SpLs. The uropod bearing lymphoid cells were of the inactive variety. It is likely that the actual percentage of uropod bearing lymphocytes among PELs was higher; uropods are detectable in electron micrographs only when they lie within the plane of section.

Discussion

We undertook the morphologic comparison of NPLs and PELs as a first step in understanding the factors responsible for the accumulation of lymphocytes in the peritoneal cavity in its normal and inflamed state. In the preceding section, we had presented evidence that NPLs and PELs are functionally different populations. Were there, then, morphologic correlates for these functional differences? Indeed, not only were the lymphoid (nonadherent) populations morphologically quite different, but the unfractionated freshly harvested populations were also different. Thus, the exudate population contained far more PMNs that the normal population and contained eosinophils, which were not observed in the normal population. On the other hand, mast cells were almost found exclusively in the normal peritoneal population. It was predictable that plasma cells were not found among PEL, a B cell poor population. However, it was surprising that NPLs, which consist of at least 50% surface Ig^+ cells, contained no plasma cells. Plasma cells were readily identifiable among SpLs.

Lymphocytes bearing neither surface Ig or theta (θ) antigen have been described in mouse spleen (Stobo et al, 1973). These have a characteristic morphology, being inactive lymphocytes with a segregated macronucleolus (Stobo et al, 1973). In the preceding section we demonstrated that there was a large percentage of NPLs unaccounted for

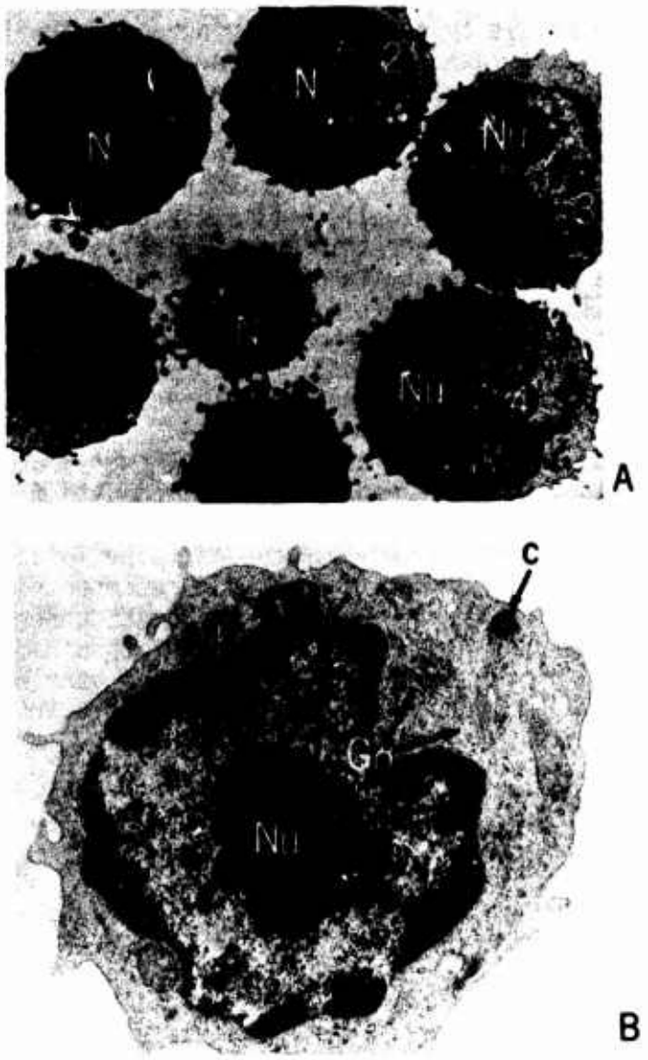

Figure 22. A. Survey photomicrograph of several inactive normal peritoneal lymphocytes. Note absence of prominent nucleoli in cells numbered 1 and 2. Nu denotes nucleolus in cells 3 and 4. Other cellular profiles include only a small part of the nucleus in the p plane of section. N denotes nucleus. 5,200X. B. Normal peritoneal lymphocyte classified as an inactive lymphocyte. Mitochondria are seen in the plane of section; ribosomes occur singly and profiles of rough endoplasmic reticulum are not seen. The plane of section includes a prominent Golgi complex (Go) and centriole (C); the nucleolus (Nu) is quite prominent. 14,900X.

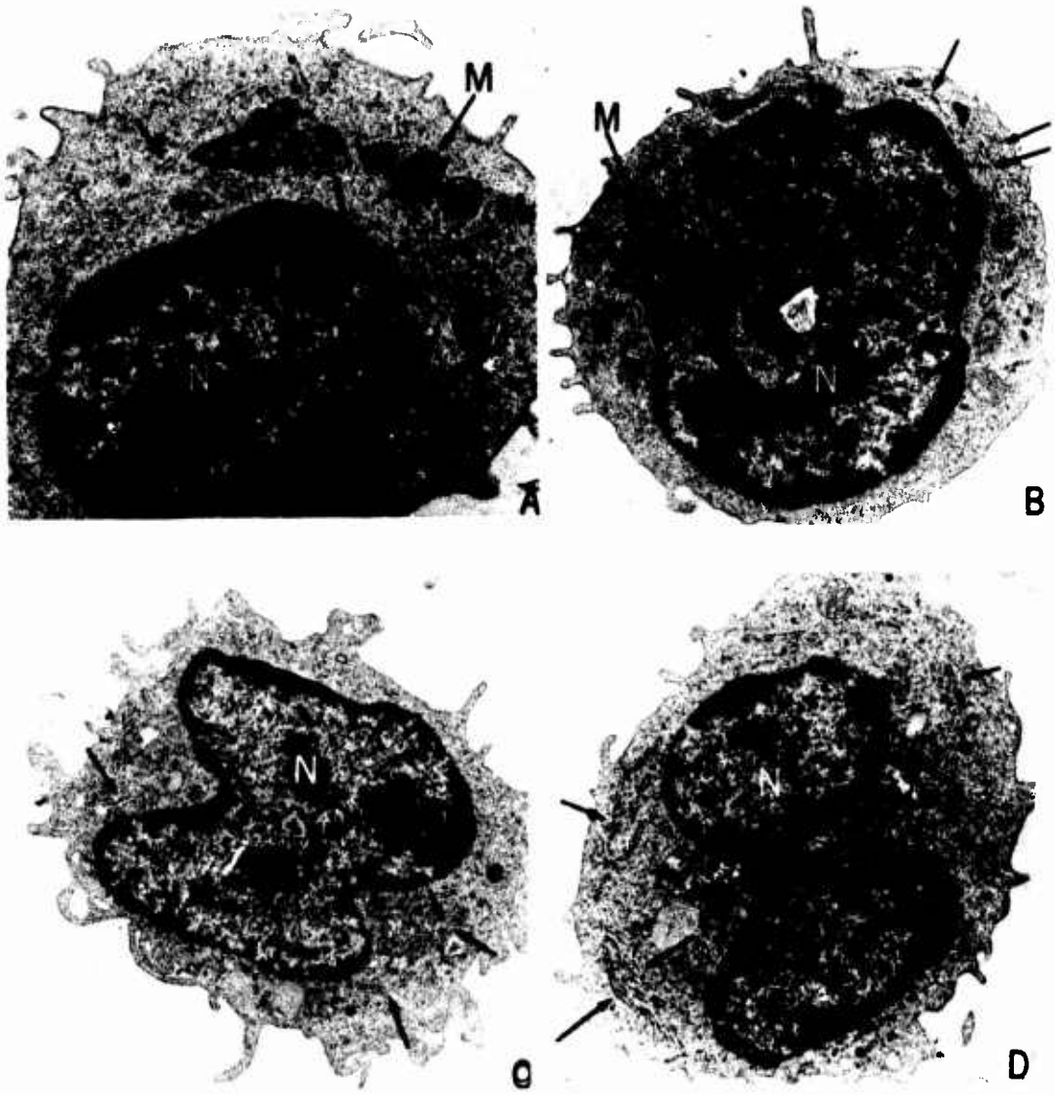

Figure 23. Lymphocytes classified as intermediate cells. A. and B. Normal peritoneal lymphocytes. C. and D. Peritoneal exudate lymphocytes. Note a decrease in nucleus/cytoplasm ratio, the increase in the number of mitochondria (M) and profiles of rough endoplasmic reticulum (arrows). The plasma membrane contours are interrupted by a variety of villous projections. Perinuclear microfilaments (Mf) are seen in A. A. 16,400X; B. 16,400X; C. 12,000X; D. 12,000X.

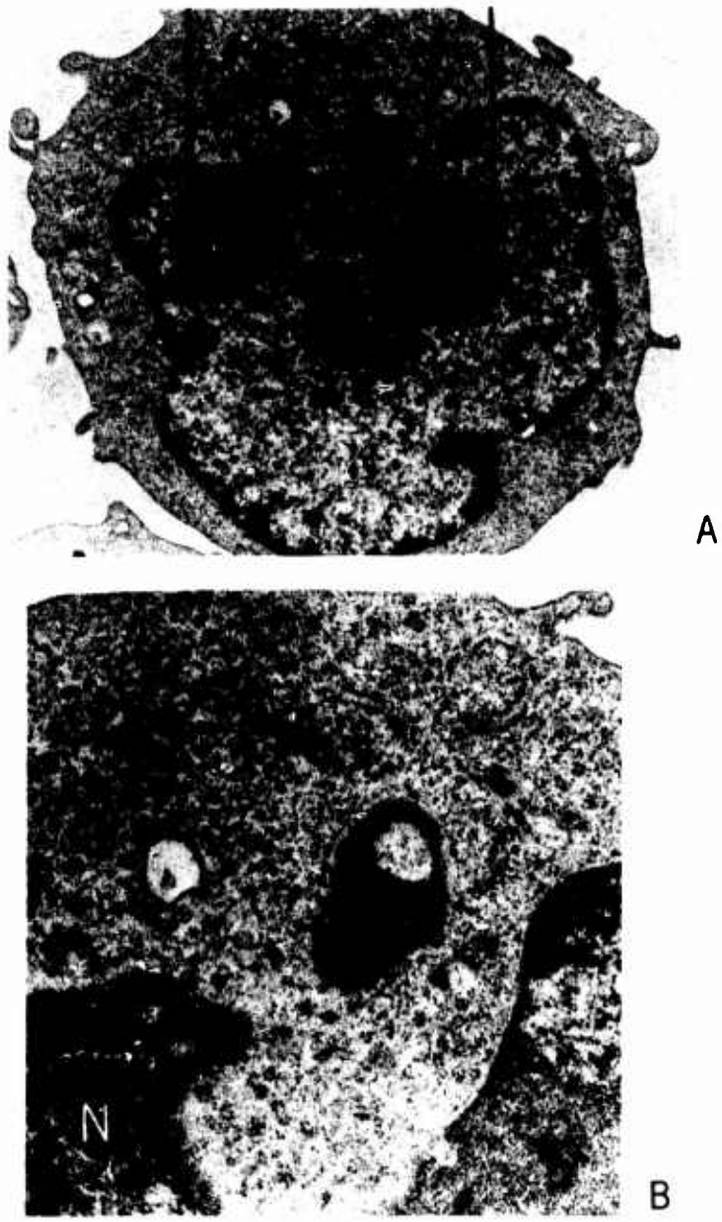

Figure 24. A. Intermediate peritoneal exudate lymphocyte exhibiting a low nucleus/cytoplasm ratio, frequent profiles of rough endoplasmic reticulum and numerous polyribosomes. N indicates nucleus. 10,600X. B. Higher power micrograph of the demarcated area in A demonstrating numerous clusters and rosettes of polyribosomes. 21,900X.

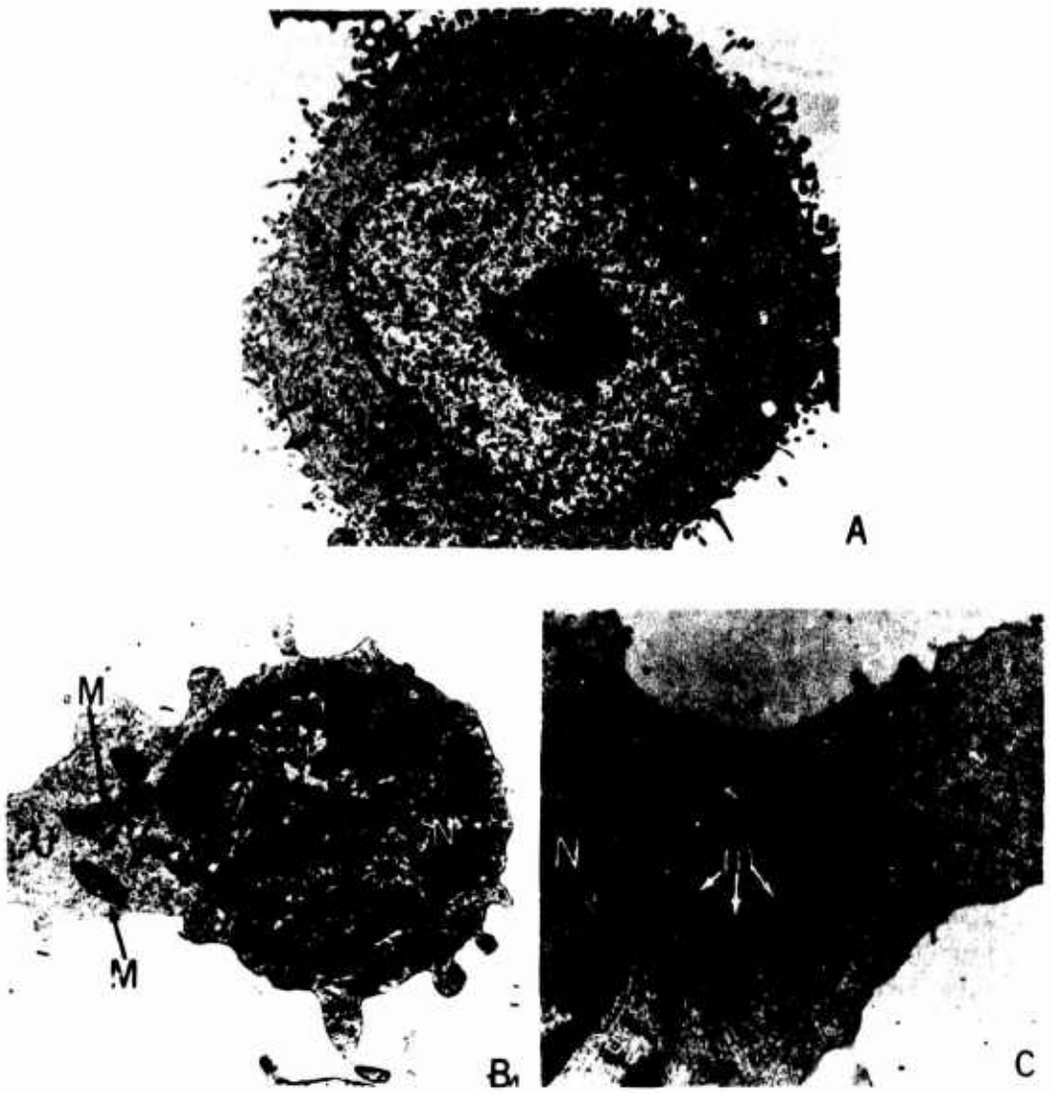

Figure 25. A. Lymphoblast from exudate lymphocytes with low nucleus cytoplasm ratio, commonly seen here, but only rarely in normal peritoneal lymphocytes. Nuclear chromate is rarefied with only a thin peripheral rim of heterochromatin; prominent nucleolus (Nu). The cytoplasm contains numerous profiles of rough endoplasmic reticulum (arrows) and many mitochondria. Note many villous-like projections of the cytoplasmic membrane. 7,700X. B. Inactive lymphocyte from exudate lymphocytes exhibiting uropod (U) opposite the "hof" of the nucleus (N) never observed among normal lymphocytes. Mitochondria (M), vesicular profiles and an occasional profile of rough endoplasmic reticulum (arrow) are seen in the uropod. 15,280X. C. Higher power view of another uropod-bearing exudate lymphocyte. There is a large number of mitochondria (M) and frequent profiles of rough endoplasmic reticulum (arrows). N denotes nucleus. 20,500X.

as either a T cell (response to mitogen or allogeneic cells) or B cells (surface Ig^+). Further NPLs contained a substantial percentage of inactive lymphocytes with segregated macronucleoli identical in morphology to those described by Stobo et al (1973). It appears that NPLs are a rich source of such Ig^- and θ^- lymphocytes. Experiments are presently in progress with the appropriate anti Ig and anti θ sera to establish this point. Such lymphocytes were quite rare among PELs.

There were two cell types seen almost exclusively among PELS, the lymphoblast (Fig.25-A)and the uropod-bearing lymphocyte (Figs. 25-B,C). It was surprising that NPLs which contained so many intermediate cells (Figs. 23, 24), contained no blast-like forms. Certainly NPLs contain cells capable of undergoing mitosis. Thus, it was shown in the preceding study that NPLs contained a small percentage of T cells capable of blast transformation; also, Shands, et al (1974) have shown that nonadherent peritoneal cells give a blastogenic response to bacterial lipopolysaccharide, a B cell mitogen (Peavey, et al, 1973). That lymphoblasts were freqently seen among PELs was consistent with reports by McGregor et al (1971) on PELs from rats.

It has been shown in mice (Berke et al, 1971; Brunner et 1971) and in guinea pigs (Rosenstreich et al, 1971) that PELs are a rich source of thymic dependent effector cells. Further, Rosenstreich et al (1972) showed that uropod bearing lymphocytes from guinea pigs were indicative of an immunologically active T-cell population. It was consistent with the above that uropod bearing lymphocytes were identified in mouse PELs. Also, their rarity of uropod bearing lymphocytes in NPLs suggested that it was a T-effector cell poor population. This is consistent with functional evidence presented in the previous section, showing that NPLs contained relatively few histocompatibility antigen reactive cells and few mitogen reactive cells. NPLs also are a far less reactive source of cells capable of transferring a local (dermal) graft versus host reaction, as shown in the subsequent section.

We conclude, therefore, that there exist morphological correlates to the functional distinctions between NPLs and PELs described by our laboratory and others. Thus, while PELs are a rich source of T-effector cells, NPLs contain a large number of B cells and cells of uncertain nature which may be Ig^-, θ^- lymphocytes.

3. Differential *in vivo* reactivity of mouse peritoneal exudate lymphocytes and normal peritoneal lymphocytes

Lymphocytes are normally present in the peritoneal cavity of a mouse in the absence of induced inflammation (normal lymphocytes) (Shelton and Rice, 1959) and also accumulate following the injection of an irritant such as mineral oil (Catanzaro and Graham, 1970). Exudate lymphocytes have been shown to contain immune effector cells which afford host protection against infectious agents (Koster *et al*, 1971) and neoplasia (Burke *et al*, 1972). Further, they are a rich source of antigen reactive cells following immunization (Rosenstreich *et al*, 1971, 1973). Much less is known about normal lymphocytes and indeed some investigators make no distinction between the two peritoneal populations. Since the ability to mobilize effector cells to or away from target tissues is important, we were interested in whether the physiologic state of a tissue could affect the *in vivo* functional capabilities of the lymphocytes which accumulated therein. For example, does non-specific inflammation enhance a tissue's capacity to destroy a tumor, resist an infection, etc. To investigate this, we decided to concentrate both peritoneal lymphoid populations into the skin of histoincompatible recipients by intradermal injection and compare the resulting dermal inflammatory response, i.e., a lymphocyte transfer test as described by Brent and Medawar (1963, 1964, 1966). This type of *in vivo* assay allows a daily evaluation of the inflammation and thus an appreciation of the kinetics of the response.

Although others have described such transfers between histoincompatible mice (Shelton and Rice, 1959), we found the dermal injections difficult to administer accurately and, more importantly, quite difficult to evaluate quantitatively once administered. For this reason, we modified our system from an allogeneic lymphocyte transfer to a xenogeneic transfer by injecting donor mouse lymphocytes into guinea pig skin in which erythema and induration were easily evaluated. The guinea pig hosts were rendered immunologically inert by prior lethal whole body x irradiation and in some cases were pretreated with cobra venom factor to deplete complement (Ballow and Cochrane, 1969; Cochrane *et al*, 1970). Where appropriate, the response of both peritoneal lymphoid populations were compared with mouse peripheral blood lymphocytes (PBLs) and splenic lymphocytes. The data establishes that normal lymphocytes and exudate lymphocytes exhibit both qualitative and quantitative differences in their behavior in tissue.

Materials and methods

Conventionally maintained male BALBC/C mice (Flow Laboratories, Rockville, MD), 18 to 22 wks of age and male guinea pigs (250-400 g) of the Hartley strain were used according to the experimental situation. Guinea pig hosts were given 700 to 800 γ of whole body x irradiation from 2 to 5 hrs prior to cell transfer. The radiation

was administered from a Gamma Cell 40 (Atomic Energy of Canada, Ltd.) with a Cesium 137 source (140 ± 4 per minute for 5 minutes). In some experiments host guinea pigs were depleted of complement. A purified low molecular weight (130,000-150,000) fraction of cobra venom (kindly supplied by Dr. George Naff) which is anticomplementary in guinea pigs (Ballow and Cochrane, 1969; Cochrane et al, 1970) was prepared according to the method of Ballow and Cochrane (Ballow and Cochrane, 1969). A dose of 250 units/kg was injected intraperitoneally into guinea pigs in four equally divided doses spaced over a 24-hr period on the day before transfer. Daily intraperitoneal injections were given thereafter for 3 additional days. Complement activity of guinea pig serum was determined by a hemolytic assay (CH_{50}) (Mayer, 1961). No complement activity was demonstrated after the first day and for 6 successive days.

In cell preparation and separation into adherent and non-adherent cells, we routinely used RPMI 1640 (Grand Island Biologic Co., Grand Island, NY) containing 0.005 M Hepes buffer (Calbiochem, San Diego, CA) and 1% v/v of an antibiotic/antimycotic solution (100 X; Grand Island Biological Co.). This will be referred to in the text as culture medium. When 10% v/v heat inactivated (56°C for 45 min) fetal bovine serum (Flow Faboratories) was added to culture medium, it was referred to as FBS/culture medium.

Peritoneal lymphocytes: Cells were lavaged with culture medium from the uninflamed peritoneal cavity or from 3-day-old exudates induced by the prior intraperitoneal injections of 3 ml of sterile light mineral oil. The cells were washed three times in culture medium and adherent cells were removed by two procedures. Adherence columns were prepared in 10 ml disposable plastic syringes. To the bottom of these was added 600 mg of nylon wool (Fenwal Laboratories, Morton Grove, IL) which was preboiled and then rinsed exhaustively with culture medium and finally with warm FBS/culture medium. Approximately 10^8 cells in 5 ml of warm culture medium were added to the column maintained for 30 min at 37°C. The non-adherent cells were forcefully eluted, using the syringe plunger with an excess of the same medium. The eluted cells were then added to plastic T-flasks (75 cm^2, Falcon Plastics, Oxnard, CA) and allowed to adhere an additional 2 hours. The non-adherent cells were designated normal lymphocytes or exudate lymphocytes and were greater than 95% and 93% lymphocytes, respectively, by morphologic criteria.

Spleen lymphocytes: Spleens were minced through a 60-mesh stainless steel screen in culture medium and passed through a 27 gauge needle to remove clumps and produce a single cell suspension. Red blood cells, macrophages and polymorphonuclear leukocytes were separated from lymphocytes on a Hypaque-Ficoll gradient (sp. gr. 1.080) (Perper et al, 1968). This resulted in greater than 98% lymphocytes by morphologic criteria.

Peripheral blood lymphocytes: Mice were anesthetized by CO_2 narcosis and exsanguinated by incision of the right axillary artery. The blood was immediately diluted with culture medium containing heparin without preservative (50 μ/ml; Flow Laboratories) and exposed to plastic T flasks to remove adherent cells. Red blood cells were separated from other non-adherent cells by the Hypaque-Ficoll technique (Ballow and Cochrane, 1969) (sp. gr. 1:085). This resulted in greater than 95% lymphocytes by morphologic criteria.

In some experiments, donor mouse cells were exposed to 1200 r of x-irradiation from a Gamma Cell 220 (Atomic Energy of Canada, Ltd.) cobalt 60 source.

Treatment of cells with anti-light chain serum: 25×10^6 nucleated cells/ml were suspended in culture medium. Rabbit anti-mouse light chain serum (kindly provided by Dr. Rose Mage, USNIH) was added in a 1/10 v/v final concentration. Cells were incubated for 30 minutes at 4°C and 30 minutes at 20°C, diluted with TCM 20/1, v/v, centrifuged and resuspended in guinea pig complement (previously absorbed with agarose and murine cells) at a final dilution of 1/4 v/v. Incubation continued for 40 minutes at 37°C in the presence of DNAase (5 μg/ml; Worthington Biochemical Co., Freehold, NJ). The cells were washed and resuspended for injection. When used with splenic lymphocytes, the resultant population contained no more than 4% of cells bearing surface immunoglobulin by fluorescent criteria. Also 39% specific release of ^{51}Cr from suitably labelled SpLs followed treatment with anti-light chain serum and complement.

Guinea pig spleen cells were harvested in the manner described above for mouse spleen cells. Potential donor mice were immunized with 2×10^7 nucleated guinea pig spleen cells by a single subcutaneous injection in the subnuchal region. Modifications of this procedure will be noted in the results section.

The skin of the flank of guinea pig recipients was closely shaved with animal clippers and depilated with Nair (Carter Products, NY City). The standard test inoculum varied from 5×10^6 to 4×10^7 donor mouse lymphocytes in 0.1 ml of TCM. This was injected intradermally with a 27 gauge needle. The animals were examined daily and the reactions were scored for the diameter of erythema (perpendicular to the needle tract) in millimeters and induration assessed on an arbitrary numerical scale; 0 = no induration, +1 = slight induration, +2 = induration, +3 = central blanching, +4 = hemorrhage or necrosis. Each experimental group contained at least 8 guinea pig host animals and results were expressed as a mean $\pm$ standard error (S.E.).

Mixed lymphocyte culture: One way xenogeneic and allogeneic mixed lymphocyte cultures were carried out was according to the method of Phillips et al, 1973. Briefly, 0.5×10^6 responding lymphocytes were cultured in microtiter plates (Falcon Plastics) with equal numbers

of target cells. The target cells received 1200 r of x irradiation. The responding cells were always from BALB/c ($H\text{-}2^a$) and the target cells included $C_{57}BL_6(H\text{-}2^a)$ and guinea pig spleen cells. Cultures were assayed for blastogenesis daily from day 1 to day 5 after initiation of culture. Four hours before the termination of culture, one microcurie of tritiated thymidine (New England Nuclear Corp., Boston, MA; 6.7 uCi/mM) was added. The cells were harvested using a MASH II Cell Harvester (Microbiological Associates, Bethesda, MD) onto glass filter discs. The discs were airdried and dissolved in a liquid scintillation mixture (Aquasol, New England Nuclear Corp.). Samples did not vary more than 15% from the mean. Results were expressed as mean counts per minute (CPM) of incorporated thymidine or stimulation ratios, i.e., CPM experimental/CPM control.

Results

General: The intradermal transfer of lymphoid cells from preimmunized donor mice into host guinea pigs was referred to as the immune lymphocyte transfer test. In the normal lymphocyte transfer test, donor mice had not been preimmunized with host guinea pig cells. In initial experiments, we injected 10^7 heat killed SpLs, 0.1 ml of culture medium and 0.1 ml of culture medium containing 5% v/v Ficoll Hypaque. At most these resulted in mild transient erythema which was undetectable by the second day and no induration. These responses were collectively referred to in the text as background.

Peritoneal lymphocytes: The normal lymphocyte transfer test with 10^7 transferred exudate lymphocytes differed from that with 10^7 transferred normal lymphocytes in several ways (Fig. 26). First, at each day post transfer, erythema and induration due to exudate lymphocytes were significantly greater than were found with normal lymphocytes. Second, peak erythema was achieved one day earlier with exudate lymphocytes (24 hrs vs 48 hrs). Finally, while the dermal inflammatory response was undetectable at five days after transfer of normal lymphocytes, significant erythema and induration could be found at eight days after the transfer of exudate lymphocytes, at which time most of the lethally x-irradiated guinea pig hosts were beginning to die. If donor normal or exudate lymphocytes were x-irradiated (1200 r) prior to transfer, there was no difference in the normal lymphocyte transfer test.

Effect of T-cell enrichment: We have shown elsewhere by immunofluorescence criteria that normal lymphocytes were a B cell rich population (Adler et al, 1970). Perhaps these differences between normal exudate lymphocytes in the normal lymphocyte transfer test noted above were due solely to the higher content of T cells in exudate lymphocytes. To investigate this possibility, we enriched the T cell content of normal lymphocytes by treating them with anti light chain serum and complement which results in specific B cell lysis. It can be seen in Fig. 27 that such T cell enrichment resulted in an

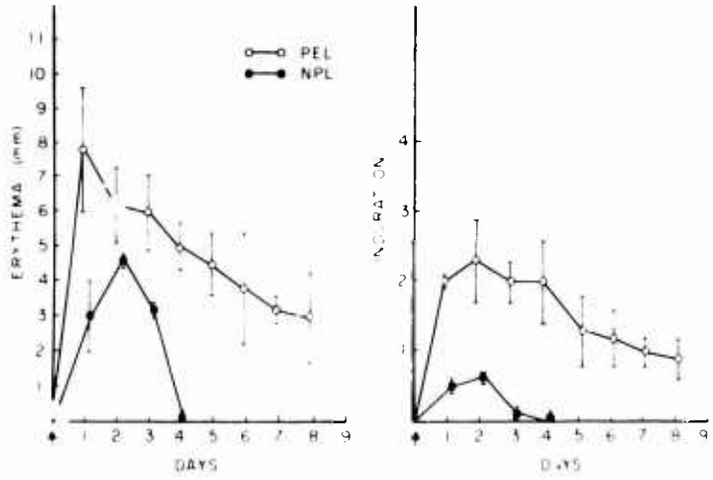

Figure 26. Comparison of the normal lymphocyte transfer test mediated by 10^7 transferred peritoneal exudate lymphocytes (PEL) and normal peritoneal lymphocytes (NPL).

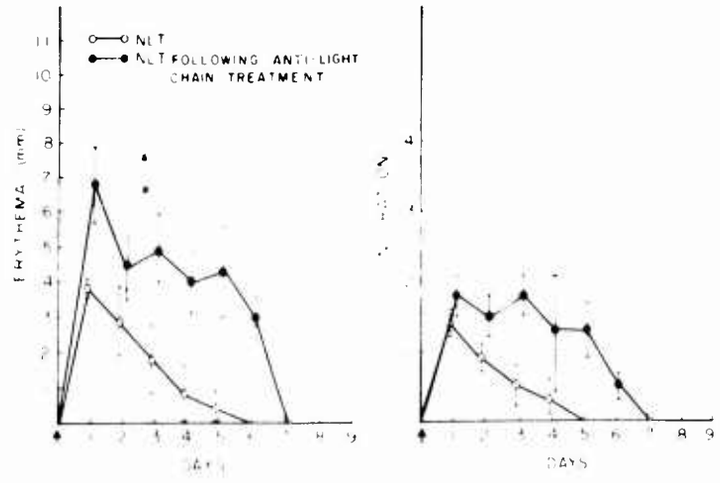

Figure 27. Enrichment of T-cell content of normal peritoneal lymphocytes by treatment with light chain serum and complement and its effect on the normal lymphocyte transfer test (NLT).

augmented response in the normal lymphocyte transfer test. Indeed, at early times after transfer, despite the fact that mean values were higher with exudate lymphocytes, they were not statistically different than T cell enriched normal lymphocytes. However, at later times after transfer, i.e., at days 7 and 8, there was no detectable inflammatory response with normal lymphocytes, while a striking response was still seen with exudate lymphocytes.

Effect of cobra venom factor on the normal lymphocyte transfer test: Schlesinger (1965) has described a guinea pig antibody which mediates complement dependent lysis in vitro of mouse thymocytes and to a far lesser degree, other mouse lymphocytes. Perhaps normal lymphocytes were more sensitive to this antibody in vivo and were being selectively killed in vivo after transfer. In some experiments, we depleted host guinea pigs of serum complement with cobra venom factor, as well as treating them with lethal X-irradiation. No augmentation of the normal lymphocyte transfer test response of normal lymphocytes, or for that matter, with any other population studies were observed. In fact, slight reductions in inflammatory intensity were occasionally noted.

Immune lymphocyte transfer test: Normal and exudate lymphocytes were harvested from mice at 3, 6, 10 and 15 days post immunization (PI) with guinea pig spleen cells. The immune test curves from 10^7 transferred normal lymphocytes at 3 or 15 days PI were almost superimposable with the corresponding normal transfer test. Differences due to immunization were detectable at 6 days PI and maximal at 10 days PI. Fig. 28 compares the normal with an immune test transferred by normal lymphocytes 10 days PI. Clear differences between the immune and normal transfer tests were more clearly appreciated with erythema than with induration. These differences included an almost twofold increase in peak erythema, occurring on day 2, and a prolongation of the erythematous response in the immune test two days longer than the normal test. Peak induration occurred at day 2 and there was a prolongation of the erythematous response in the immune test two days longer than the normal test. Peak induration occurred at day 2 in both the immune and normal transfer tests, and to the same degree in each. However, at later times, i.e., at days 3-5, the mean induration was slightly greater. Thus, the effects of immunization include not only higher peak responses but a prolongation of the response.

The immune lymphocyte transfer test with exudate lymphocytes was also maximal at 10 days PI. This response differed from the immune test with normal lymphocytes in two respects: first, the early immune test response, i.e., from 0 to 5 days (Fig. 29) was not different from the corresponding normal transfer test, unlike the increases seen with immune normal lymphocytes (Fig. 28). However, most striking was the terminal "flare" reaction from days 5 to 8 with exudate lymphocytes (Fig. 29) which was not observed in the immune test with normal lymphocytes (Fig. 28).

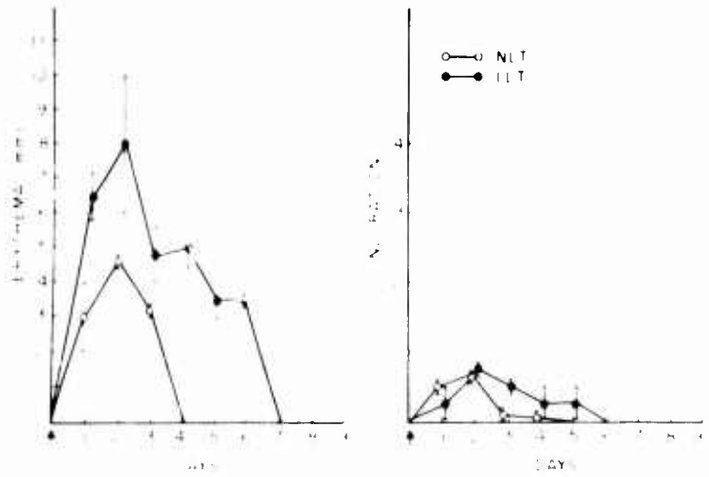

Figure 28. Comparison of the normal lymphocyte transfer test (NLT) and the immune lymphocyte transfer test (ILT) mediated by normal peritoneal lymphocytes. Donor mice had been preimmunized with guinea pig spleen cells 10 days prior to transfer.

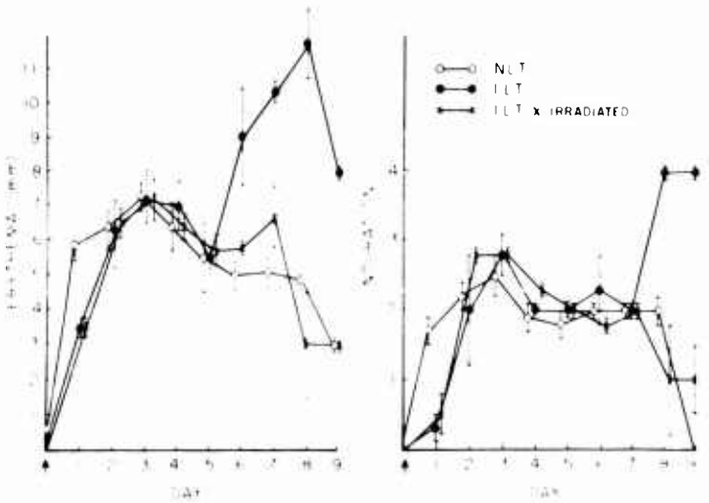

Figure 29. Comparison of the normal lymphocyte transfer test and the immune lymphocyte transfer test mediated by peritoneal exudate lymphocytes. Donor mice in the immune lymphocyte transfer test had been preimmunized with guinea pig cells 10 days prior to transfer.

We subjected immune exudate lymphocytes to 1200 r of X-irradiation before transfer to see what portion of the immune test was dependent on the proliferation of the transferred cells. Apparently during the initial 0-5 days, no mitosis of the transferred cells took place and the immune transfer test with non X-irradiated immune exudate cells was identical (Fig. 29). However, such treatment abluted the terminal flare response, indicating that this response was dependent upon mitosis. Similar irradiation experiments with immune normal lymphocytes and splenic lymphocytes (not shown) had no effect on the response.

Comparison of the immune lymphocyte transfer test vs normal exudate lymphocytes: In a separate experiment we compared the immune test, 10 days PI, of 10^7 transferred normal exudate lymphocytes. In Fig. 30 it can be seen that the differences are significant and, as was the case with the normal test, the behavior of immune exudate and normal lymphocytes in tissue are quite distinct. There was higher response at days 2 and 4 post transfer for induration and erythema, respectively, with exudate lymphocytes. Further, a flare reaction was not seen with normal lymphocytes, indicating the lack of a mitotic response of these cells in host tissues in the late stages after transfer. Notice that this experiment involves the transfer of 10^7 exudate lymphocytes, while that in Fig. 29 results from the transfer of 5×10^6 exudate lymphocytes. There is little difference in peak reactions, but with the larger cell number the early non-mitotic phase is prolonged and so the late flare is less pronounced (Fig. 30); whereas with fewer cells transferred, the early non-mitotic phase declines somewhat before the terminal flare (Fig. 29).

Splenic lymphocytes - comparison of normal vs immune lymphocyte transfer test: For sake of reference and comparison we included data obtained with the transfer of splenic lymphocytes and below with peritoneal blood lymphocytes. In terms of the ability to elicit a transfer response, splenic lymphocytes represented the least reactive of all populations studied. Thus when either 2.5×10^6 or 5×10^6 immune or non pre-sensitized splenic lymphocytes were transferred, the resulting response was no greater than background reactions. Only when 10^7 cells are transferred were reactions over background seen. Response in the normal lymphocyte transfer test was brief and undetectable after 3 days (Fig. 31). The effects of immunization were only detectable at 10 days PI and those immune transfer tests at 3, 6, and 15 days PI were not different from the corresponding normal transfer tests. While the immune test with 10^7 transferred cells resulted in a higher peak of and prolongation of erythema, induration was paradoxically lower than the corresponding normal test and lasted a day less (Fig. 31).

We attempted to see whether this hyporesponsiveness of splenic lymphocytes was a quantitative or qualitative phenomenon by increasing the transfer inoculum to 2×10^7 cells. Both the normal and immune

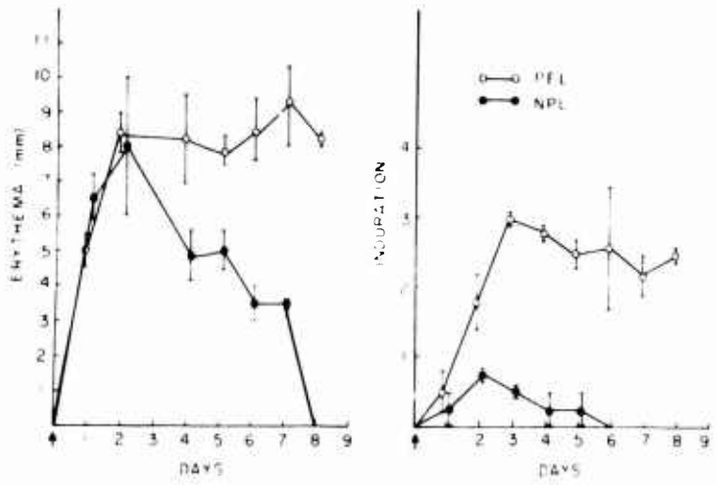

Figure 30. Comparison of the immune lymphocyte transfer test mediated by peritoneal exudate lymphocytes (PEL) and normal peritoneal lymphocytes (NPL). Donor mice were preimmunized with guinea pig cells 10 10 days prior to transfer.

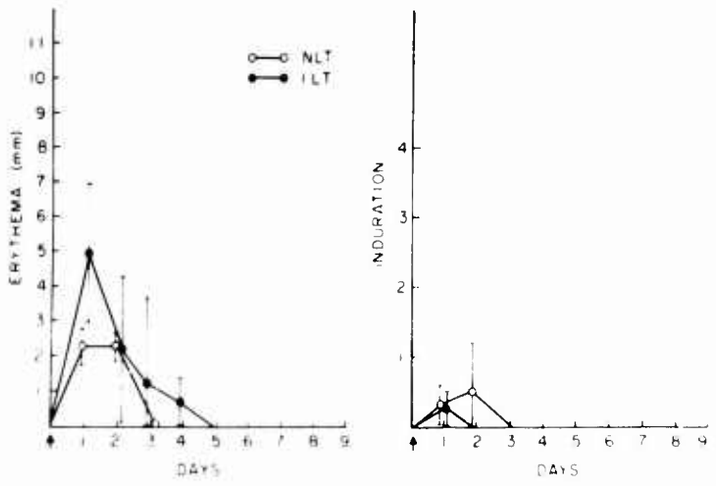

Figure 31. Comparison of the normal lymphocyte transfer test (NLT) and the immune lymphocyte transfer test (ILT) mediated by splenic lymphocytes. Donor mice in the immune lymphocyte transfer test had been preimmunized with guinea pig cells 10 days prior to transfer.

tests were increased and the reactions prolonged (Fig. 32). However, the shape of the curve was basically the same and no terminal flare was noted. Increasing the inoculum to 4 x 10^7 cells (not shown) only slightly increased the results obtained with 2 x 10^7 cells.

Marrow lymphocytes: Up to 10^7 marrow lymphocytes from immune or non-presensitized donors never gave a dermal inflammatory response over background.

Peritoneal blood lymphocytes: In many ways the response with transferred peritoneal blood lymphocytes, both in the immune and normal transfer tests, resembled that with peritoneal exudate lymphocytes. A striking early reaction was observed with only 5 x 10^6 peripheral blood lymphocytes (Fig. 33). As was the case with exudate lymphocyte transfers, differences between immunized and non-immunized donors were best seen at the later times. However, it should be noted that the initial reaction (both immune and nonimmune) with peritoneal blood lymphocytes was the most striking of all observed, even greater than peritoneal exudate cells. A slight flare was noted, perhaps masked by the already high initial values (Fig. 33).

Xenogeneic mixed lymphocyte cultures: The incubation of carefully washed X-irradiated guinea pig spleen cells in the mixed lymphocyte culture with each of the populations studied resulted in a suppression of the spontaneous DNA synthetic capacity of these when incubated alone (Table 20). Under similar conditions, the allogeneic mixed lymphocyte culture with splenic lymphocytes, BALB/c with X-irradiated C_{57} Bl_6, resulted in at least twenty-fold stimulation (Table 20). Further, if one took X-irradiated guinea pig spleen cells, carefully washed after irradiation, and cultured them overnight, the unconcentrated supernate from these cultures resulted in a similar suppression of DNA systhesis in murine splenic lymphocytes (Table 20).

Discussion

The data demonstrated striking differences in the behavior in foreign tissue of exudate and normal lymphocytes from normal and immune donors. To understand the nature of these differences, the nature of the xenogeneic transfer system should be discussed in some detail. Our first concern was the nature of the antigenic stimulus to donor mice posed by exposure to guinea pig tissues. Since the Hartley strain was not inbred, differences in reactivity may be due to variation in the nature of the antigen challenge offered by individual host guinea pigs. It has been shown by Sachs et al (1971) that in a xenogeneic system, the bulk of the response was directed against antigens shared in common by the species and to a far less degree to individual histocompatibility differences. Thus mouse lymphoid cells were in the main recognizing "guinea pig" and the degree of variation due to individual differences should not be great. The xenogeneic lymphocyte transfer tests presented above differed from

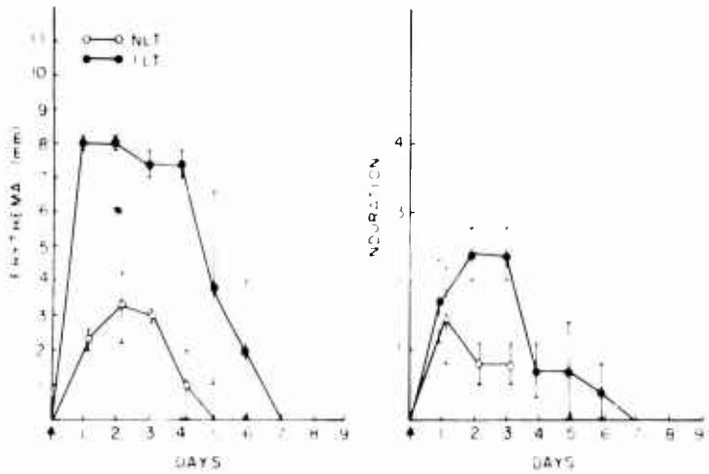

Figure 32. Comparison of the normal lymphocyte transfer test (NLT) and the immune lymphocyte transfer test (ILT) mediated by 2×10^7 splenic lymphocytes instead of 1×10^7 splenic lymphocytes as in the preceeding figure.

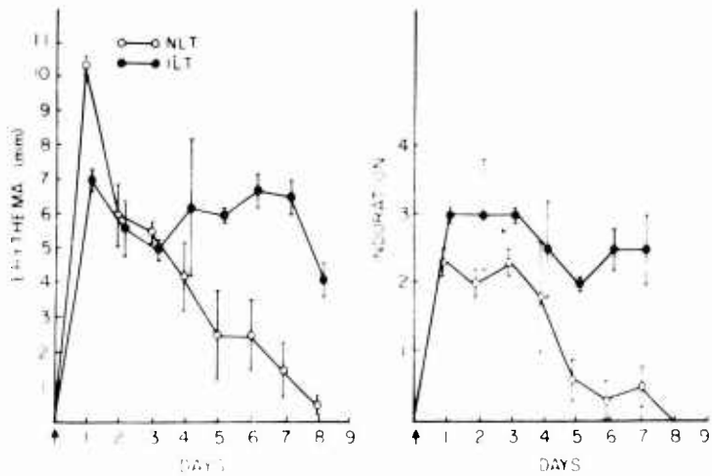

Figure 33. Comparison of the normal lymphocyte transfer test (NLT) and the immune lymphocyte transfer test (ILT) mediated by peritoneal blood lymphocytes. In the ILT, donor mice were preimmunized with guinea pig spleen cells 10 days prior to transfer.

Table 20 - Allogeneic vs Xenogeneic MLC[a]

Responding cells[b]	Control[c]	C_{51} Bl_6	Δ[e]	Guinea pig	Δ	Super-nate[f]	Δ
SpLs	952.0[d]	2,0943.0	21.9	797.0	0.8	478	0.5
PELs	6,470.8			1,111.4	0.2	-	
NPLs	6,753.0			2,435.0	0.4	-	
PBLs	909.6			475.0	0.5	-	

[a] Allogeneic and xenogeneic target cells = SpLs exposed to 1200 r prior to culture

[b] All responding cells are from BALB/c mice

[c] Control values represent the incorporation of tritiated thymidine by the responding cells when cultured alone

[d] Mean counts per minute (CPM) of tritiated thymidine incorporated per culture. These values represent the mean of quintuplicate samples and individual results vary less than 10% from the mean

[e] Δ = stimulation ratio, i.e., cpm experimental/cpm control. The data represented the maximum stimulation ratio obtained over a 5-day culture; usually reached at 72 hrs

[f] Irradiated (1200 r) target guinea pig spleen lymphocytes were carefully washed and incubated for 24 hours under standard culture conditions. One volume of the supernate from this culture was added to an equal volume of BALB/c SpLs and the culture carried out in usual fashion, i.e., as with controls

the allogeneic transfer tests originally described by Brent and Medawar (1963, 1964, 1966, 1966). In their descriptions of the normal lymphocyte transfer test an early, non-mitotic dependent response due to "recognition events" which peaked at 2 days was observed. Thereupon followed, at four days, a flare of the inflammatory response which was dependent upon the mitosis of the transferred cells. The normal transfer test with either peritoneal population (Figs. 26-28), or for that matter with any of the populations studied, never exhibited this flare. Indeed a flare was seen only with transferred immune peritoneal exudate and peritoneal blood lymphocytes, and this occurred quite late

after transfer.

We submit, for the following reasons, that the differences noted above were due to the fact that the tissues of X-irradiated guinea pigs suppressed the mitosis of transferred murine cells early in the response. One of the responses of histocompatibility antigen reactive cells to antigenic challenge is proliferation with consequent expansion of the clone of HARCs (Sachs 1971, Wilson 1967, 1968, 1971). In the normal transfer test, this lymphoid proliferation must take place in guinea pig tissues and as can be seen with the X-irradiation data, mitosis did not occur. The fading of the normal transfer test responses could be looked upon as the failure of expansion of that subpopulation of lymphoid cells reactive to guinea pig tissues. On the other hand, in the immune transfer test, proliferation of murine lymphocytes in response to guinea pig cells may take place in the mouse, resulting in an expanded clone of histocompatibility antigen reactive cells. As we demonstrated in the immune transfer test, this enlarged clone of reactive cells not only resulted in a higher peak response, but also a prolongation of the inflammatory response. The late occurrence of the flare in the immune transfer test of peritoneal exudate and blood lymphocytes suggested that this suppressive effect was temporary. It seemed that the content of histocompatibility antigen reactive cells was only sufficiently large enough in the case of peritoneal exudate and peritoneal blood lymphocytes to give a flare response. The in vitro mixed lymphocyte cultures where suppression of DNA synthesis of murine lymphoid cells by X-irradiated guinea pig cells was noted, & the fact that concentrated supernates from cultured X-irradiated guinea pig cells had a similar effect strongly suggested that the suppressive effect in vivo might be due to a soluble factor released by X-irradiated guinea pig spleen cells. Since pretreatment of the host guinea pigs with cobra venum factor which depletes complement (Ballow and Cochrane, 1969) does not change these results, the possibility that this soluble factor is an anti-mouse leukocyte antibody seems unlikely.

In the normal lymphocyte transfer test the reactions caused by exudate lymphocytes were far more intense and lasted longer than normal lymphocytes. While T-cell enrichment increased the response of normal lymphocytes, it did not bring their response up to the levels attained with exudate lymphocytes. This suggested that the differences between them with respect to the normal lymphocyte transfer test were not simply quantitative. Since the graft vs host response required the synergy of several lymphoid subpopulations (Cantor et al, 1970, 1970), perhaps a selective deficiency in a necessary T-cell subpopulation was responsible for these deficits. This aspect of the problem is currently under investigation.

The high initial reactivity of peritoneal exudate lymphocytes in the normal transfer test was hard to justify on the basis of "recognition events" alone, since exudate lymphocytes were generally regarded

as a population of T-cells committed to antigens to which the animal had been recently exposed. With this in mind, it would seem doubtful that exudate lymphocytes from non-immunized mice contained a large population of cells reactive to guinea pig tissues. Because of the suppressive effects of guinea pig cells in the mixed lymphocyte culture, it was impossible to investigate this possibility. We would suggest that the simple act of concentrating inflammatory lymphocytes in tissue was sufficient to cause a reaction of this intensity. The fact that the early stages of the immune transfer test with exudate lymphocytes was no different from the normal transfer test supported this point. Others have demonstrated the non-specificity of a large percentage of the exudate at the site of a delayed type hypersensitivity reaction (Hill, 1969). With these methods of assay, it was not possible to determine what portion of the exudate lymphocytes' early reaction was due to nonspecific factors and which were due to "recognition events". The fact that the stages of the immune transfer test early response of exudate lymphocytes did not differ from the normal transfer test (Fig. 29) suggested that the nonspecific response was indeed great and "masked" whatever specific events occurred.

The complete opposite pattern obtained with normal lymphocytes in which there was a slight initial response which was augmented by immunization. Thus, while the ability to mediate nonspecific inflammatory responses was characteristic of exudate lymphocytes, it was possible that normal lymphocytes possessed more cells potentially reactive to guinea pig tissues. It was probable that the intense early normal transfer test reaction of PBLs (Fig. 33) was due to specific recognition events. Such a view was strongly supported by Brent and Medawar (1963, 1964, 1966). However, with remote immunization, i.e., by a route other than the peritoneal cavity, it was again noted that normal and exudate peritoneal lymphocytes were functionally distinct populations. Although the ILT response of normal lymphocytes was augmented (Figs. 28, 30), it was certainly not as rich a source of immune effector cells as exudate lymphocyte (Figs 29, 30). It is of interest that in terms of pattern of response curves there were two distinct groups: peritoneal exudates with peritoneal blood lymphocytes, and normal peritoneal and splenic lymphocytes.

Such results were, of course, important in demonstrating that normal lymphocytes and exudate lymphocytes were functionally different populations. However, as we indicated in the introduction, these lymphoid cells could profitably serve as models for similar cells accumulating in normal and inflamed tissues. In this context then, we can generalize the following: The resident lymphocytes of a particular tissue contain far fewer histocompatibility antigen reactive cells capable of graft vs host activity than those circulating in the peripheral blood. Remote immunization only slightly augments this graft vs host capacity of the resident cells and it is only when inflammation calls forth an exudate that this activity is intensely increased. Thus immune protection at the site of an emerging neoplasm -

or a newly arrived metasis in the absence of inflammation may be minimal and significant protection only obtained when the tumor incites an inflammatory response. In this regard, the studies of Berke et al (1972) demonstrated that the regression of an intraperitoneal tumor paralleled its ascites-forming capacity. With these applications in mind, we are currently studying the traffic of lymphoid cells into the peritoneal cavity in the normal state and also the physiologic and non-physiologic factors which affect the functional capacity of the resident cells.

J. Immune cytolysis of LLC-MK_2 cells

The appearance of arbovirus antigens on the surface of infected cells was described in the previous annual report. Briefly, a variety of cell cultures infected with group A arboviruses could be lysed by antiviral antibody at the same time that virions were budding from the cell surface. Virus specific immune lysis of group B arbovirus infected cells was more complex. Primary chick embryo cells and continuous BHK-21 cell cultures infected with Japanese encephalitis (JE) virus could be lysed with antiviral antibody as soon as new virus was released from the cell, probably by reverse pinocytosis. However, JE infected LLC-MK_2 cells, which produce more infectious virus than the other cell types, could not be lysed until after cytopathic effects were observed, and then only minimal lysis was obtained as measured by release of absorbed radioactive chromium. LLC-MK2 cells infected with another group B arbovirus, dengue-2, could not be lysed, either, until long after the appearance of infectious virus in the culture fluid. Failure to lyse dengue infected LLC-MK_2 cells when virus was first released from the cell was not due to the absence of dengue antigens on the cell surface; peroxidase-conjugated dengue antibody was found to attach to infected cell surfaces.

We also observed a decreasing susceptibility to dengue specific lysis which appeared to be related to changes in the LLC-MK_2 cells that occurred during subculture. In support of this, preliminary experiments established that after approximately 50 subpassages, LLC-MK_2 cells could not be lysed by anti-LLC-MK_2 antiserum prepared against earlier subpassages which were susceptible to lysis by this antiserum. This decreasing susceptibility to anti-cell lysis closely paralleled the decrease in susceptibility to viral specific lysis. Of particular interest was the fact that high passaged cells productively infected with the arboviruses listed above (or with poliovirus) were again susceptible to high levels of specific lysis with anti-cell antibody, although not with dengue antibody. We repeated these observations with another lot of anti-cell antibody. We felt that productive infection may influence: a) cell fragility; b) the re-expression of cellular antigens previously deleted by subpassage; c) accessibility of membrane antigens by glycocalyx changes; d) any combination of the above.

In the following experiments, specific lysis refers to the percent chromium release in experimentals minus the controls. Maximal lysis represents the largest percent lysis observed in a particular series of experiments. There were essentially two experimental situations: the first when antiserum gave maximal lysis (usually 65 to 70 percent) and the second case when antiserum gave submaximal lysis. When maximal anti-cell lysis was obtained, then there was no increase in lysis following productive infection, and dengue specific lysis was obtained by 48 hours post infection. When anti-cell lysis was submaximal, there was an increase in anticell lysis with productive viral infection, and dengue specific lysis was reduced and delayed up to 96 hours post infection. LLC-MK_2 cells during subculture will revert spontaneously in just a few passages at unexpected times from one condition to the other. Thus, at the present time, high passaged LLC-MK_2 cells with a previously low lytic potential have "transformed" back to maximal specific release to both lots of anti-cell antiserum. As a result, several of the experiments are incomplete at this time.

Cell fragility: Chromium release due to rapid freeze-thawing or gradual decreases in culture fluid osmolarity was the same in both normal and dengue infected LLC-MK_2 cells. This was true when the uninfected cells were exhibiting both high and low specific chromium release to the anti-cell antibody. The results indicated that there was no increase in cell fragility following infection, by these parameters.

Expression of cell antigens (quantitative): In an attempt to determine if infected LLC-MK_2 cells re-express cellular antigens deleted or somehow covered during subculture, we examined the binding of anti-cell antibody to infected and uninfected cells. Normal and dengue-infected cells were incubated with anti-cell antibody prepared in mice, washed extensively, and further incubated with ^{125}I-rabbit anti-mouse globulin. It can be seen in Table 21 that the binding of anti-cell antibody decreased with infection (column 1) even though complement dependent lysis of these cells by the same antibody remained constant up to 72 hours post infection (column 2). The number of cell antigens above a certain density may be unimportant for lysis, and productive infection, while causing a decrease in antibody binding, ensures access to antigens critical to the integrity of the plasma membrane. Unfortunately, these experiments were carried out when uninfected cells were lysed by anti-cell antibody; as with many previous experiments, we had expected to show that uninfected cells could not be lysed by anti-cell antibody until they became productively infected. We are presently testing for reversion to submaximal lysis to complete these results.

Nevertheless, the results suggested that qualitative factors in cellular antigenic expression may be of importance. In contrast to antibody mediated lysis, T cell mediated lysis requires not only a certain antigenic density on the target cell but an antigenic configuration very similar to the immunizing cell (Brunner and Cerrottini, 1971). Subtle changes in antigenic configuration can be detected by this method.

Table 21 - Binding of anti-LLC-MK_2 cell antibody to normal and dengue-2 infected cells by the indirect technique as compared to complement-dependent lysis by the same antibody

	Antibody binding (counts per min.)[a]	Cell lysis (percent chromium release)[b]
Uninfected cells	18,987	64.5
24-hr DEN infected	17,448	66.7
48	10,805	64.3
72	10,838	65.4

[a] CPM due to ^{125}I-rabbit anti-mouse globulin

[b] Percent ^{51}Cr release = CPM supernate/CPM supernate + CPM cells after incubating the cells with guinea pig complement and anti-cell antibody prepared Nov 1971; highest background with antibody alone or complement alone = 7 percent

T effector cells were obtained from peritoneal exudates of mice immunized 9 to 15 days previously with 2 x 10^7 LLC-MK_2 cells. Macrophages were separated by adherence techniques. Lymphoid cells were applied to chromium labeled LLC-MK_2 cells in a ratio of at least 50:1. In initial experiments (July 1973) we obtained 10 percent specific lysis. This value was maintained in four separate experiments over a two-month period. During that time we showed that no increases in specific chromium release could be obtained by any of the following: 1) inclusion of macrophages with the lymphocytes; 2) reduction of killer cell target/cell ratio to 30:1 or an increase to 100:1; 3) a booster immunization with LLC-MK_2 cells in a similar schedule that produced the anti-LLC-MK_2 cell antibody. We began to observe (Sept 1973) a decrease in specific chromium release to 5% and finally to undetectable levels by Oct 1973. Unlike antibody mediated lysis which dramatically increased after virus infection in these experiments, no increase in lysis by LLC-MK_2 cell sensitized T cells was observed post virus infection. The passage level of the cells relative to the source of anti-cell antibody and sensitization of the T cells was quite different. Antibody to the LLC-MK_2 cells was made greater than 50 subpassages earlier and did not lyse the higher passaged cells until they were infected; the T cells were sensitized with the same passage cells

aliquots of which were held in culture until they were used as target cells. This implies that antigenic drift of the cell surface is not the reason for decreasing lytic ability. If antigenic drift were true, then T-cell mediated lysis should not decline as the antibody mediated lysis declines. The increased lysis of productively infected cells by antibody but not by T cells strongly suggested qualitative antigenic changes were taking place in the cultured cells. Further these changes apparently could take place in one or two subpassages.

In view of the differences between T cell and complement-mediated lysis by antibody, we attempted to determine if the differences were due to the antibody by using the lymphocyte antibody-dependent lysis test, a form of noncomplement lysis observed with normal B lymphocytes and specific antibody. Antibody binds to the target cell (specific interaction) and B lymphocytes have a receptor for the Fc piece of the immunoglobulin. This nonspecific reaction brings the lymphocyte into contiguity with the target cell and results in lysis, a phenomenon not completely understood (Yust et al, 1973). Using this test, no lysis (chromium release) was obtained with the mouse anti-LLC-MK_2 cell antibody using either human peripheral blood lymphocytes or mouse spleen cells. Only with human peripheral blood lymphocytes and rabbit antibody could lysis or chromium release be obtained. Dengue infected LLC-MK_2 cells used as target cells for the lymphocytes (usually 150:1) were even more susceptible to lysis by the rabbit anti-cell antibody, an increase from 40 percent to 70 percent. It was of interest that complement-dependent lysis by rabbit antibody was the same before and after infection. The phenomenon of decrease in susceptibility to C^1 mediated anti-cell lysis during cell subpassages, and then an increase following virus infection, is restricted to antibody made in the mouse (at the present time).

Surface coat changes: The glycocalyx or surface coat consists of an epimembranous layer of glycoprotein. This layer if increased during subculture may mask membrane antigens, and possibly be decreased again following productive viral infection. We took advantage of the observation that concanavalin A binds to mannosyl residues in the surface coat of cells and that ^{125}I (CON A) is commonly used to estimate surface coat material. It can be seen in Table 22 that the amount of CON A bound to LLC-MK_2 cells decreased by 48 hours after infection with dengue-2 virus as compared to the controls, and it was substantially decreased by 72 hours post infection. These data indicate only that the surface coat material per culture dish of LLC-MK_2 cells has decreased. Since the monolayer remained confluent, the changes observed are probably not due to the minimal cytopathic effects observed. However, the decrease may reflect changes in the surface area of the cell or decrease in the thickness of the surface coat, or both. We are presently examining these aspects by electron microscopy of cells with specific surface coat stains (e.g., ruthenium red and cationized ferritin). Again, to complete these results, we are testing for reversion to submaximal lysis.

Table 22 - Binding of ^{125}I - concanavalin A to normal and dengue-2 infected LLC-MK_2 cells

^{125}I CON A prep	Uninfected	Dengue-2 infected cells		
		24	48	72
No. 1	34,370[a]	34,469	26,624	20,826
No. 2	16,344	14,473	11,416	9,881

[a] Counts per minute

We have tentatively shown that LLC-MK_2 cells during subpassages decrease or increase in their susceptibility to lysis by anti-cell antibody. When in a decreased state of susceptibility, productive viral infection renders the cells susceptible to lysis again. Their viral induced lytic susceptibility is probably not due to increased fragility, nor in quantitative antigenic changes, but in more subtle qualititative antigenic presentation, perhaps mediated by changes in the surface coat of the cell.

K. Dengue vaccine progress

1. Preparation of a dengue-2 vaccine seed pool in certified primary green monkey kidney (PGMK) cells

As previously described (Annual Report, 1973), small and large plaque virus clones were isolated from a dengue-2 human isolate, PR-159. At the 14th passage level, these clones were tested for plaque morphology, temperature sensitivity, and suckling mouse and monkey virulence. The small plaque clones, S-1 through S-8, had reduced in vivo virulence while the large plaque clones, L-1 through L-5 were similar in virulence to the parent, passage 6 virus. The S-1 clone was purified by three cycles of direct plaque picking resulting in a virus pool at passage 18. After characterization of this pool for homogeneous small plaque virus and low in vivo virulence, a large volume vaccine seed pool was prepared at the 19th passage level. The passage and cloning history for the S-1 seed pool is listed in Table 23. The S-1, p19a seed pool was tested for adventitious bacterial and viral agents by inoculation of bacterial culture media, cell cultures, and adult and suckling mice. No evidence was found for the presence of any bacterial or viral contaminant.

At the 14th passage level, the small plaque clones appeared to contain homogeneous small plaque virus populations. The large plaque clones, however, purified by the same procedures of direct plaque picking, always contained small plaque virus after growth of the final plaque pick in PGMK cells. This may have been due to a rapid reversion to a small plaque virus populations upon passage. The small plaque clones also demonstrated what appeared to be reversion, however, at least two passages in PGMK cells were required to see a detectable rise in a large plaque population. Reversion of S-1 was also dependent on multiplicity of infection (MOI). Passage of S-1 and other small plaque clones at a high MOI, i.e., a passage using undiluted virus stock as the input inoculum, resulted in a reversion to mixed virus populations. After three passages at a high MOI, the resultant virus also appeared to be similar to the parent virus in that mouse virulence returned. The small plaque character of the original stock could be maintained by passage of diluted virus at a low MOI as shown in Table 24. Two small plaque clones at the 14th passage level, S-1 and S-6, were stable for as many passages (6) as we tested under these conditions. In vivo stability of the S-1 clone was tested after one passage in the brains of suckling mice. Only virus capable of producing small plaques was recovered from mice inoculated with S-1 while the parent virus-inoculated mice yielded progeny virus that produced a plaque mixture.

Table 23. Passage and cloning history for plaque variants of dengue-2, PR159

Procedure	Result	Passage	Plaque size in [illegible] cells (mm)	PFU/ml
Source: DEN-2, PR-159 acute human serum				1×10^{3}
Passage in PGMK cells, consecutive 5 day harvests		P-1		$[illegible] \times 10^{4}$
		P-2		$[illegible] \times 10^{4}$
		P-3		$[illegible] \times 10^{4}$
		P-4		$[illegible] \times 10^{5}$
		P-5		$[illegible] \times 10^{5}$
	[illegible] prepared [illegible]	P-6	[illegible]	$[illegible] \times 10^{6}$
P-6 parent virus [illegible] plaqued in PGMK [illegible]	[illegible]			
Virus pools prepared from selected clones		P-7		
P-8 virus pools plaqued in PGMK cells	[illegible]	P-8		
Virus pools prepared from selected clones, P-9	[illegible]		[illegible]	[illegible]
	[illegible]	P-9	[illegible]	[illegible]
Clones 5 and 8 plaque purified 3X in PGMK cells		P-11		
		P-12		
		P-13		
Virus pools prepared from 8 sublines of clone 8 and 5 sublines of clone 5	S-1, 2 and 3 of P-13 sublines from clone 8	P-14	[illegible]	[illegible]
	S-1, 2 and 3 of P-13 sublines from clone 5	P-14	[illegible]	[illegible]
S-1 line plaque-purified 3X in PGMK cells		P-15		
		P-16		
		P-17		
	Virus pool prepared	P-18	[illegible]	[illegible]
S-1, P-18 virus passaged 1X at a low multiplicity of infection	Virus pool prepared	P-19	[illegible]	[illegible]

2. Antigenic properties of the small plaque variants of D-2 virus in lower primates

Subsequent to three direct plaque picks and at the 14th passage level, eight small plaque clones were selected for study in lower primates. Assays for the determination of viremia and antibody levels of these clones were performed in rhesus monkeys. Consistent with the data obtained for small plaque variants in earlier studies (Annual Report, 1973), the eight plaque-purified clones, designated S1/p14 through S8/p14, respectively, showed a marked reduction in virulence (negligible to total absence of viremia) and high antigenicity with significant levels of CF, HI and N antibody being produced (Table 25). To equate these serological findings with protection, two pairs of rhesus monkeys which had received inoculations of S1/p14 and S2/p14 respectively, were held 90 days and then challenged with a subcutaneous injection of the wild-type parent virus PR159/P6. Two non-immunized monkeys were

Table 24. Multiplicity-dependent reversion of the S-1 and S-6 clones

Clone, passage	Input MOI	PFU/ml	SMIC LD_{50}/PFU	Plaque morphology
S-1, p-14		9×10^5	.003	Small
S-1, p-17	4×10^{-5} to 8×10^{-6}	5×10^5	0.008	Small
S-1, p-17	4×10^{-1} to 8×10^{-2}	3×10^5	0.1	Mixture
S-6, p-14		2×10^6	0.0008	Small
S-6, p-17	1×10^{-5} to 1×10^{-6}	1×10^6	0.006	Small
S-6, p-17	1×10^{-1} to 6×10^{-1}	1×10^5	0.2	Mixture
S-6, p-20	5×10^{-4} to 4×10^{-5}	1×10^5	ND[a]	Small

[a] Suckling mouse titration not done.

Table 25. Virulence and immunogenicity of selected small-plaque clones of the dengue-2 virus in the rhesus monkey

MONKEY NO.	CLONE NO.	INOCULUM PFU/ML	VIREMIA ON DAY POST INOCULATION (PFU/ML) 1	2	3	4	5	6	7	8	9	10	1/CF 30d.	60d.	1/HI 30d.	60d.	90d.
866	S1/p.14	$15x10^5$	0	0	0	0	0	0	0	0	0	0	128	128	160	80	770
859			0	0	0	0	0	0	0	0	0	0	<4	128	80	80	410
861	S2/p.14	$25x10^5$	0	0	0	0	0	0	0	0	0	0	128	<4	160	20	1420
825			0	0	0	0	0	0	0	0	0	0	128	256	160	<40	1440
826	S3/p.14	$25x10^5$	0	0	0	0	0	0	0	0	10	0	128	128	320	40	1350
880			0	0	0	0	0	0	0	0	0	0	128	256	160	80	540
868	S4/p.14	$25x10^5$	5	10	0	0	0	0	0	0	0	0	128	<4	80	40	370
858			0	0	0	0	0	0	0	0	0	0	128	<4	160	40	<80
885	S5/p.14	$5x10^5$	0	0	0	0	0	0	0	0	0	0	<4	32	160	40	480
020			0	0	0	0	0	0	0	0	5	35	256	128	<40	80	380
025	S6/p.14	$5x10^5$	0	0	0	0	0	0	0	0	0	0	<4	32	80	40	160
382			0	0	0	0	0	0	0	0	0	0	128	32	160	40	440
384	S7/p.14	$5x10^5$	0	0	0	0	0	0	0	0	0	0	16	8	80	40	350
397			0	0	0	0	0	0	0	0	0	0	<4	16	80	40	ND
558	S8/p.14	$5x10^5$	0	0	0	0	0	0	0	0	0	0	128	32	<40	80	ND
572			0	0	0	0	0	0	0	0	0	0	<4	32	320	40	ND
238	PR159/p.6	$9x10^5$	0	0	5	85	150	55	10	0	0	0	128	<4	160	80	810
988			0	0	5	30	375	400	80	0	0	0	128	32	320	80	ND

* Fifty percent plaque-reduction neutralising antibody test.

included as controls. Following challenge, all monkeys were bled on 10 consecutive days for the detection of viremia and at intervals of 15, 30 and 60 days post-inoculation for the determination of CF and HI antibody levels. None of the previously immunized monkeys developed a detectable viremia, whereas, both control monkeys were viremic for 3 and 4 days, respeccively. Also, the absence of a rise in the CF and HI tests performed on the immunized monkeys subsequent to challenge, indicated that the S1/p14 and S2/p14 clones conferred a solid immunity. Results of these tests are shown in Table 26.

Table 26. Protective efficacy of selected small-plaque clones in rhesus monkeys inoculated 90 days later with the wild-type PR159/p.6 dengue virus

MONKEY NO.	ORIGINAL INOCULUM (PFU/ML)	CHALLENGE VIRUS & DOSE (PFU/ML)	VIREMIA *	PRE-CHALLENGE 1/CF	1/HI	POST - CHALLENGE 1/CF 15d.	60d.	>60d.	1/HI 15d.	30d.	>60
866	S1/p.14 (15×10^5)	PR159/P_6 (7×10^5)	N	1.2	1.6	..	..	..	80	80	80
859			N	..	..	..	..	..	160	80	40
861	S2/p.14	PR159/p.6	N	..	..	..	..	..	40	40	..
825	(25×10^5)	(7×10^5)	N	..	..	1.2	..	..	..	..	..
826			YES	..	..	..	..	..	..	..	..
880		PR159/p.6 (7×10^5)	YES	..	..	..	1.2	1.2	..	..	..

* Both control monkeys had viremias of 3 and 4 days duration, respectively.

During the course of passage work with the small plaque variants, it was frequently observed that at high multiplicity of infection (MOI), resulting seed pools contained both large and small plaques whereas with low MOI harvests, only small plaques were obtained. On the basis of this information it was considered appropriate to determine if there was any difference in the expression of virulence between the "mixed" and the "pure" small plaque virus pools. For this determination a number of rhesus monkeys were inoculated with virus pools containing mixed large and small plaque progeny and pure small plaque progeny, respectively. Although there was no significant variation observed in the serologic response between monkeys receiving mixed or pure plaque progeny, it is clearly evident from the viremia data that the mixed plaque populations were associated with enhanced virulence (Table 27). This observation clearly establishes the necessity of passing vaccine candidate strains of virus at the lowest permissible MOI in order to maintain the attenuated virulence characteristic. Further investigation of this "reversion phenomenon" is being made and will be reported on later.

Under normal conditions the body temperatures of rhesus monkeys will fluctuate between 37.5 and 39.8 C, and since the small plaque variant of PR159 virus has shown some evidence of temperature sensitivity, it was suspected that following inoculation with the attenuated strains, viremia levels might be abnormally suppressed at the elevated body temperatures. To test this hypothesis, it was decided that inoculations of selected small plaque clones be made in a primate whose body temperature more closely approximated that of man. Chimpanzees were considered to be a suitable host since their body temperature under normal conditions will vary only from 37.5 to 38.5 C. Utilizing the PR159/P6 wild-type virus pool, which contains both large and small plaque variants, and the S1/P19a pool containing only small plaque progeny, two pair of chimpanzees were inoculated by the subcutaneous route with each of these suspensions, respectively. Each animal was bled on 10 consecutive days for the detection of viremia and on days 15 and 30 post-inoculation for antibody studies. The results are presented in Table 28. It is clearly evident from the data that S1/P19a virus is associated with reduced virulence in a primate host whose body temperature approximates that of man. Both chimpanzees receiving the wild-type PR159/P6 virus developed potent and persistent viremia of several days duration. Significant levels of CF and HI antibody were elicited by both the attenuated and wild-type parent virus.

Table 27. The relative virulence and immunogenicity of mixed large and small plaque populations vs. a pure small plaque pool when inoculated into rhesus monkeys

MONKEY NO.	INOCULUM AND DOSE(PFU/ML)	VIREMIA ON DAY POST INOCULATION(PFU/ML) 1	2	3	4	5	6	7	8	9	10	1/CF 30d.	60d.	1/HI 30d.	60d.
283	S1/p.17 (mixed)	5	[illegible]	100	210	140	20	0	0	0	0	32	128	320	160
300	7×10^5	25	55	30	85	10	0	0	0	0	0	512	[illegible]4	160	160
392	S6/p.17 (mixed)	0	5	10	5	0	5	0	0	0	0	32	[illegible]4	160	160
383	5×10^4	0	10	10	30	20	0	0	0	0	0	1.8	[illegible]4	160	160
384	S6/p.17a (pure)	5	10	0	0	0	0	0	0	0	0	1.8	32	160	80
004	5×10^5	0	0	0	0	0	0	0	0	0	0	1.8	[illegible]4	160	80
398		0	5	0	0	0	5	0	0	0	0	[illegible]4	[illegible]4	160	160
391	S1/p.18a (pure)	0	0	0	0	20	0	0	0	0	0	1.8	1.8	320	80
396	5×10^4	0	0	0	0	0	0	0	0	0	0	[illegible]4	1[illegible]	[illegible]0	40
397		0	0	0	0	0	0	0	0	0	0	1.8	[illegible]4	40	80

Table 28. Relative virulence and immunogenicity of a plaque-purified clone vs. the wild type parent strain of dengue-2 virus when inoculated into chimpanzees

CHIMP NO.	INOCULUM AND DOSE(PFU/ML)	VIREMIA ON DAY POST INOCULATION(PFU/ML) 1	2	3	4	5	6	7	8	9	10	1/CF 15d.	30d.	1/HI 15d.	30d.
381	S1/p.19a	0	0	0	0	5	0	0	0	0	0	[illegible]4	1.8	80	160
8[illegible]	3×10^4	0	5	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]4	10	40
380	PR159/p.[illegible]	5	15	50	800	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0	512	512	1/80	[illegible]40
383	30×10^5	0	5	100	10[illegible]	1[illegible]	10	[illegible]	[illegible]	0	0	1.8	1.8	160	320

3. Evaluation of the neurovirulence of the S1/P19a attenuated strain of D-2 virus in lower primates

In collaboration with the Division of Pathology, a comparative study of the neurovirulence of the non-attenuated PR159/P6 wild-type virus and the attenuated S1/P19a strain was made in rhesus monkeys.

Two monkeys from each group of five inoculated intracerebrally with the attenuated S1/P19a strain and PR159/P6 wild-type, respectively, were bled on 10 consecutive days for the determination of viremia, and on the day of sacrifice (day 19 post-inoculation) for CF and HI antibody levels. The results are presented in Table 29.

From the data presented it appears that S1/P19a virus exhibits a lower level of virulence than the PR159/P6 wild-type. These data support the following histopathological findings, wherein, those monkeys inoculated with the PR159/P6 wild-type virus exhibited a more diffuse dissemination and twice as many microscopic lesions as the group inoculated with the attenuated S1/P19a strain.

Table 29. Viremia and serological data on monkeys used in neurovirulence studies

MK #	Inoculum (D-2 strain)	Viremia on Day Post-Inoc. (PFU/ml) 1	2	3	4	5	6	7-10	Day 19 CF	Serology HI
970	PR159/P6	0	10	5	0	0	0	0	256	160
972		0	5	135	235	300	55	0	512	320
962	S1/P19a	0	0	0	0	0	0	0	64	160
979		0	0	0	5	0	0	0	64	160

To compare the neurovirulence of the attenuated S1/P19 strain of dengue-2 virus with that of the nonattenuated parent, PR159/P6, twelve conditioned, tuberculin-negative, juvenile rhesus monkeys were used. Following anesthesia and surgical preparation, a midline skin incision was made over the frontal and parietal sutures of the skull. After locating the injection site by stereotoxic methods, a burr hole was made in the right parietal bone. A 22 gauge, 3-1/2 inch needle was then passed through the hole and into the brain until the desired intrathalamic depth was reached. A volume of 0.5 ml of fluid was then injected and the needle removed. Five animals received 5 X 10(5) PFU of viral strain PR159/P6 in 0.5 ml of vehicle consisting of African Green monkey cell culture fluid containing 50% fetal bovine serum. These animals also received 2 X 10(5) PFU of strain PR159/P6 via intrathecal injection of the lumbar spinal cord at level between vertebrae L1 and L2. Five other animals received intrathalamic injections with 5 X 10(3) PFU of strain S1/P19 in 0.5 ml of vehicle. These also were given 2 X 10(3) PFU of strain S1/P19 by intrathecal injections at lumbar cord level L1-L2. The remaining 2 monkeys served as controls and were given intrathalamic injections of 0.5 ml of vehicle alone and intrathecal injections of 0.2 ml of vehicle at lumbar cord level L1-L2. Identification numbers of monkeys receiving the two strains of virus are shown below in Table 30.

Table 30. Identification numbers of rhesus monkeys inoculated with the parent strain (PR159/P6) and a small plaque clone (S1/P19) of dengue-2 virus

PR159/6	S1/P19	Controls
970	962	967
972	966	969
978	968	
980	979	
988	984	

After 19 days of clinical observation, with serologic and virologic studies, the animals were sacrificed. Each monkey was first sedated with phencyclidine HCL, then anesthetized by intravenous injection of pentobarbital sodium. The heart was surgically exposed and a cannula introduced into the beating left ventricle. An 0.85% saline solution was then introduced into the ventricle and allowed to flow by gravity. The vena cava was severed thus allowing the animal to exsanguinate. After perfusion with 300-500 ml of saline, the animal was perfused with 500-700 ml of 10% neutral buffered formalin. A complete autopsy was performed on each animal. Brains and spinal cords were removed in toto and preserved in 10% neutral buffered formalin. Representation samples of the remaining organs were also removed and stored for reference in formalin. After formalin fixation for 10 days, each brain was sectioned transversely into 4-5 mm thick slides. Following gross examination, 6 sections of brain were selected for histopathologic examination from the following anatomic sites:

Right and left hemispheres at level of optic chiasma - 2 sections

Right and left hemispheres at level of middle portion of cerebral peduncles - 2 sections

Pons and cerebellum at rostral end of brachium pontis - 1 section

Pons and cerebellum at caudal end of brachium pontis - 1 section

Five sections of spinal cord were selected from the following approximate levels: C-4, T-6, T-12, L-1, and L-2. In some instances, because the desired level of cord was damaged during post mortem removal, sections were taken from the nearest intact portion. After processing, 2 thin sections were cut from each of the 11 blocks of

brain and cord and stained with hematoxylin and eosin. Thus, a minimum of 22 sections from each animal was examined microscopically. The histopathologic grading system shown below is a modification of that used by Nathanson et al., 1966.

Grade 1 - Occasional (usually 1 or 2) small perivascular cuffs or glial nodules; no neuronal changes.

Grade 2 - Moderate number of small cuffs or glial nodules; neuronal changes may be seen, but less than 30% involved.

Grade 3 - Marked number of perivascular cuffs; extensive neuronal changes.

All sections were numerically coded and the histopathologic observations made without the investigator's prior knowledge of the treatment group to which each animal belonged. Gross examination of the brains revealed resolving needle tract lesions in the right thalamus of all 12 monkeys. There were no grossly detectable differences in the severity of these induced lesions in any of the animals. None of the animals had gross lesions in their spinal cords.

None of the lesions in either treatment group or in the 2 control monkeys exceeded Grade 1 in severity. In the 5 monkeys treated with strain PR159/P6, there were 20, grade-1 brain lesions (mean of lesions per animal), and 18, grade-1 spinal cord lesions (mean of .6 per animal). In the 5 monkeys treated with strain S1/P19, there were 10, grade-1 brain lesions (mean of 2.0 per animal), and 5, grade-1 spinal cord lesions (mean of 1.0 per animal). The 2 control animals had 2 brain lesions (mean of 0.5 per animal) and 2 spinal cord lesions (mean of 1.0 per animal).

The microscopic lesions in both groups of virus-inoculated monkeys were more numerous and widespread than those in the 2 control monkeys. The microscopic lesions in those monkeys inoculated with the wild strain, PR159/P6, were more numerous and widespread than those in monkeys inoculated with the attenuated strain S1/P19. From the results of these histopathologic observations, we conclude that the neurovirulence of the attenuated strain. S1/P19 is clearly lower than that of the wild strain P159/P6.

4. Time of effect of the non-permissive temperature

Temperature-shift experiments were carried out in primary African green monkey kidney cells to determine when the non-permissive temperature exerted its effect. A concentrated virus preparation was needed for experiments where high MOI's were required. Concentrated seeds usually had higher reversion frequencies compared to unconcentrated seeds. Reversion frequency (RF) for a seed is defined as the ratio of PFU formed at 39.5 C/PFU formed at 35 C, where 39.5 C is the non-permissive temperature for S-1 in LLC-MK_2 cells and 35 C is the permissive temperature. Multiplicity-dependent leakiness may account for the rapid reversion of the S-1 clone at a high MOI. The S-1, p-19a seed was examined for possible isolation of a subclone that had more stable characteristics. The S-1 seed was inoculated at a high dilution (10^{-3}) onto PGMK cells contained in 96 wells of a Microtest (Falcon) cell culture plate. After seven days, each well was harvested and tested for virus by plaquing in LLC-MK_2 cells. Sixteen clones were isolated by this technique. One of these subclones labeled M-4 contained virus that produced very small and faint plaques. The M-4 subclone, concentrated by pelleting at 30K rpm for 3 hr had a lower RF (4×10^{-4}) than another of the subclones, M-12 (10^{-1}) or the original S-1 seed (2×10^{-3}). The M-4 subclone will be used for all high MOI experiments.

In order to locate the temperature sensitive defect in the S-1 clone, shifts were made at various times during the latent period from the permissive (35 C) to the non-permissive (39.5 C) temperature and vice versa. Harvests taken at 24 hrs post inoculations were low in titer and the results of the 48 hr harvest are shown in Table 31. Reduced virus yields result when cultures infected with S-1 are kept at 39.5 C for greater than 9 hrs. Conversely, those shifted from 35 C to 39.5 C before or at 9 hrs post inoculation also show reduced virus yields. The temperature sensitive event appears to occur late in the latent period (9-13 hr) and may involve inhibition of maturation of dengue virions. Although it appears that the S-1 clone is an RNA+ mutant, this must be demonstrated by recovery of infectious or labeled RNA from cultures held at the non-permissive temperature.

5 Inactivation kinetics of the S-1 and parent viruses

The S-1 and parent viruses were diluted in EBSS with the normal concentration of Mg^{++} and Ca^{++} and also in EBSS containing no Mg^{++} or Ca^{++} cations. Inactivation of approximately 1000 PFU of each virus seed was carried out at 27 C or 35 C. Controls held at 4 C indicated that the GM-6 parent was unstable at this temperature over a period of 2 hr if the cations were not present. A loss of

Table 31. Effects of temperature shifts on replication of the S-1 clone

Shift	Yield (48 hr) PFU/ml
35 C → 39 C, 5 hr	350
9 hr	300
13 hr	1200
39 C → 35 C, 5 hr	2500
9 hr	1200
13 hr	350
17 hr	100

37 to 73% of the original viral infectivity was seen for GM-6 while S-1 showed no loss of infectivity. At 27 C, inactivation of the GM-6 seed was more rapid than S-1 in the presence of cations as shown in Figure 34a. The activation phase for S-1 seen in the first hour of heating at 27 C was more marked at 35 C as seen in Figure 34b. This may be due to a deaggregation phenomenon that is dependent on temperature and which is characteristic for the S-1 clone. The inactivation rate for S-1 at 35 C in the second hour of heating after the activation phase, is actually greater than the inactivation rate for GM-6 over this same time period. Thermal stability of the S-1 clone as measured by rate of inactivation is therefore less than the parent virus after an initial deaggregation activation phase at 35 C.

6. Characteristics of dengue-2 strains passaged in fetal rhesus lung (FRhL) cells

Since primary African green monkey kidney cells may not be acceptable as a virus substrate for vaccines for peripheral inoculation in humans, an alternate cell line was investigated. The most promising substrate appears to be a diploid cell line derived from fetal rhesus lung tissue by Petricciani at NIH, and designated FRhL-2. This cell line has been exhaustively tested and found free from adventitious agents, tumorigenicity and karyological

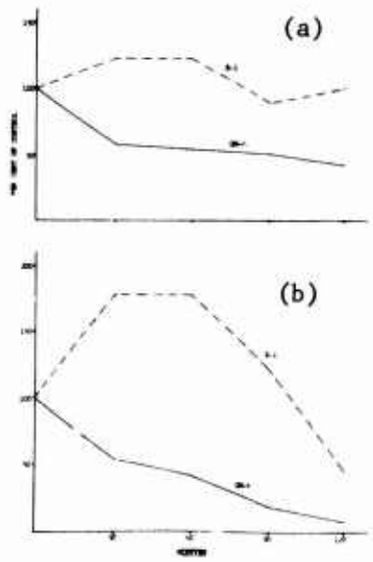

Figure 34. Inactivation kinetics of S-1 and GM-6 (parent) virus seeds: (a) 27 C, with cations; (b) 35 C, with cations

abnormalities. Attempts were made to pass and adapt the S-1, p-14 or S-1, p-19a virus seeds for growth in FRhL cells. A low yield of 2 to 3 logs of virus was found after one passage of the p-14 or p-19a virus in FRhL cells. With S-1, p-14, reversion to a mixed large and small plaque population was evident after one passage as listed in Table 32, while the S-1, p-19a virus was stable after one passage but showed reversion to a virus plaque mixture after two passages in FRhL cells. Further passage of S-1, p-19a in FRhL cells as listed in Table 33 yielded a high titer seed that had a mixed large and small plaque population and was not temperature sensitive. The S-1, FRhL-4 seed also produced viremia in two monkeys inoculated with it (Table 34). However, when the first AGMK passage of the PR159 strain was passed through FRhL cells four times, and each harvest was delayed until the 14th day or later (see Work Unit 177, Annual Report, 1973), we found that the progeny virus contained only small plaques, that it was temperature sensitive, and that it produced transient viremia in one of two monkeys (Table 11). By comparison, the parent virus produced a viremia that reached relatively high titers and lasted for 5 days. The observation that late harvest produced a small plaque-forming and temperature sensitive virus is currently being repeated.

Table 32. Replication of the S-1 clone in FRhL cells from an early and late passage in PGMK cells

Virus: S-1, p-14			Virus: S-1, p-19a		
Day	Yield PFU/ml	Plaque morphology	Day	Yield PFU/ml	Plaque morphology
2	1.3×10^2	Small	1	50	Small
4	2.2×10^3	Mixture	3	1×10^2	Small
6	4.5×10^2	Mixture	5	8×10^2	Small
			7	1.2×10^3	Small
			8	6×10^2	Small

Table 33. Passage of S-1, p-19a seed in FRhL cells

FRhL passage	PFU/ml	Plaque morphology	Monkey viremia	Temperature sensitivity
0	1×10^5	Small	-	+
1	1.1×10^3	Small	ND	ND
2	3×10^4	Mixture	ND	ND
3	3×10^6	Mixture	ND	ND
4	2×10^6	Mixture	+	-

Table 34. Virulence and antigenicity of two strains of D-2 virus passaged in fetal rhesus lung (diploid) cells (FRhL-2)

MK #	INOCULUM (DOSE)	VIREMIA ON DAY POST-INOCULATION (PFU/ML.) 1 2 3 4 5 6 7 8 9 10	IMMF. FIX. 15 30 45 60	HEMAGG.INHIB. 15 30 45 60
271	PR15-GMK-1 ($\approx 10^6$)	0 0 0 0 0 0 0 0 0 0	1+ 1+ 1+ 1+	1+ 1+ 40 40
265		0 0 0 0 0 0 0 0 0 0	1+ 1+ 1+ 1+	40 40 80 80
274	S1 P14 FRhl 4 (2×10^6)	0 0 0 0 0 0 0 0 0 0	1+ 1+ 1+ 1+	0 40 40 40
270		0 0 0 0 0 0 0 0 0 0	1+ 1+ 1+ 1+	40 80 40 40

7. Passage of dengue-3, CH53489 in PGMK cells

A human serum isolate of dengue-3, CH53489 from Bangkok was typed by neutralization tests and screened for hepatitis B antigen by radioimmunoassay. Replication of this isolate in PGMK and other certified cells will determine the usefulness of this isolate as a candidate source for a dengue-3 vaccine seed. Passage history of the isolate in certified (Lederle) PGMK cells is listed in Table 35. From the 1st passage a virus harvest was obtained

Table 35. Passage history of dengue-3, CH53489, in PGMK cells

D-3 seed	SM death* Und	-1	PFU/0.2 ml + morphology 35 C/morph	39.5 C/morph	Efficiency of plating: 39.5 C/35 C
p-1, day 10	2/13(15%)	4/10(40%)	1×10^4 /S	13/S	0.001
p-2, day 5	2/9(22%)	5/14(36%)	4×10^4/L+S 4×10^{-2}	4×10^2/S	0.01
p-2, day 10	4/14(28%)	1/13(8%)	5×10^5/L+S	ND	
p-3, day 5	5/13(39%)	non-specific deaths	1×10^4/L+S	4×10^2/L+S	0.013
p-3, day 10	ND	6/8(75%)	3×10^4/L+S	ND	
p-4, day 5	10/13(77%)	1/14(7%)	2×10^4 L+S**	3×10^2/L+S	0.015
p-4, day 10	ND	ND	2×10^4 L+S**	2×10^1/L+S	0.028
D-3, SMB -27 H-87	SMIC LD_{50} = 5.0/0.02 ml		1×10^6/S	4×10^5/S	0.4

* Deaths scored from day 10 - 25

** Approximately 1/2 and 1/2 S

after 10 days that contained small plaque virus. Upon passage, emergence of several virus populations appeared, finally resulting in a ratio of 50% small (1-2 mm) and 50% large (3-5 mm) plaque-forming virus at the 4th passage level. The efficiency of plating (EOP = PFU titer at 39.5 C/PFU titer at 35 C) also increased upon passage along with a slight increase in suckling mouse virulence. A mouse-adapted strain of dengue-3 (SMB-27, H-87) was also tested for plaque size, mouse virulence, and ts^+ characteristics. In this case the dengue-3, H-87 seed contained small plaques but had high suckling mouse virulence and an EOP value of 0.4. The dengue-3, CH53489 strain is also being passaged in mouse brain for development of similar characteristics.

8. Clone selection from dengue-3, CH53489 seed

The 4th passage, day 5 harvest of dengue-3, CH53489 was prepared as a large volume seed (50 X 2 ml, 50 X 4 ml) and will be used as the parent virus for isolation and purification of virus clones with suitable characteristics of attenuation. The parent virus was plaqued in PGMK cells at a terminal dilution and well isolated plaques picked and grown in PGMK cells under fluid culture media. Of 14 picks made, only three contained virus in seven day harvests as shown in Table 36. One of these three, pp-6 appears to be ts^+ at 39.5 C and will be purified and characterized further.

The dengue-3 parent virus seed was also mutagenized by replication in the presence of 5-azacytidine (5-AC) for the selection of chemically derived mutants. The mutagen was tested for cytotoxicity at concentrations of 25, 50, 100, and 150 μg of the drug per ml of cell culture maintenance media. The 5-AC was added after virus adsorption and remained in the culture media until the day of harvest (day 4). As listed in Table 37, 25 to 50 μg/ml of 5-AC reduces the virus titer 1 to 2 logs while being minimally cytotoxic for the PGMK cells. There is some evidence for selective inhibition of replication of large plaque virus by 5-AC. In the control harvest that received no 5-AC, there existed a 31/240 ratio of L/S plaques while the virus grown in the presence of 25 μg/ml of 5-AC had 5/280 ratio.

Table 36. Dengue-3, CH53489 naturally-occurring clones at the passage 6 level

Dengue-3 clone	PFU/ml 35 C	39.5 C	Plaque morphology
pp-5	1×10^4	1.5×10^3	Small, 1-2 mm
pp-6	2.5×10^3	0	Small, 1-2 mm
pp-12	2×10^3	1×10^2	Small, 1-2 mm

Table 37. Effect of 5-azacytidine (5-AC) on the replication of dengue-3 virus

Conc 5-AC µg/ml	Cell toxicity[a]	Virus yield PFU/ml
25	trace	1.5×10^4
50	1 to 2 +	1×10^3
100	3 +	5
150	3 to 4 +	0
Control	0	1×10^5

[a] Toxicity measured as % cell sheet destroyed.

L. Japanese encephalitis (JE) virion and soluble antigens

As previously reported (Annual Report, 1972, 1973), JE virions contain at least one complement fixing (CF) antigen that is associated with the major envelope polypeptide. This antigen cross reacts in CF and immunodiffusion (ID) tests with other members of the JE subgroup. The antigen can be purified by solubilizing JE virions with sodium sulfate (SLS) and followed by gel filtration chromatography. The major envelope antigen has a molecular weight (mw) of 58 X 10^3 daltons.

A naturally-occurring soluble CF (SCF) antigen is found in JE subgroup virus-infected mouse brain homogenates rendered free of hemagglutinins by centrifugation. These antigens have mw's ranging from 45 to 67 X 10^3 and show increased type specificity over crude antigens in CF and ID tests. Dengue SCF antigens prepared by the same techniques also have increased type specificity upon purification (Russell, Chiewsilp, and Brandt, 1970). The antigenic, biophysical, and biochemical relationships of the JE SCF antigen to the JE major envelope antigen have been investigated. Further evidence that the JE SCF antigen has common similarities with the dengue SCF antigens broadens understanding of the immunologic response to group B arboviruses.

Isoelectric focusing and biophysical characterization of the JE SCF antigen. Further purification of the JE SCF antigen by isoelectric focusing (IEF) was carried out in ampholyte gradients ranging from pH 3 to 10 and stabilized in sucrose (0 to 70%) with sulfuric acid as the cathode and ethanolamine acting as the anode. The sample was loaded in the 10 to 20% sucrose layer and focused with a current of 850 volts. Focusing at a controlled temperature of 20 C continued for approximately 48 hrs until the amperage dropped to 3 milliamps. Fractions (2 ml) were collected and the pH and CF antigen activity determined. Dr. Joel Dalrymple directed the IEF operation.

The JE SCF antigen from Sephadex columns and purified by IEF contained a major CF antigen with an isoelectric point (pI) of approximately 5.3 while several minor CF antigen zones were found at higher pH values as shown in Figure 35. The pI 5.3 antigen was concentrated by freeze drying and further characterized by treatment with SLS and 2-ME. Dengue SCF antigens could be distinguished from antigens of the virion and the slowly sedimenting hemagglutinin by this treatment (Brandt, Cardiff, and Russell, 1970). The JE SCF antigen was stable under these conditions, however, JE virion CF antigens were reduced in titer 8-fold as shown in Table 38. These results paralleled those obtained with dengue SCF antigens.

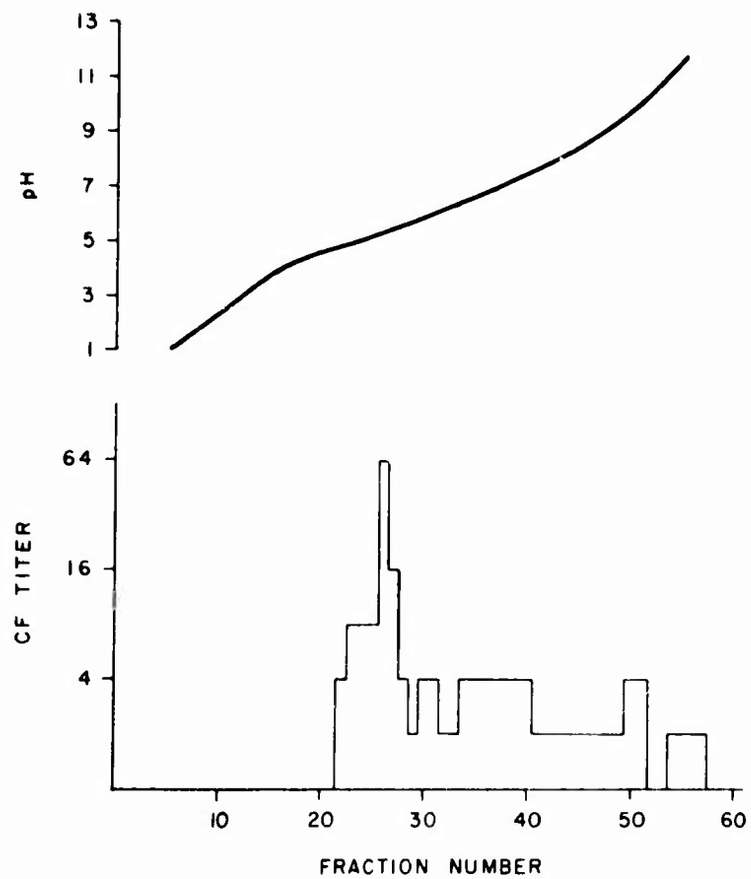

Figure 35. Isoelectric focusing of a Sephadex-purified JE SCF antigen.

Table 38. JE virions and JE SCF antigen treated with SLS and 2-ME

Antigen	Control	SLS/2-ME[a]
JE-SCF	16[b]	16
JE virion	16	2

[a] Antigen was treated with 0.1% and 0.1% 2-ME at 35 C for 30 min; following 18 hr dialysis against 500 volumes of 0.01 M PBS, the antigens were titrated for CF activity; controls were treated with 0.01 M PBS in place of SLS and 2-ME.

[b] Reciprocal of the CF titer per 25 µl.

Antigenic characterization of the JE SCF antigen. The IEF-purified JE SCF antigen also showed increased type specificity over the Sephadex-purified SCF antigen in CF tests. In Figure 36 pre- and post-IEF purification indicates a reduction in cross reactivity of the JE SCF antigen with closely related Murray Valley and West Nile virus antibody. The same antigens in ID tests demonstrated identical reactions with the formation of two bands of precipitation against homologous hyperimmune ascitic fluid. These two bands, also seen in JE virion and envelope ID reactions with homologous antibody (Annual Report, 1972, 1973) appear to differ in cross reactivity with other JE subgroup viruses. The minor band 1 closest to the antigen well appears to be type specific. Preliminary data show that two immunoglobulins, probably IgM and IgG, are responsible for the group and type specific reactions respectively. The two immunoglobulins, in different concentrations, react with two determinants on the same antigen molecule or determinants located on separate molecules. If the determinants are on separate molecules, separation techniques so far have not demonstrated that the antigen preparations are heterogeneous. The antigenic relationship between JE envelope antigen and JE SCF antigen by ID test is shown in Figure 37. The precipitin line

closest to the antibody well was continuous for both antigens, however, spur formation in the major precipitin band clearly demonstrated the presence of an additional determinant on the SCF antigen indicating some molecular difference between the envelope and SCF antigens in JE virus preparations. The sharing of antigenic determinants between these two JE antigens has not been demonstrated for dengue SCF antigens and dengue hemagglutinins (Cardiff et al., 1971). The JE SCF antigen being associated with a non-structural protein as is the case with dengue has not been clearly demonstrated and depends on further characterization of this antigen.

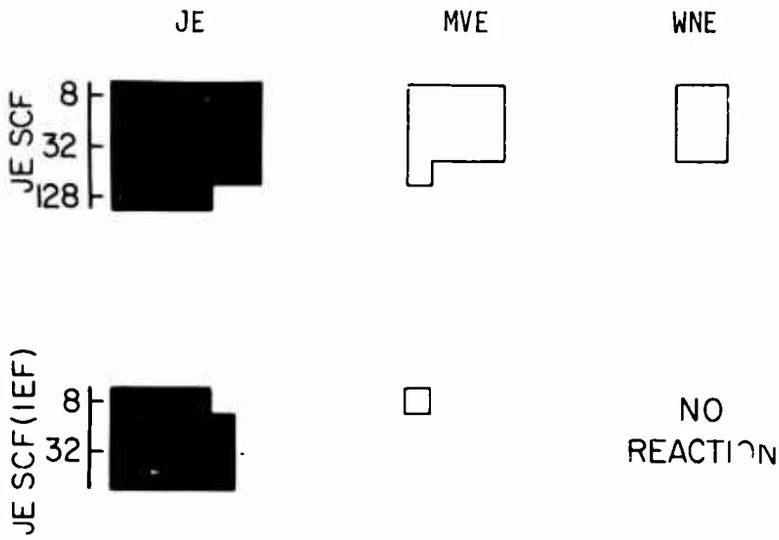

Figure 36. Block CF titrations of JE SCF and JE SCF(IEF) antigens against Murray Valley encephalitis (MVE) and West Nile encephalitis (WNE) antibody.

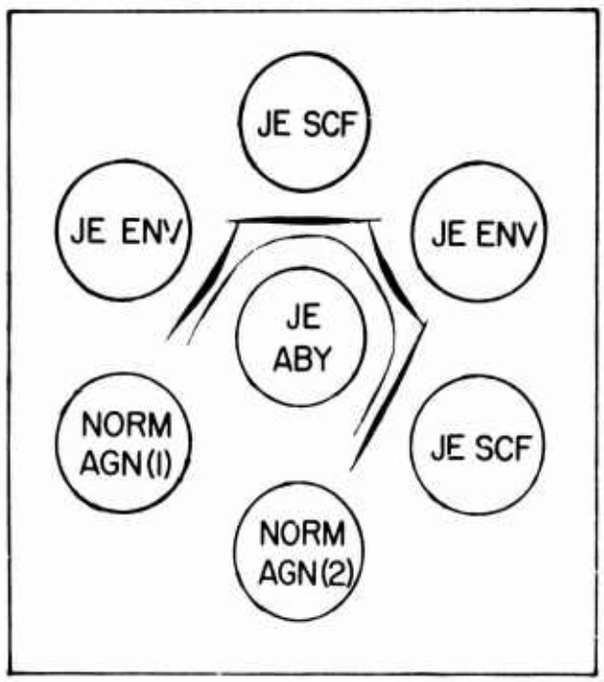

Figure 37. JE envelope and SCF antigens reacted against homologous antibody in an immunodiffusion test. Normal antigens consisted of uninfected mouse brain prepared identically to JE virions and solubilized with SLS (NORM AGN (1)), and uninfected mouse brain prepared identically to JE SCF antigen (NORM AGN(2)).

M. Structural proteins of BFS-283, California encephalitis virus

The polypeptides of BFS-283 CEV have been analyzed by polyacrylamide gel electrophoresis. Six radioactive peaks were detected in purified virions grown in two different cell types. Five of these radioactive peaks contain glucosamine, two of which appear to be glycoproteins in nature. When virions grown in the two different cell types were compared by coelectrophoresis, host dependent differences were detected in the electrophoretic mobility of some glycoproteins but not with the nonglycoprotein. This may suggest that the carbohydrate moiety of these proteins may be host cell specified. The molecular weights of the six radioactive peaks, coelectrophreesed with Sindbis virus marker proteins, ranged from 82,000 to 17,500, with a total molecular weight of 269,500.

Treatment of virions with the non-ionic detergent, NP-40, allowed detection of a light sedimenting material which contained all of the polypeptides except the smallest, VP-1 ≅ 17,500 daltons.

BFS-283 virus was originally isolated from a pool of Culex tarsalis mosquitoes collected in Kern County, CA in June of 1944 (Hammon and Reeves, 1952). Subsequently, it was serologically grouped into the California encephalitis virus complex (Hammon et al, 1952). Electron microscopic observations show that the virion has a mean diameter of approximately 98-100 nm, and contains an electron-dense core with spiked outer projections (Murphy et al, 1968). Recently Rosato et al (1974) reported the detection of three proteins of two different CEV complex members, including BFS-283. A detailed study of another member of the CEV complex (La Crosse), showed that the virion contained three structural proteins and a complex of six RNA species, and had a molecular weight of 2.8×10^8 daltons (McLerran and Arlinghaus). A virus of this size, weight, and RNA complexity should have a greater coding capacity than three polypeptides with a total m.w. of 156,000 daltons. However, the number of nonstructural proteins specified by this virion has not been determined. Studies on the biochemical and biophysical properties of the more closely related viruses within the CEV complex, such as BFS-283, Snowshoe Hare, Jamestown Canyon, should aid in understanding some of the basic characteristics of what seems to be a very complex and heterogeneous group of viruses. In this present study, we have analyzed the polypeptide compositions of purified BFS-283 virions produced in BHK_{21}-Clone 13 and $LLC\text{-}MK_2$ cells. The polypeptides and carbohydrate moiety of the virus have been studied by using radioactive amino acids and sugars.

Materials and methods

Virus: A lyophilized BFS-283 strain of CE virus was obtained from ATTC (Rockville, MD) in the 24th mouse passage. Seed stock was prepared in 1-3-day-old mice; suckling mouse brain (SMB) was ground with a pestle in a sterile mortar and diluted 1/5 (w/v) in medium 199 with

5% fetal calf serum, and frozen at -70°C.

Cells: Baby hamster kidney (BHK-21, Clone-13) cells were grown in 32 oz stationary prescription bottles or 64 oz roller bottles, using a modified Eagles' basal medium (BME) with 10% tryptose phosphate broth and 10% heat-inactivated fetal calf serum. The LLC-MK_2 cell lines were grown in 32 oz stationary prescription bottles or 64 oz roller bottles in medium 199, containing 5% heat-inactivated fetal calf serum.

Virus assay: Plaque assays were carried out in BHK_{21} and LLC-MK_2 monolayers (Shapiro et al, 1971) and hemagglutination titrations with 0.30% goose RBCs were done as previously described (Clarke and Casals, 1958).

Preparation and purification of virions: To prepare purified virus for analysis of proteins, LLC-MK_2 and BHK_{21} cells were inoculated with seed virus at a multiplicity of 1-2 pfu/cell and allowed to adsorb for 60 minutes at 36°C. Unadsorbed virus was then removed and cells were washed twice with Hank's balanced salt solution (HBSS). Medium consisted of one part normal reinforced medium 199 or basal medium and three parts of the same medium from which the metabolite to be used as a radioactive precursor was omitted. Eight hours after infection cells were labeled with 10 μCi/ml of ^{3}H(G)-L-amino acid mixture containing 15 amino acids (specific activity 23.6 Ci/mmol), 2-10 μCi/ml of ^{3}H(6)-D-9 glucosamine hydrochloride (Sp. Act. 7.3 Ci/mmol) 2-10 μCi/ml, ^{14}C(u)-L-amino acid mixture (Sp. Act. 3.5 Ci/mmol) or 5-10 μCi/ml [5-C^{14}]-uridine (Sp. Act. 20 Ci/mmol). Labeled amino acid mixtures and uridine were obtained from New England Nuclear, and glucosamine from Amersham/Searle. The pH was adjusted to approximately 7.3 with bicarbonate when necessary. Released virus was harvested 24-28 hrs after infection in BHK_{21} cells and 40-48 hours in LLC-MK_2 cells. The virus was centrifuged at 3,000 g for 20 min to remove debris, then pelleted at 205,000 g for 2 hrs at 4°C. The resulting pellet was resuspended in 1-2 ml of TSE buffer (0.01 M Tris-HCl, 0.15 M, NaCl, 0.001 M EDTA), pH 8.2. The virus suspension was sonicated by two 1-minute cycles in a 10-Kc Raytheon Sonic Oscillator and purified through a sucrose gradient.

Sucrose gradients: Linear sucrose gradients were prepared with ribonuclease-free sucrose (Schwarz/Mann) in TSE buffer, using a mixing chamber. Rate-zonal centrifugation was done by layering 0.5 ml virus suspensions on a 4.5 ml, 15-50% sucrose gradient over a 0.1 ml 70% sucrose cushion. The gradient was centrifuged for 45 minutes at 205,000 g in a Spinco SW-50L rotor. Isopycnic centrifugation was carried out with pooled radioactive virus peaks from rate zonal runs diluted to 0.5 ml, then applied to 4.5 ml 15-50% sucrose in deuterium oxide (D_2O) and centrifuged at 205,000 g in a SW-50L rotor for 18 hrs. Density was determined by the direct weighing of ice-cold 20 μl samples of each fraction collected.

Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis was done by the method of Shapiro et al (1971). Proteins were dissociated by mixing purified virions with sodium dodecyl sulfate (SDS) and 2-Mercaptoethanol (2-ME), each to a final concentration of 1% and boiling at 100°C for 10 min. In coelectrophoresis experiments, samples were mixed prior to treatment with SDS and 2-ME. Procedures employed in the preparation of polyacrylamide gels, electrophoresis, staining with coomassie blue, and counting of radioactive samples, have been described previously (Rosato et al, 1974).

Results

Growth curves of BFS-283 CE virus in BHK_{21}, and LLC-MK_2 cells: The growth curves of BFS-283 CE virus in BHK_2 and LLC-MK_2 cells are shown in Fig. 38. Virus appeared in the medium by 14 hrs after infection and reached maximal titers by 18-28 hrs in both cell lines. Infection virus began to decline in BHK_{21} cells by the 28th hr, probably due to thermal inactivation of released virus and the lack of virus-producing cells. However, virus persisted in LLC-MK_2 medium at an approximately constant titer throughout the 120 hr test period. Maximal intact virions were detected at 40-48 hrs after infection in LLC-MK_2 cells, as determined by a coincident peak of radioactivity and hemagglutinating activity. Therefore, radioactively labeled virus was harvested at 24-28 hrs from BHK_{21} cells and at 40-48 hrs from LLC-MK_2 cells.

As seen in Fig. 39, BFS-283 virus grown in BHK_{21} and LLC-MK_2 cells was recovered from sucrose gradients in a single radioactive peak containing hemagglutinating activity. Virions from the single fraction containing the peak radioactivity were used for PAGE.

The proteins of BFS-283 virion: Six radioactive peaks were detected on electrophoretic analysis of purified BFS-283 virions grown in two different cell types (Figs. 40-43). Although every protein was not resolved as a discrete peak in identical fashion in both cell types, six radioactive peaks were clearly identified when virions were labeled with both amino acid mixtures and glucosamine (Fig. 40).

As seen in Fig. 41, proteins 3 and 4 are seen as low radioactive peaks in a rather high radioactive background when the virion is labeled with only an amino acid mixture, regardless of the cell line the virus was grown in. The electropherograms of BHK_{21} grown virus labeled with ^{14}C-AA + ^{14}C-glucosamine + ^{3}H-A.A (Fig. 42), and of BHK_{21} grown virus labeled with ^{3}H-glucosamine, coelectrophoresed with LLC-MK_2 grown virus labeled with ^{14}C-AA (Fig. 43), indicate that 5 of the 6 radioactive peaks (2-6) contain significant amounts of glucosamine. Glucosamine is absent from protein 1. It was frequently observed that the BHK grown virions labeled with glucosamine yielded higher CPMs in radioactive peak #5 than virions grown in LLC-MK_2 (Fig. 44).

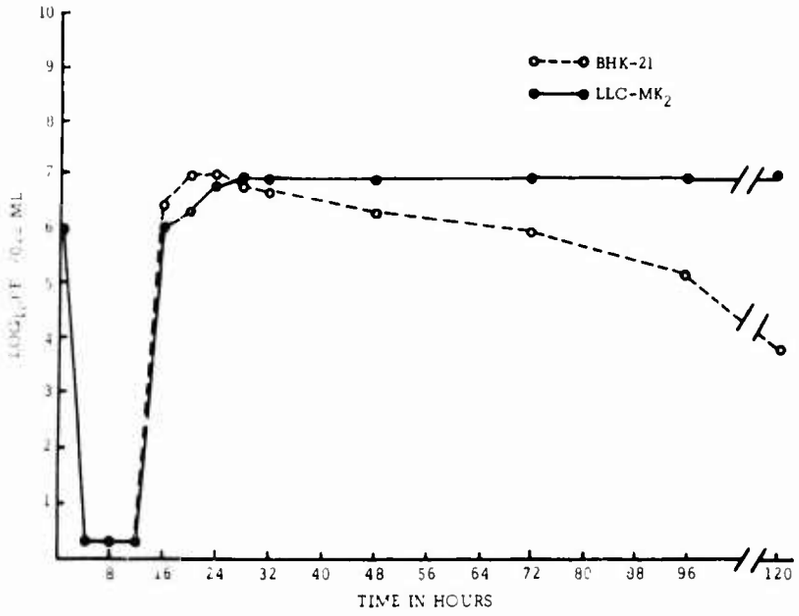

Fig. 38. Growth curve of extracellular BFS-283 CEV in BHK_{21} and $LLC\text{-}MK_2$ cell cultures of a multiplicity of infection (MOI) 1. Infectivity assays were made on $LLC\text{-}MK_2$ monolayers.

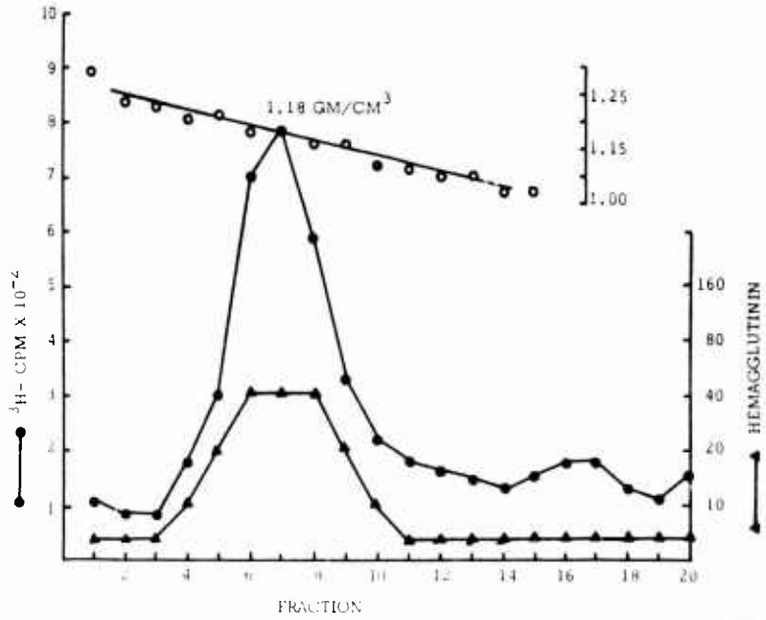

Fig. 39. Isopycnic centrifugation of radioactive BFS-283 CEV; ^3H-amino acid labeled virus from BFS-283 infected BHK_{21} cells was layered over a 15-50% linear sucrose gradient, with a cushion of 0.1 ml 70% sucrose in D_2O containing TNE, pH 8.2. The gradient was centrifuged in a SW-50L rotor at 50 000 rpm for 18 hours; fractions were collected and assayed for radioactivity and hemagglutination.

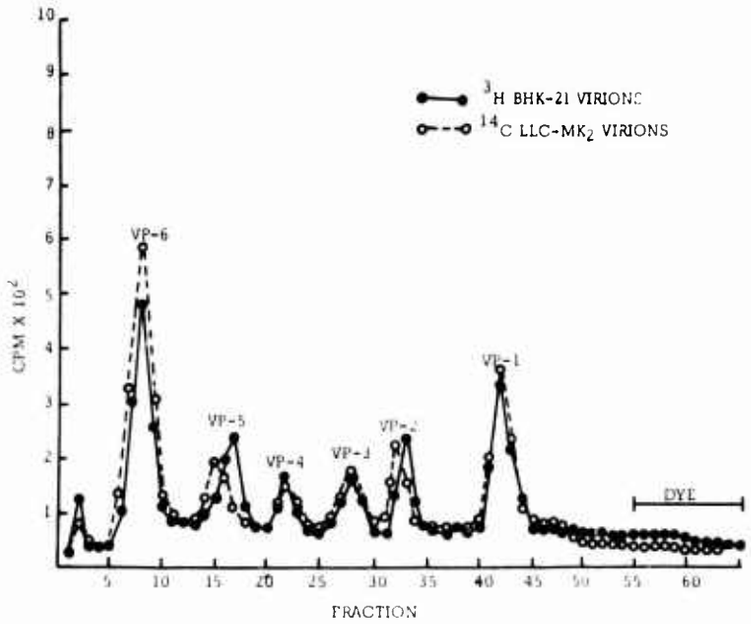

Fig. 40. Polyacrylamide gel coelectrophoresis of BFS-283 CEV grown in BHK$_{21}$ and LLC-MK$_2$ cells in medium containing both radioactive amino acids and glucosamine.

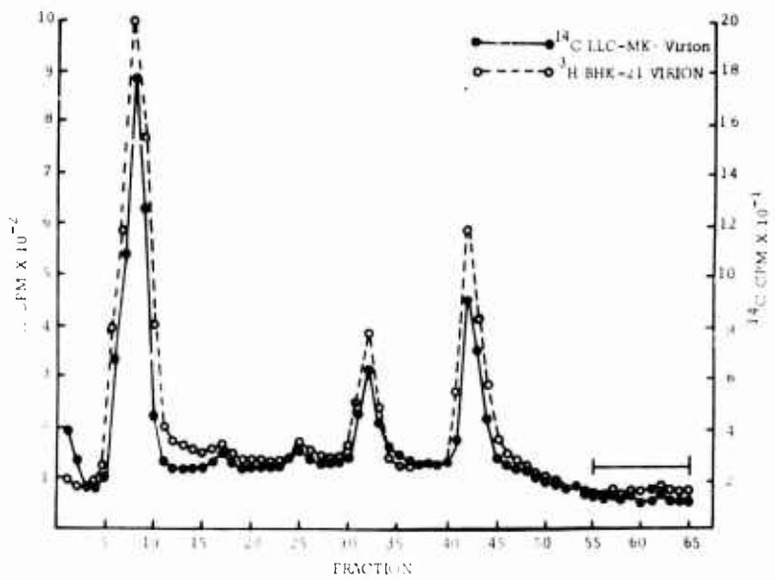

Fig. 41. Polyacrylamide gel coelectrophoresis of BFS-283 CEV grown in BHK$_{21}$ and LLC-MK$_2$ cells. Virions were labeled with radioactive amino acid mixtures.

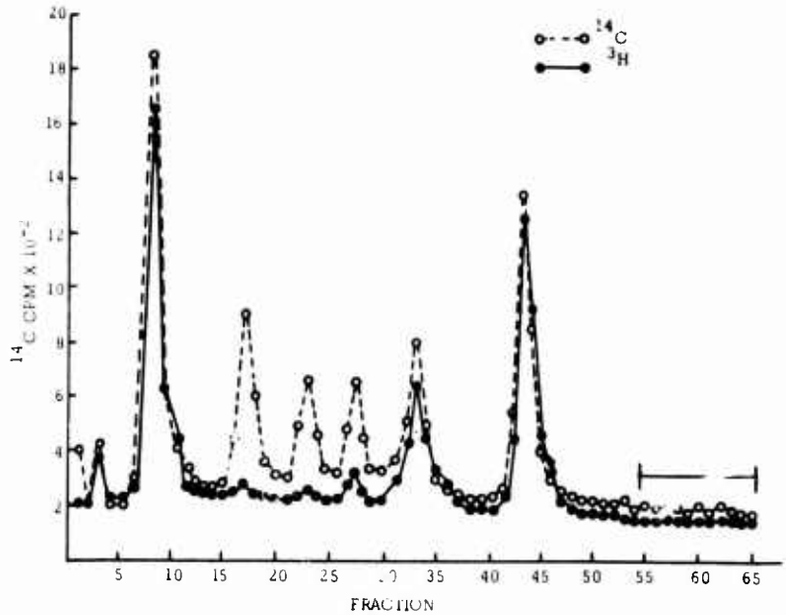

Fig. 42. Polyacrylamide gel electrophoresis of BHK_{21} grown virus labeled with ^{14}C-AA + ^{14}C-glucosamine, coelectrophoresed with BHK_{21} grown virus labeled with ^{3}H-AA mixture.

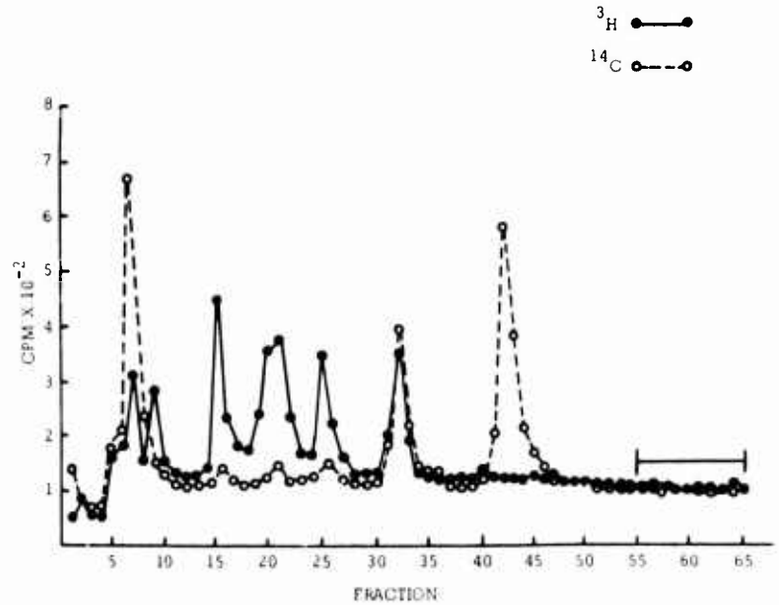

Fig. 43. Polyacrylamide gel coelectrophoresis of BFS-283 virions from LLC-MK_2 cells labeled with ^{14}C-amino acids and from BHK_{21} cells, labeled with ^{3}H-glucosamine.

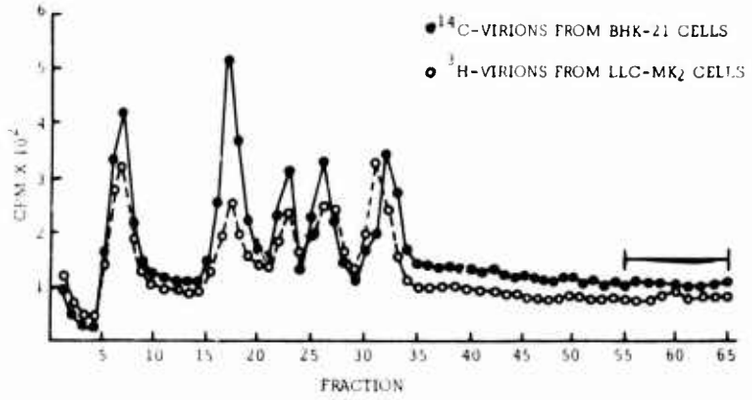

Fig. 44. Polyacrylamide gel coelectrophoresis of BHK_{21} grown BFS-283 virus labeled with ^{14}C-glucosamine and LLC-MK_2 grown virus labeled with ^{3}H-glucosamine.

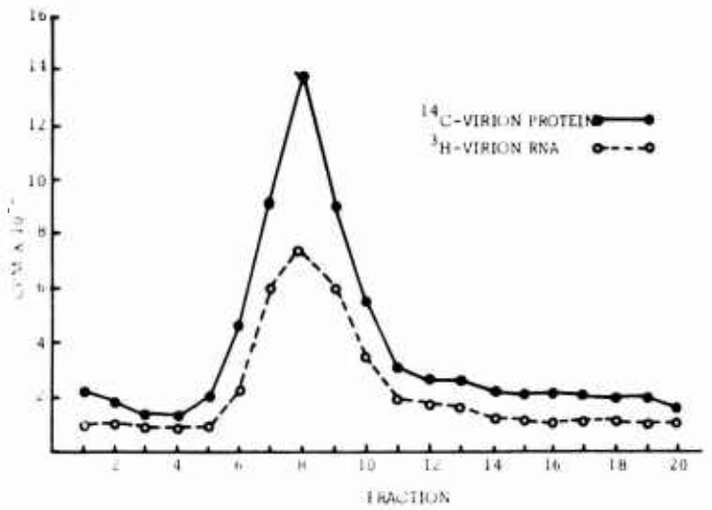

Fig. 45. Isopycnic centrifugation of BFS-283 virus triply labeled with ^{14}C-amino acid mixtures and ^{14}C-glucosamine, plus ^{3}H-uridine. Virus was centrifuged at 50,000 rpm in a SW-50L rotor for 18 hrs at 4°C.

Identification of core protein: Virions triply labeled with ^{3}H-AA, ^{3}H-glucosamine and ^{14}C-uridine were centrifuged through a sucrose gradient and banded at a density of 1.18 gm/cc (Fig. 45). When the peak fraction of this material was treated with the nonionic detergent NP-40 (1.0% for 15 min at 4°C), and rebanded on a 15-50% sucrose gradient, two radioactive peaks were obtained. One was a dense uridine containing material, the other a light amino acid and sugar labeled material (Fig. 46). Co-electrophoresis of the light material with intact virions revealed an electrophoretic pattern which contained all of the popypeptides except P-1 (Fig. 47). It therefore appears that polypeptide #1 may be "core" protein or nucleoprotein.

The molecular weights of the polypeptides of BFS-283 were estimated by coelectrophoresis with Sindbis virus marker proteins. The values obtained ranged from 82,000 for P6 to 17,500 for P-1. Cumulative molecular weights are shown in Table 39.

Table 39 - *Molecular weights of BFS-283 CE virion proteins. Estimated by coelectrophoresis with Sindbis virus marker proteins

Protein	Estimated m. w.	Range of values
1	82,000	80-84,000
2	54,000	53-55,000
3	45,000	45-46,000
4	38,000	37-39,000
5	30,000	29-31,000
6	17,500	16-18,000
TOTAL	266,500	

* Tentative molecular weights, including radioactive peaks 3, 4 & 5 as glycoprotein with high sugar: protein ratios

The percentage of each protein in the virion was calculated from the electropherograms of BHK-grown virus labeled with amino acid mixtures plus carbohydrates (Table 40).

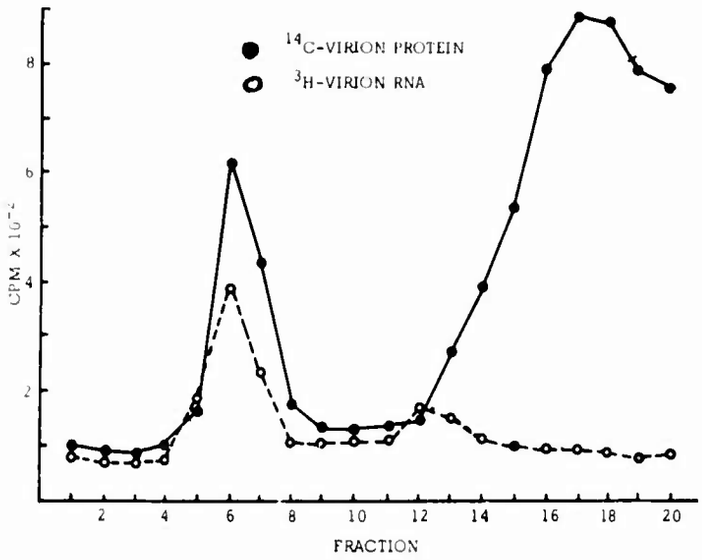

Fig. 46. Isopycnic centrifugation of BFS-283 virus in Fig. 45 following treatment with NP-40 at a final concentration of 1.0%. After 15 minutes at 4°C, the aliquot was layered onto a 15-50% sucrose gradient and centrifuged in a SW-50L rotor at 50,000 rpm for 18 hrs.

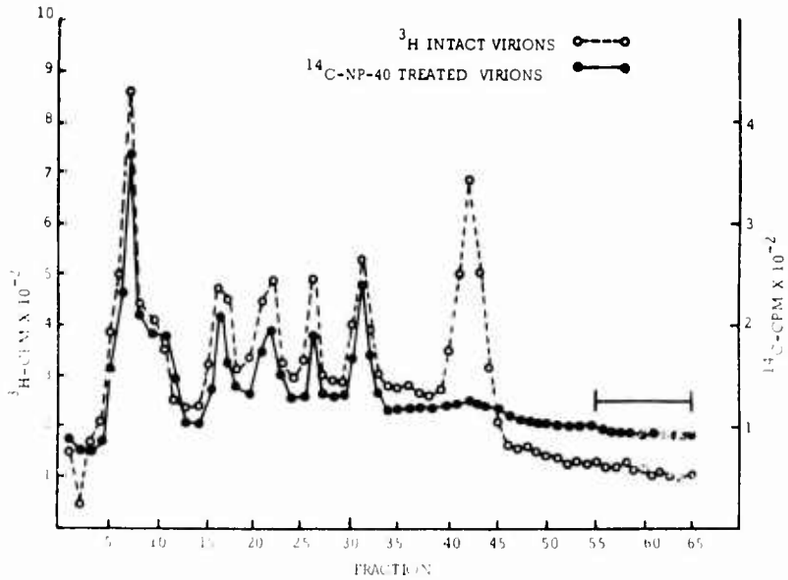

Fig. 47. Coelectrophoresis of intact BFS-283 virions labeled with ^{3}H-amino acids and ^{3}H-glucosamine and ^{14}C-fractions 17 and 18 of NP-40 treated virions shown in Fig. 46.

Table 40 - *Protein composition of BFS-283 CE virions

Protein	% of virion protein
1	22.7
2	11.7
3	9.7
4	11.1
5	15.6
6	29.2
TOTAL	100.0

* Tentative molecular weights, including radioactive peaks 3, 4 & 5 as glycoprotein with high sugar: protein ratios

Discussion

We have found four distinct polypeptides in BFS-283 purified virions; in addition, two radioactive peaks are found by labeling with glucosamine, but not with amino acids. These peaks are tentatively labeled NV-4 and NV-5. Although the previous reports (Rosato et al, 1952; McLerran & Arlinghaus, 1973) have shown only three structural proteins associated with virions, close examination of some of the electrophoretic patterns (McLerran & Arlinghaus, 1973) suggest that at least five radioactive peaks may be present if minor components are included. The addition of a radioactively labeled sugar, such as glucose of fucose may have permitted identification of the additional proteins since these aren't strongly labeled with amino acids.

Three of the proteins detected in our study appear similar to those previously reported (McLerran & Arlinghaus, 1973). Viral protein six (VP-6) was found to be a glycoprotein with molecular weight of 82,000 and appears similar to VP-3 described by McLerran for La Crosse virus, and to VP-3 for the glycoprotein found by Rosato et al, and has the same molecular weight of BFS-283 and Tahyna California viruses. Viral protein (VP-2) #2, found to be a glycoprotein of molecular weight 20,000, appears to be similar to the VP-2 glycoprotein described by Rosato et al (1974). Viral protein #1(VP-1), with a molecular weight of 17,500, was not labeled with glucosamine, but was labeled

with uridine; thus, it is likely the nucleoprotein, or core protein, of BFS-283.

Radioactively labeled peaks 3, 4 and 5 have not previously been described. VP-3 is labeled with both glucosamine and amino acids, and would appear to be a glycoprotein. It appears to migrate in polyacrylamide gel electrophoresis system similar to the minor components reported by McLerran (1973). Peaks 4 and 5 cannot be identified as proteins since they did not incorporate amino acids under the conditions used in these experiments. This might indicate that these peaks were glycolipids, rather than glycoproteins, or that they were host glycoproteins translated before, but glycosylated after labeling. Experiments are in progress to define whether radioactive peaks 3, 4 and 5 are structural components of the virion, cellular glycoproteins adsorbed onto the virion, or on cellular glycoproteins migrating with virions throughout the procedures used for purification.

N. Arbovirus ecology

1. General introduction and background

Investigations in the Pocomoke Cypress Swamp (PCS), MD, made during recent years, have established the presence of one or more members of the California encephalitis (CE) group viruses through isolations from mosquitoes. The vast majority of the CE virus isolates have been from a single mosquito species, Aedes atlanticus. This species is present in the swamp in large numbers only after heavy summer rains (2-3" in 24 to 48 hrs) have flooded the depressions in the densely shaded upland forests where its eggs are deposited. Previous collections have shown that this mosquito rapidly develops through the larval and pupal stages and is often the first species to appear as adults resulting from these summer rains. Adults are present for a relatively short period, and the majority have usually disappeared after 3-4 weeks. The extremely heavy rains needed to flood these breeding sites often occur only once a year, giving this species the appearance of being univoltine. Previous studies have also shown adult female A. atlanticus readily feed on sentinel rabbits and a variety of small mammals. They have also been shown to feed on cold-blooded vertebrates, especially turtles. Birds do not appear to be a major blood source.

Based on the numerous CE virus isolations made from A. atlanticus and known characteristics of this species, the following objectives were established for the 1973-74 study period. First a method had to be perfected to accurately identify by CE subtype the mosquito virus isolations made previously to determine the exact strains present in the swamp, and then to identify by subtype the CE antibody detected from wild vertebrate sera collected. Expanded serological studies of small

mammals and turtles were planned in attempts to detect the primary vertebrate hosts involved in maintenance of these viruses in nature. Viremia studies were planned to demonstrate the ability of selected vertebrate hosts to infect feeding mosquitoes through high titered viremias. Finally, a program of weekly bleedings of sentinel rabbits exposed in the Pocomoke Cypress Swamp was begun. Objectives of this program were to detect the onset and magnitude of virus transmission to sentinel animals throughout the warmer months. Much information directly relating to the general question of arbovirus maintenance in the swamp is presented in the Department of Entomology Annual Report covering this same study period and should be reviewed in conjunction with data presented herein.

2. Identification of California encephalitis virus subtypes

As previously stated, numerous virus isolations had been made from wild caught mosquitoes collected in the PCS during 1971. These isolates had been tentatively classified as members of the CE group, but no capabilities existed at that time to identify the CE subtype. Characterization of CE subtypes by others has shown that hemagglutination inhibition tests are not useful in typing CE strains. Most characterization of CE subtypes has been based on complement fixation tests. Unfortunately this test is often difficult to interpret, due to significant cross reaction among subtypes. Since considerable success was realized using the plaque reduction neutralization test (PRNT) to differentiate other closely related viruses, i.e., dengue subtypes, it was felt this test had the most promise as a routine laboratory tool for CE subtyping. Initial objectives were to a) produce specific antibody against each of the recognized CE subtypes, b) determine whether each subtype could be readily distinguished from other CE subtypes using the PRNT, and c) determine whether CE antibody from wild caught vertebrates or exposed sentinel hosts could be identified to subtype.

All reference seed viruses of the various CE subtypes were obtained from Dr. Robert Shope, YARU, through the American Type Culture Collection, Rockville, MD, with the exception of the LaCrosse subtype, which was supplied by Dr. Wayne Thompson, Univ. of Wisconsin. Mouse hyperimmune ascitic fluid (MHAF) was made from these seeds (after one passage in suckling mice at WRAIR) by the Brandt method (1967). A continuous line of LLC-MK$_2$ cells was used for all PRNTs. Cells were grown to confluency in 25 cm^2 flasks at 37°C. For determining the cross reactivity of strains within the CE group, two-fold dilutions of each immune ascitic fluid were made beginning at a 1:20 dilution. Diluted viruses calculated to contain 50-150 plaque-forming units (pfu) were added to equal amounts of immune ascitic fluid dilutions. These were incubated at room temperature for 30 minutes, returned to an ice bath and inoculated onto confluent cell culture flasks. Inoculated flasks were overlayed with Difco purified agar, incubated for 7 days at 37°C, then stained. Plaques were counted within 24 hours after staining and

a 50% reduction point was calculated by plotting the observed reductions on probit graph paper.

Table 41 presents results of PRNT tests with strains and hyperimmune ascitic fluid raised to CE group viruses. Although all cells of this comparison are not complete, certain conclusions are possible. High titered hyperimmune ascitic fluid was produced without difficulty and with few exceptions readily distinguished homologous from heterologous strains. Cross reactions between La Crosse and Snowshoe Hare subtypes are potentially troublesome. Even so, the value of the PRNT as a diagnostic tool to differentiate CE subtypes is clearly established.

Results of PRNTs with selected field isolates tested against the CE subtypes thought most likely to be in the PCS are shown in Table 42. Two subtypes were found to be present in the swamp, Keystone and Jamestown Canyon. Based on these observations, the remaining mosquito isolates were tested only against the Keystone and Jamestown Canyon subtypes of CE. All strains recovered from A. atlanticus were found to be members of the Keystone subtypes. Two strains were recovered from A. canadensis. One was identified as Keystone and the other as Jamestown Canyon subtype CE.

3. California encephalitis serology in wild vertebrates

Based on the avid feeding of A. atlanticus mosquitoes on turtles, reported elsewhere, and the known relationship of A. atlanticus mosquitoes with Keystone virus, the role of turtles in the maintenance of CE in the swamp was considered. Serum samples were collected from turtles caught in the PCS during the spring and summer of 1973. These sera were tested for neutralizing antibody using the PRNT against a representative swamp mosquito isolate of the Keystone subtype (isolate # '72-1736). Virus isolations were attempted from these sera by inoculation of 0.1 ml sera into duplicate tubes of 80-90% confluent BHK-21, Clone 13 cells. Tubes were observed for 5-7 days for CPE. Suspect positive pools were passed a second time in cell culture and the harvest plaqued in LLC-MK_2 cells.

No virus was isolated from the 148 turtle and snake sera collected in 1973, and of the 98 turtle sera tested to date, none have shown significant neutralizing antibody against the Keystone subtype of CE. Tests using the Jamestown Canyon subtype are pending. Table 43 presents turtle sera collected and tested to date.

Data presented elsewhere in this Annual Report by the Department of Entomology clearly established that A. atlanticus mosquitoes will feed on turtles. The high rate of Keystone infection of A. atlanticus mosquitoes implies that if turtles are infected by the Keystone subtype, a sample of 98 sera should contain individuals having been fed upon by infected mosquitoes. Thus, our failure to detect antibody or virus in

Table 41 - Cross reactivity of CE subtypes as shown by plaque reduction neutralization tests

Antigen	Keystone	Jamestown Canyon	Snowshoe Hare	La Crosse	San Angelo	BFS-283	Trivit-tatus	Tahyna	Melao
Keystone	1500*	45	70	750	80	50	70		
Jamestown Canyon	70	2500	50	120	130	300	< 20		
Snowshoe Hare	20	30	1550		150	600	< 20		
La Crosse	90	40	940	7400	250	250	60		
San Angelo	540	30					280	180	< 20
BFS-283	90	65	700	320	280	4000	< 20	200	< 20
Trivittatus		40	240		110	170		< 20	< 20
Tahyna	160	160			410		150		< 20
Melao	370	600	600		210		90	< 20	1000

* Reciprocal of 50% reduction point as calculated by probit analysis

Table 42 - Results of PRNTs using selected PCS isolates as antigens against MHAF to local and reference strains of CE

Antigen	Antibody (MHAF)				
	Keystone	Snowshoe Hare	La Crosse	Pool 4**	Pool 84***
Keystone	1:1500	70	750	NT	NT
Snowshoe Hare	20	1550	(> 640)	< 20	< 20
La Crosse	90	940	1:7400	< 20	< 20
Pool 4**	75	NT	150	2300	NT
Pool 84***	850	NT	NT	20	450
71-560	850	40	1000	< 100	100
71-477	1500	100	800	< 20	100
71-577	1300	130	900	25	65
T 73-17*	500	140	1300	30	120
T 73-80*	920	220	1000	80	240
T 73-83*	760	240	1000	40	180
T 73-86*	1000	NT	800	40	200
T 73-87*	1100	140	700	240	200
T 73-95*	1500	65	560	35	200
T 73-96*	1100	500	1000	50	250
T 73-100*	800	200	900	30	90
T 73-104*	800	100	800	40	100
T 73-107*	900	200	800	70	200
T 73-108*	550	200	1400	40	160
Sentinel Rabbit #764	700	120	600	100	NT

* T 73 series strains represent those isolates recovered from A. atlanticus larvae collected in 1973

** Local strain of Jamestown Canyon

*** Local strain of Keystone

Table 43 - Poikilothermic vertebrate sera collected and tested for virus and N antibody to CE from the Pocomoke Cypress Swamp, Maryland, 1973

Species	Tocal collected	Virus recovered / # tested	N ab$^+$ / # tested
Painted turtle	91	0/91	0/61
Mud turtle	19	0/19	0/0
Snapping turtle	21	0/21	0/0
Box turtle	3	0/3	0/3
Musk turtle	8	0/8	0/3
Other turtles	3	0/3	0/3
Black & white kingsnake	2	0/2	0/0
Black rat snake	1	0/1	0/0
TOTAL	148	0/146	0/70

this sample of sera suggests that turtles may not be susceptible to infection with the Keystone strain of CE. Experimental infection of turtles with CE is planned in an attempt to demonstrate this hypothesis.

As in previous years, deer sera were collected at the Snow Hill, Maryland deer check station (about 15 km from the swamp) during hunting season (24 Nov to 2 Dec 73). These samples were collected from deer carcasses at the time of weighing by aspirating free blood from the gutted carcass using a 5 or 10 cc syringe without a needle. Samples were allowed to clot at room temperature and the serum separated by centrifugation and then stored frozen until assayed for antibody. No virus isolations were attempted.

Table 44 represents the results of 121 deer sera tested against both the Jamestown Canyon and Keystone subtypes of CE. Of these 121 sera, 28 reduced Jamestown Canyon by greater than 80%, but did not reduce the Keystone subtype appreciably, while only 2 reduced Keystone

Table 44 - Results of PRNTs, using deer sera collected during 1973 hunting season and tested at 1:10 dilution against local strains of Keystone and Jamestown Canyon CE; N = 121

		Keystone		
		> 80%	50-79%	No reduction
Jamestown Canyon	> 80%	22	19	28
	50-79%	1	5	8
	No reduction	2	7	29

Keystone by greater than 80% while not reducing the Jamestown Canyon subtype. An additional 29 sera did not reduce either subtype substantially. Twenty-two sera reduced both the Jamestown Canyon and Keystone subtypes by greater than 80%, but in most instances the reduction was greatest against the Jamestown Canyon subtype.

These data clearly establish the involvement of deer with a CE group virus and suggest more frequent infection by Jamestown Canyon subtype rather than Keystone on the Del Mar Va Peninsula. These results also emphasize the importance of assaying for all known subtypes of CE present in an area when attempting to establish an accurate antibody prevalence estimate. This association of Jamestown Canyon virus with deer is not surprising since a) all Jamestown Canyon strains recovered from the swamp have been from A. canadensis mosquitoes, b) A. canadensis have previously been shown to feed avidly on deer, and c) deer have been implicated as a primary vertebrate host for Jamestown Canyon virus elsewhere (Issel et al, 1972).

4. Viremia studies

In order to define vertebrate species capable of acting as amplyfying hosts of CE virus in nature, selected potential vertebrate hosts were experimentally infected with a local Keystone strain of CEV and the resulting viremia monitored.

Initially, a laboratory model was needed to establish valid experimental techniques. For this purpose young (6-10 week) hamsters were

prebled to eliminate those with existing CE antibodies, then inoculated with 0.5 ml of varying dilutions of virus. A local isolate of Keystone subtype CE was used in all experiments (Isolate #172-1735, TC-2, isolated from A. atlanticus and passaged twice in BHK_{21} cells).

Three groups of five hamsters each were inoculated subcutaneously (s.c.) with 155 pfu, 1550 pfu and 12,400 pfu, respectively. Hamsters were bled via cardiac puncture at 24, 48, 72 and 96 hours post inoculation, 0.1 ml of whole blood being diluted directly into 0.9 ml LLC-MK_2 growth media (Medium 199 with 20% FBS, P & S). Blood samples were then assayed for virus by direct plaquing of diluted blood on confluent monolayers of LLC-MK_2 cells grown in flasks. Samples producing more plaques than could be accurately counted were titrated in subsequent plaque assays.

Table 45 presents results of these hamster viremia studies. With the exception of a single individual in the first group, all hamsters developed viremia. While the magnitude of each individual's viremia was generally comparable, it appears that the onset of viremia was directly related to the amount of virus inoculated, i.e., the greater the virus inoculum, the more rapid the onset of viremia.

Based on these studies it was assumed that the techniques used would a) adequately infect susceptible hosts, and b) detect circulating virus from viremic hosts. Wild caught raccoons from Assateague Island, Virginia were then infected with the same local isolate of Keystone CE as used in the hamster studies. Four raccoons were inoculated s. c. with 1 ml containing approximately 4.5×10^5 pfu. A fifth raccoon was inoculated s. c. with approximately 2.25×10^5 pfu in 0.5 ml. All raccoons were prebled for detection of existing CE antibodies by cardiac puncture before exposure; however, the actual testing for antibody was not done until the viremia study was completed.

An additional four raccoons and a single opossum were inoculated with approximately 670 pfu of Jamestown Canyon subtype CE (local isolate '71-4, isolated from A. canadensis, SM-4, TC-1, and passed 4 times in suckling mice and once in BHK_{21} cell cultures). Prebleeds were taken as described above.

All animals were then bled via cardiac puncture at 24, 48, 72 and 96 hrs post inoculation. Approximately 2.0 ml was taken at each bleeding, of which 0.2 ml was diluted immediately in 1.8 ml LLC growth medium and frozen. The remaining blood was allowed to clot at room temperature, separated by centrifugation, and the sera stored at -70°C.

Daily bleedings were assayed for circulating virus (by the direct plaque technique) as described for the hamster experiment. Results of these tests are shown in Table 46. Results of screening tests for neutralizing antibody are also presented in the table, as are the results of PRNTs to detect antibody at four days post inoculation (p.i.).

Table 45 - Viremia resulting from experimental infections of hamsters with various amounts of Keystone subtype CE (PCS isolate '72-1736)

Hamster	Total inoculation	Viremia (pfu/1.0 ml) on days p.i.			
		1	2	3	4
I-P	155 pfu	0	0	0	0
I-LR	"	0	60	$4.3\text{-}5.0 \times 10^4$	$1.2\text{-}1.5 \times 10^5$
I-RR	"	0	5	5.5×10^2	DEAD
I-LF	"	0	0	35	$1.6\text{-}2.0 \times 10^5$
I-RF	"	0	25	$6.0\text{-}6.5 \times 10^4$	$1.0\text{-}2.0 \times 10^5$
2-P	1550 pfu	0	0	5	2.2×10^5
2-LR	"	0	40	$3.0\text{-}4.5 \times 10^5$	2.0×10^5
2-RR	"	0	5	$3.6\text{-}5.0 \times 10^4$	$7.1\text{-}9.0 \times 10^5$
2-LF	"	0	5	$1.3\text{-}2.0 \times 10^4$	$0.7\text{-}1.0 \times 10^5$
2-RF	"	0	0	5.6×10^2	$4.0\text{-}5.5 \times 10^4$
3-P	12400 pfu	5	1.1×10^3	$2.4\text{-}2.5 \times 10^5$	0
3-LR	"	0	570	$3.1\text{-}6.5 \times 10^5$	280
3-RR	"	15	DEAD	DEAD	DEAD
3-LF	"	0	215	695	50
3-RF	"	30	N. S.	6.5×10^5	0

N.S. = not sampled

Unfortunately all raccoons died with severe diarrhea between day 4 and day 7 p.i., so that subsequent bleedings for sero-conversions could not be accomplished. Whether or not fatality was caused by the CE virus strains was not determined. The single opossum was bled on day 10 and did have detectable antibody to Jamestown Canyon. It was not tested against the Keystone subtype.

If viremia does occur in raccoons, apparently the time from exposure to onset of viremia must be longer than that in hamsters. In this experiment only two raccoons showed any virus during the immediate 4 days post infection, and both in low titer. Obviously subsequent

Table 46 - Results of experimental exposure of wild-caught raccoons from Assateague Island, VA to local strains of Keystone and Jamestown Canyon subtypes of CE

Raccoon number	Pre-existing N Ab*		Inoculum	Viremia				N Ab	
				Days p. i.				Day 4 p.i.	
	K	JC		1	2	3	4	K	JC
K-NM	-	-	Keystone 2.25×10^5 pfu	-	-	-	-	-	-
K-LF	-	±	4.5×10^5 pfu	-	-	-	-	±	-
K-RF	-	-	"	-	-	-	10 pfu/ 1.0 ml	-	-
K-LR	+	+	"	-	-	-	-	+	+
K-RR	±	-	"	-	-	-	-	-	-
4-LF	-	-	Jamestown Canyon 670 pfu	-	-	-	-	-	-
4-RF	-	-	"	-	-	-	5 pfu	±	-
4-LR	-	-	"	-	-	-	-	-	-
4-RR	-	-	"	-	-	-	-	±	±
Opossum	-	-	"	-	-	-	-	-	-*

* 90% reduction against JC in day 10 p. i. serum

\+ ≥ 80% reduction in number of plaques formed by a 1:10 serum dilution.

\- < 50% reduction in number of plaques formed by a 1:10 serum dilution

± 50-79% reduction in number of plaques formed by a 1:10 serum dilution.

experiments should monitor for viremia at least through day 7 p. i. Unfortunately, little else can be concluded regarding the onset or duration of viremia. Additional experiments are planned to clarify the

role of raccoons in the maintenance of CE in the swamp.

Before isolation and serology was begun on the infected raccoon, 11 gray squirrels were collected from the Shad Landing State Park at Snow Hill, Maryland, prebled for antibody titers and inoculated with Keystone and Jamestown Canyon CE (same seed viruses as in previous experiments), and inoculated s.c. as in previous experiments. Daily samples of whole blood were diluted as described earlier, but no serum samples were taken. Final bleeds for antibody conversions were done on day 10 p.i.

Results of these experimental infections are shown in Table 47. At least one squirrel (#82) produced a high titered viremia. Several other squirrels produced what appeared to be viremia; however, those showing 5 pfu/ml must be viewed with caution, since this figure represents a single plaque on only one of two flasks exposed.

With the exception of #29, which escaped before its final bleeding, and #87, animals without pre-existing CE antibody responded serologically to the virus inoculated; 4/5 adequately tested developed N Ab to the heterologous strains.

The low titered inoculum given to those squirrels exposed to Jamestown Canyon was not intended. With the 50 pfu inoculum, the 3 squirrels lacking N Ab did respond serologically, suggesting a low threshhold of infection. Further, the viremia detected in #34 may have been sufficient to infect feeding mosquitoes.

Again, it was unfortunate that the daily bleedings were not extended past day 4 p.i. Future experiments are planned to sample for viremia at least through day 10.

5. Sentinel rabbit program

A program of periodic bleedings from sentinel rabbits exposed in the swamp was begun in early May and continued through mid October 1973. The objectives of this program were to determine the onset, magnitude and ecological distribution of CE virus transmission within the various habitats of the swamp. Laboratory rabbits were prebled to detect pre-existing CE antibody, then exposed in the swamp in wire mesh cages approximately 1 to 1.5 M above ground. Exposure sites were established in three basic habitats within the swamp (site designations refer to Saugstad et al, 1972); closed rootmat swamp (four separate sites at site 7 and one at site 3), upland forest (three sites near site 10 and two at site 11), and transitional areas between the swamp proper and the upland forests (near site 15, site 12 and at the bus). Two rabbits were exposed at each site for a maximum total of 26 rabbits exposed at any given time. Most rabbits were replaced at least once during the summer.

Table 47 - Results of experimental exposure of wild caught gray squirrels from Snow Hill, MD to local strains of Keystone and Jamestown Canyon subtypes of CE

Squirrel number	Pre-existing N antibody		Inoculum (0.5 ml)	Viremia, days p.i. pfu/1.0 ml				Neutralizing antibody day 10 p.i.	
	K	JC		1	2	3	4	K	JC
30	-	-	1.7×10^3 pfu K	-	-	-	-	+	n.s.
82	-	-	1.7×10^3 pfu K	-	-	5	1.2×10^5	+	+
35	-	-	8.5×10^2 pfu K	-	-	-	-	+	-
33	±	-	8.5×10^2 pfu K	-	-	-	-	+	-
32	+	±	1.7×10^3 pfu K	-	-	5	-	+	+
36	+	-	1.7×10^3 pfu K	-	-	-	5	+	+
29	-	-	50 pfu JC	-	-	-	5	n.s.	n.s.
31	-	-	50 pfu JC	-	-	-	-	+	+
34	-	-	50 pfu JC	-	-	-	325	+	+
84	-	-	50 pfu JC	-	-	-	-	±	+
87	±	-	50 pfu JC	-	-	-	-	±	-

n.s. = not sampled

+ = ≥ 80% reduction in number of plaques formed; - = < 50% reduction in number of plaques formed; ± = 50 to 79% reduction in number of plaques formed.

Rabbits were bled via cardiac puncture at weekly intervals beginning on 15 May and continuing through 12 October 1973. No samples were taken during the two weeks between 12 September and 4 October. Blood samples were allowed to clot at room temperature, then separated by centrifugation. Serum samples were stored at -70°C, pending assay.

Neutralizing antibody was detected by PRNT using local isolates of Keystone (Isolate # '72-1736) and Jamestown Canyon (Isolate # '71-4), and tested against 1:10 serum dilutions. Each initial bleeding showing seroconversion was titrated to determine the specific CE strain causing infection. Results of these conversions and titrations are provided in Tables 48 and 49, respectively. The cululative total of seroconversions by date of onset is presented in Fig. 48.

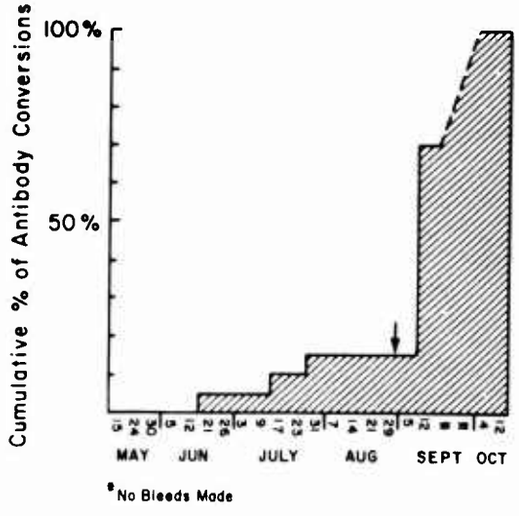

Fig. 48. Acquisition of N antibody against Keystone virus by sentinel rabbits exposed in the Pocomoke Cypress Swamp, MD, May through October, 1973. Total conversions = 20. Arrow indicates date at which larval A. atlanticus mosquitoes were collected from which Keystone virus was subsequently recovered.

Table 48 - Onset of seroconversion in sentinel rabbits exposed in the Pocomoke Cypress Swamp, Maryland, May to October, 1973, as determined by PRNT

Sentinel rabbit	Conversion date	Exposure site	Habitat
84	21 June	12	Swamp/forest
286	17 July	7	Swamp
440	31 July	7	Swamp
777	12 Sept	10	Forest
274	12 Sept	10	Forest
772	12 Sept	10	Forest
760	12 Sept	near 15	Swamp/forest
784	12 Sept	near 15	Swamp/forest
81	12 Sept	bus	Swamp/forest
69	12 Sept	bus	Swamp/forest
587A	12 Sept	12	Swamp/forest
587B	12 Sept	7	Swamp
612	12 Sept	7	Swamp
615	12 Sept	3	Swamp
598	12 Sept	3	Swamp
316	04 Oct	11	Forest
594	04 Oct	11	Forest
614	04 Oct	12	Swamp/Forest
436**	04 Oct	7	Swamp
345	04 Oct	7	Swamp

* Site designations refer to Saugstad et al, 1972

** Moved from site 10

All rabbits that converted serologically in the swamp, and those which expired during their exposure, were assayed for circulating virus. Only a single virus strain was recovered, that from rabbit #764 exposed at Slab Road 4 on September 1973. This rabbit expired before we detected CE antibody. Characterization of this strain is presented in Table 42.

Table 49 - Identification of strain-specific N. antibody to CE in sera of sentinel rabbits exposed in the Pocomoke Cypress Swamp, Maryland, October 1973; sera tested represent earliest N. antibody positive samples taken

Sentinel rabbit	Keystone (local isolate '72-1736)	Jamestown Canyon (local isolate '71-4)
84	200	10
268	ND*	20
440	470	100
777	75	< 10
274	260	30
772	ND	140
760	ND	< 10
784	270	10
81	310	30
69	240	100
587A	220	< 10
587B	ND	~200
612	320	100
615	200	100
598	95	20
316	ND	70
594	340	50
614	180	70
436	ND	ND
345	250	< 10

* ND = not done

The earliest conversion was 21 June, and only two other conversions occurred before 12 September. Of 20 susceptible rabbits exposed from 29 August to 12 September, 10 converted serologically between the 5 and 12 September bleedings. Heavy summer rains fell during the week of 21 August, and adult Aedes atlanticus mosquitoes first appeared in large numbers in the swamp between the 30th of August and 1 September. This mosquito species has previously been shown to emerge infected with the Keystone subtype of CE (Department of Entomology Annual Report). The strong correlation between the emergence of A. atlanticus mosquitoes and seroconversions of sentinel rabbits suggests that transovarially infected A. atlanticus are capable of transmitting Keystone virus at their first blood feeding. Further, the low level of virus transmission detected previous to the emergence of A. atlanticus implies that transovarial transmission is an important means of Keystone virus maintenance in the swamp. The conversion of several rabbits exposed in different ecological habitats within the swamp simultaneously suggests that the distribution of A. atlanticus is widespread throughout the greater swamp, and that transovarially infected mosquitoes are probably emerging from several different breeding sites. This is based on the suggested short flight range of A. atlanticus presented elsewhere (Dept of Entomology Annual Report). The number of seroconversions seen suggests that transovarial transmission of Keystone strain CE in A. atlanticus mosquitoes may be common.

Ten sentinel rabbits were exposed at five separate sites in Fort George Meade, Maryland and periodically bled via cardiac puncture during the period 9 May through 25 July, 1973. Exposure sites sampled habitats containing permanent water, hardwood forests, floodwater pools and stagnant ponds. Routine adult mosquito collections were made by personnel from the Entomology Section, Army Area Laboratory, Fort Meade, Maryland and results of these collections can be found in their annual report. No virus isolations were attempted from these mosquitoes. This pilot program was an attempt to detect transmission of CE virus at Fort Meade. Rabbit sera were tested against swamp isolates of both Keystone and Jamestown Canyon subtypes of CE by PRNT. Results detected no neutralizing antibody in any of the rabbits exposed against either of the CE subtypes tested. Unfortunately this project was terminated in late July due to difficulties in maintaining the rabbits in the field; consequently virus transmission in late summer could not be detected.

II. RESPIRATORY VIRUSES

A. Influenza Virus

1. Neuraminidase assay - Myxoviruses have two major surface antigens; hemagglutinin (HA) and neuraminidase (NA). These antigens are on different proteins and vary independently with different strains of virus. Although antibody to NA does not neutralize influenza virus, it has been shown to protect against disease by reducing transmission of virus from cell to cell in vivo. Since evaluation of the potential pathogenicity of a novel influenza strain or of protective efficacy of influenza vaccine requires knowledge of the antigenicity of both envelope proteins and antibody induced to both proteins, a test for influenza NA activity has been introduced. 50 ul of virus solution is mixed with 50 ul of 0.2 M Tris-maleate-NaOH buffer, pH 5.9, containing 8×10^{-3} M CaCl. Then 100 ul of a 24 mg/ml solution of fetuin (Grand Island Biological Company) in water is added. The mixture is immediately stirred and placed at 37°C for 30 min. The reaction is stopped by addition of 0.1 ml of Warren's periodate reagent (Warren, 1959). Released neuraminic acid (NANA) is measured by the method of Warren as modified by Aminoff (Aminoff, 1961). A standard of 50 ul of a 100 ug/ml solution of pure NANA will give an optical density (OD) of 0.19 to 0.22 at 549 nm in this test. One unit of NA is defined as that amount required to yield sufficient NANA to produce an OD of 0.1 in 30 min. A preparation of virus with an HA titer of 1:1280 should contain approximately 50 units of NA in 1 ml. The concentration of fetuin must be adjusted so that straight line kinetics are obtained for the 30 minute incubation period. New lots of fetuin must be tested to assure adequate substrate concentration in this assay.

For the anti-NA assay, twofold dilutions of antiserum in 0.05 ml of buffer are mixed with 4-6 N-ase units of virus in 0.05 ml and incubated at room temperature for 30 min. 100 ul of fetuin is added and the standard NA assay carried out. Titers are expressed as the initial serum dilution giving 50% inhibition of enzyme.

2. Role of the Viral Carbohydrate - The surface proteins of all enveloped viruses thus far investigated contained covalently bound carbohydrate. The carbohydrate moiety of many serum glycoproteins has been shown to be responsible for the binding of these molecules to certain cells. A study has therefore been undertaken to determine if the carbohydrate moieties on the surface of myxoviruses have a biological role.

For these studies, the WSN strain of influenza virus and its host MDBK cells were obtained from Dr. R. Compans at the Rockefeller University. The virus was grown in plastic flasks, radiolabelled with ^{3}H and ^{14}C amino acids and purified according to the methods of the Rockefeller

group (Choppin, 1969 and Compans et al., 1970).

An attempt to produce radiolabelled, carbohydrate free virus was made using the inhibiter of glycosylation, (glucosamine, Courtney et al., 1973). MDBK cells were inoculated at an MOI of 1-10 pfu/cell. After 60 minutes at 37°C the inoculum was removed and the cell sheet washed twice with balanced salt solution. The cells were fed with 10 ml of the standard reinforced Eagles Medium which was modified to include half the usual amino acid concentration, no calf serum (to prevent cleavage of HA0 to HA1 and HA2), only 1 g/L glucose (to reduce competition with glucosamine), 1% glucosamine, 20 u Ci/ml ^{3}H amino acids and extra bicarbonate to achieve a pH of approximately 7.

At 48 hours after infection the control without glucosamine showed 4+ cytopathic effect (CEP) and a 1:160 HA titer. Glucosamine containing flasks showed a 3+ CPE and an HA titer of 1:10. The media was removed from the cells, and the presumptive carbohydrate free virus concentrated by centrifugation at 52,000 x g for 1 hr onto a 60% potassium tartrate cushion. Through purification by two tartrate gradient centrifugations, virus from glucosamine treated cells was noted to band in the same location as normal virus. Fractions of purified virus from glucosamine tested cells had an HA titer of only 1:10 and no detectable NA activity compared to HA titer of 1:1280 and NA titer of 49.2 u/ml of control virus.

Studies are proceeding to characterize further this defective virus. The relatively pure carbohydrate free virions can be used as a starting material to purify carbohydrate free hemagglutinin and N-ase molecules. Peptide mapping and amino acid analysis are planned to determine whether defective virions differ from biologically active molecules only in their lack of carbohydrate.

3. Effect of trypsin on viral growth. The recent report of Tobita and Kilbourne (1974) noted that growth of influenza B viruses in chicken embryo fibroblast (CEF) cells was markedly enhanced by the presence of a low concentration of L-1-tosylamide-2-phenylethyl-chloromethyl ketone-treated trypsin (obtained from Worthington Company) in the media. Since this phenomenon might be of use in the adaption of new influenza isolates for vaccine production, a systematic study of this phenomenon has begun. Preliminary studies have indicated that trypsin (obtained from Grand Island Biological Company) does not permit growth of influenza A Port Chalmers/1/73 and B Hong Kong/5/72 in the continous diploid WI-38 and HR-6 cell lines. Studies are being directed at reproducing and determining the mechanism of action of trypsin on virus growth in CEF cells. The effects of other proteolytic enzymes on the growth of influenza virus in various cells will also be tested.

4. Immune Cytolysis as a Tool for Studying Virus Entry. For some years, a controversy has occured between groups who believe myxoviruses enter cells by pinocytosis and those who believe cell entry occurs by fusion of viral and cellular envelopes. Immune cytolysis may provide a definitive answer to this question. If virus enters by fusion with the cellular membrane the envelope of the virus should be incorporated into the cellular membrane shortly after viral penetration. This might make the cell susceptible to immune cytolysis by anti-viral antibody and complement within one hour after infection. If virus enters by pinocytosis, the cells should not be susceptible to immune cytolysis shortly after infection. Preliminary studies have shown that 51 Cr labelled BHK-21 cells infected with a high MOI of A/England/42/72 are susceptible to lysis by antiviral antibody and complement at 24 hours post infection when maturing virus buds through the cell membrane. Experiments are proceeding to determine if immune cytolysis will occur within one hour after viral infection. Necessary controls will include studies on Sendai virus grown in eggs (which attaches to cells but can not infect them due to a nonfunctional fusion component), and adenovirus (which probably does not enter by fusion) (Dales, 1973).

5. Liposome binding experiments - A project was undertaken with LTC John Mooney, Department of Exp. Path. to determine if influenza virus bound to various kinds of liposomes. These experiments would determine whether influenza virus used additional receptors besides the NANA containing glycoprotein described by others.

WSN influenza virus was grown, radiolabelled, purified and tested for binding to liposomes consisting of the neutral lipid fraction of sheep red blood cells obtained by published methods (Mooney et al., 1974).

Significant binding did not occur although the virus did agglutinate sheep red cells. Experiments are proceeding using human neutral lipids and glycolipids.

6. Tests for Cap formation - When flourescent antibody directed towards lymphocyte fibroblasts is added to these cells at 4°C and the cells warmed to 37°C, the antibody and antigen migrate in the fluid membrane to one portion of the cell to form a "cap" (Eddidin et al., 1972). The possibility that viral infected cells may cap on addition of antibody directed towards the budding virus was considered. If cap formation did occur, it might be a mechanism by which antibodies to HA or N-ase inhibit viral release (Dowdle et al., 1974). Cap formation is also of interest since it may make virus infected cells more or less susceptible to immune cytolysis. Monolayers of BHK-21, primary monkey kidney cells, CEF cells, HR-6 diploid human fibroblasts and WI-38 diploid human fibroblasts were infected

with A/Port Chalmers/1/73 virus. Twenty four hours later, when hemadsorption was 4+ the cells were washed and dispersed with 0.25% trypsin containing 1:5000 versene. The cells were centrifuged at 180xg for 15 min and resuspended in 0.8 ml of ice cold Ca++ and Mg++ free phosphate buffered saline (PBS) with 0.8 ml of ice cold dilutions of human anti A/Port Chalmers serum in 0.8 ml of Ca++ and Mg++ free medium 199. The tubes were incubated at 4°C or 37°C for 1/2 hour. The cells were washed twice in ice cold or room temperature PBS and resuspended in 50 ul of undiluted flourescent goat antihuman Ig G serum with 50 ul of PBS and 100 ul of Ca++ and Mg++ free medium 199 to determine if the anti-viral serum alone caused capping. After 30 minutes at 37°C or 4°C the cells were washed 3 times in PBS at 4°C or room temperature and resuspended in ice cold Eagles medium. The cells were either fixed immediately in an equal volume of 2.5% paraformaldehyde and 0.1 M sucrose, pH 7.3, or incubated at 37°C for various lengths of time and then fixed. Staining with neutral red showed most cells were living. Appropriate controls were included. Only about 1% to 5% of the flourescence stained cells showed caps; the remainder of the cells showed "ring flourescence". No increase in capping was obtained by varing incubation temperatures or duration of incubation.

7. Influenza virus isolation and identification - During Jan - Feb 1974, scattered outbreaks of febrile respiratory disease occurred in schools and families in the Washington Metropolitian Area. Influenza B isolates along the Eastern Seaboard, among other areas, were reported by CDC. From Jan - Mar 74, 21 Throat wash specimens from patients with acute respiratory tract infections were recieved by the Department of Virus Diseases and inoculated into embryomated eggs and/or primary Rhesus monkey kidney cell monolayers. Four isolates were obtained; three were Influenza A strains similar to A/Port Chalmers/73 and one an Influenza B strain similar to B/HongKong/5/72.

In May 1974, strains were received from the Department of Virology US Army Medical Component, SEATO. These viruses were isolated in embryonated eggs from throat washings obtained from Karen hill tribe villagers residing near Mac Saeriang, Thailand during a March 1974 epidemic of Influenza. One strain from each 3 villagers, MaeTia, Huay Makfai, and Mai La Ngui, were tested and found to be similar to A/Port Chalmers/73. Our identification of these strains was verified by the WHO International Influenza Center for the Americas of the CDC in Atlanta which was informed of both Washington DC and Thailand outbreaks and strains shortly after isolation and identification of strains as Influenza viruses.

B. Adenovirus surveillance program, Jul 73 - May 74

Fiscal year 1974 was distinguished by the lowest ARD rates encountered in basic combat trainees (BCTS) during the history of the

adenovirus surveillance program. This was due to administration of potent adenovirus vaccines throughout the year. In previous years, Wyeth Laboratories had experienced problems with vaccine virus stability due to inadequate evaporation of solvent in the process of coating tablets with enteric coating. Although some instability was noted in titer of the ADV-7 vaccine (Lot 5302) used until Feb 74 (These tablets lost 2 $\log_{10}$ in titer within one month of manufacture but thereafter titered 3.7 $\log_{10}$), the titer remaining was adequate for control of type 7 disease. The initial type 4 tablet (Lot 4902) and the vaccines recieved in Jan 74 and used in the last half of the fiscal year were stable and high titered (Type 4 Lot 5501, 5.2 $\log_{10}$ $Tcid_{50}$ and Type 7 Lot 5303, 6.3 $\log_{10}$ $Tcid_{50}$).

The following reports include adenovirus surveillance program data from 1 Jul 73 - 1 May 74.

FORT DIX: Adenovirus vaccines were used in BCTS from 1 Sep 73 - 1 May 74. Median ARD rates by month were below 1.5/100 men/week except for March when a peak of 2.1 was noted (An adenovirus was not isolated from Fort Dix BCTS in March). Of the 352 trainees sampled from 1 Sep - 1 May, adenoviruses were isolated from only 10 (3%); these included 8 type 7 and 2 type 4 viruses.

FORT JACKSON: Vaccines were given from 1 Oct 73 - 1 May 74. ARD rates were below 1.0 except for Feb - Mar 74 when they were 1.1. From 1 Oct 73 to 1 May 74, only 2 of 151 BCTS sampled (1%) had adenovirus isolates (Type 7).

FORT KNOX: Adenovirus Type 4 and 7 vaccines were given from 1 Jan - 1 May 74. Prior to vaccine administration, a median weekly rate of 1.5 occurred at Ft. Knox in December 1973. During that month, 39 of the 111 BCTS sampled (35%) had adenovirus in throat swabs; 37 of the isolates were adenovirus type 7 with one isolate each type 3 and type 4. The median rate in Jan 74 (the first month of vaccine administration) was 1.2 with a 24% adenovirus Type 7 isolation rate. Thereafter median weekly median and rate by month was less than 1.0 and only 9 of 281 BCTS sampled (3%) yielded adenoviruses.

FORT ORD: Adenovirus vaccines were administered to BCTS throughout the year. The median ARD rate was 1.5 or below except for a rate of 2.3 in Jan 74 coincident with Influenza B. From 1 Jul 73 - 1 May 74, 19 of 453 BCTS (4%) sampled had adenovirus isolated from throat swabs; 11 isolates were type 4 and 8 type 7.

FORT POLK: As usual, Fort Polk had little adenovirus ARD from Jul - Dec 73 even through adenovirus vaccines were not administered. With vaccines used from 1 Jan - 1 May 74, ARD rates were less than 1.5 and only one adenovirus was isolated from the 73 trainees sampled (1%).

FORT WOOD: Vaccines were administered from 1 Oct 73 - 30 Jun 74. Ft. Wood had ARD rates between 2.6 - 3.0 from Jan - Apr 74. In Jan - Feb, much of the ARD was Influenza B documented by viral isolation and serology. Adenovirus isolation rates during the 4 peak months were low (5% - 15 isolates from 301 BCTS sampled) and similar to the other 5 BCT posts during periods of Adenovirus vaccine administration. Both type 4 and 7 adenovirus were isolated at Fort Wood.

In summary, ARD rates at 5 of the 6 BCT posts were considerable lower than in past years. Only at Fort Wood did rates approach 3.0/ 100 men/week. At least past of ARD at Wood was Influenza B and the remainder could not be attributed to adenovirus. Indeed during periods of adenovirus vaccine administration, less than 5% of all BCTS hospitalized with upper respiratory infections at all posts yielded adenovirus.

C. Titration of ADV 7 and ADV 4 in Porcine Kidney Cells

Due to increasing difficulty in obtaining human embryonic kidney cell lines for research, an attempt was made to study the growth pattern of adenovirus types 4 and 7 in porcine kidney cell lines. No final conclusion can be drawn from the preliminary data, but the results appear on the surface to show promise for the future in the use of porcine kidney cells as an adjunct or substitute for human embryonic kidney cells in the isolation and vaccine titration of adenovirus Types 4 and 7.

The porcine kidney cell line, PS (Y-15), used in this study was obtained in passage 847 from Gulf South Research Institute, in 1973. In our laboratory it has been maintained in Medium 199 supplemented with 10% fetal bovine serum with antibiotics (100 units/ml of penicillin and 100 µg/ml streptomycin). The cells used in these experiments were in passage 868.

Five samples of ADV-4 have been titered in the PS (Y-15) cell line by cytopathic effect (CPE) or by plaque assay. All adenovirus type 4 sampled yielded a reproducible PFU titer comparable with that produced in HEK using CPE endpoint. Titers ranged to 10^{-5} in 3 separate experiments. Comparable plaque assays in HEK are underway. Four samples of ADV-7 have been studied for PFU and CPE. The results of all samples studied in Y-15 cell lines were reproducible. The available viruses for testing were low in titer and gave no CPE or plaques in the Y-15 cell lines or CPE in HEK cells. After serial passage in HEK or Y-15 cell lines, ADV Type 7 gave a reproducible plaque formation in Y-15 cell lines at a titer of 10^{2} with comparable titers in HEK using CPE end point. Experiments are presently in progress to attempt to determine if CPE and PFU are as promising as for Type 4 Adenovirus.

III. DIAGNOSTIC VIROLOGY

A. Cytomegalovirus infections in WRGH hemodialysis patients and personnel.

Because of reports in the literature of a high incidence of cytomegalovirus (CMV) disease patients in hemodialysis units, we decided to study serologically the incidence of CMV infection in patients on the hemodialysis ward (Ward 38) of Walter Reed General Hospital. Since sequential sera were also available on numerous staff members on that ward, these specimens were also surveyed by serologic means to determine if in fact infection by the cytomegalovirus could be considered an occupational hazard in personnel attending patients on hemodialysis.

The sequential sera used were those being obtained on a regular basis from patients and personnel on Ward 38 for prevalence and incidence of HB_sAg. Patient and staff sera had been collected since 1970 on a regular basis and kept frozen at -40°C; in all there were sera on 108 patients and 43 staff members during years 1970-1973. Sera were run in CF tests with 4u. CMV antigen using 5 CH_{50} units of complement in microtiter plates as standard.

During fiscal years 1970 - 1973, sera were collected on 108 patients. Of these 108 patients, 77 had had sequential sera (at least 3 specimens) collected, and 31 had single serum specimens. Twenty-three of the 31 (74%) with single sera had CMV CF titers $\geq$ 1:4 and 64 of the 77 (83%) patients with multiple serum specimens had equivalent titers in at least one specimen. Of the 67 patients on whom sequential sera were obtained, 40% showed $\geq$ 4-fold rise in CMV titer and thus had infection with CMV during the period of study.

Of the 43 staff members surveyed, 40 had sequential serum specimens. Nine (40%) had $\geq$ 4-fold CMV titer rises during the study. Twenty-one (53%) had CMV antibody. An attempt was made to correlate incidence of CMV infection with patient contact or involvment in hemodialysis procedure. (Table 50). Physicians had a low prevalence of CMV antibody and no CMV infections were detected. Although no CMV infections were documented in nurses, the prevalence of antibody was high and similar to technicians and patients. Dialysis technicians had a high prevalence of antibody and an incidence of infection similar to patients. These results would tend to incriminate blood as a source of CMF infections.

TABLE 50: Incidence of CMV infections and Prevalence of CMV antibody in Personnel of Ward 38.

Group	Number Standard	CMV Infection No	(%)	CMV CF Antibody No	(%)
Dialysis Technicians	26	9	(35)	22	(85)
Nurses	8	0	(0)	6	(75)
Physicians	9	0	(0)	2	(22)

B. Enteroviral Meningitis Survey

Experience in this laboratory has been that during the summer months asceptic meningitis symdrome due to enteroviral infections are at peak incidence. Two basic isolation patterns have emerged in past years. Either there has been a mixed pattern with several different viral strains participating in the overall rise in incidence, or there has been an increase in incidence or epidemic due to a single enteroviral strain. Because of our experience last year when a strain of Coxsackie B-5 virus acted almost alone as the major culprit, we decided to trace the etiologic path of viral meningitis again in 1973.

All cases of aseptic meningitis with materials presented to this laboratory for isolation attempts between June 1, 1973 and August 30, 1973 were seen by the Infectious Disease Service and a clinical confirmation of meningitis or meningoencephalitis, was obtained. Specimen of various types (throat wash, stool, CSF, urine, tissue) were obtained by either the WRGH house staff, or a member of the Infectious Disease staff. Although requested by our laboratory, not all patients had a complete set of specimens obtained. Aliquots of the specimens which were obtained were inoculated on cell lines of Rhesus monkey kidney, human embryonic kidney, WI-38 and HR-6 cell lines. Following initial isolation of a virus strain from a specimen typing was carried out by tube neutralization studies. No attempt to prove viral infection in patients by a specific isolated viral strain was done due to the fact that paired sera were unavailable on most patients.

Forty-eight specimens were obtained on 22 patients with confirmed clinical meningitis or meningoencephalitis. Of the 22 patients, 3 died. Two of the fatal cases had Herpesvirus Type 1, recovered from brain at autopsy. Of the remaining 19 patients 4 had Echovirus Type 6. Three of these patients had ECHO-6 virus isolated from 2 or more sources (Table 51). The low recovery rate was unexplained but was probably due

to a combination of factors involving initial handling of specimens. Also since specimens were not inoculated into suckling mice, Coxsackie Virus A infections may not have been detected. In addition to the ECHO Type 6 isolates, one stool specimen contained a second enterovirus different from ECHO Type 6.

TABLE 51: Summary of results of enteroviral meningitis survey

Specimen	Number	Number Positive	Viral Type
CSF	20	0	-
TW	10	1	Echo-6
Stool	14	5	Echo-6 (4) & 1 untypable
Brain	4	2	Herpes Type 1(2)

C. Herpesvirus Neutralizing antibody in Cerebrospinal Fluid of Encephalitis Patients

Herpesvirus hominis, Type 1, accounts for a significant proportion of clinically significant viral central nervous system disease and is the most common cause of sporadic fatal encephalitis in the Unites States. Although it is known that Herpesvirus hominis infections occur in the presence of pre-formed circulating anti-Herpes virus antibody, little is known about cerebrospinal fluid neutralizing antibodies. In 1969, MacCallum reported that a rising titer of neutralizing and complement fixing antibodies had been found in the cerebrospinal fluid in the acute and convalescent stages from several patients with encephalitis caused by Herpes simplex virus. In collaboration with Gostling & Ross, a larger series of results was obtained and published in 1972. Comparative tests for poliovirus and other antibodies present in the patient's serum confirmed that this antibody rise was not due to passive leakage of antibodies through an inflamed membrane.

Juel-Jensen and MacCallum have assumed that the finding of herpes neutralizing antibodies in the cerebrospinal fluid in a titer of 1:4 or greater is probably diagnostic in a patient with encephalitis ten days or more after disease onset, even if no rise in antibody is found.

Spinal fluid samples from patients with lymphocytic meningitis have been screened for neutralizing activity and detailed investigations have been performed on the nature of this CSF neutralizing activity from one patient with fatal encephalitis.

MacIntire VR3 HSV-H Type 1 and MS HSV-H Type 2 were obtained from Dr. Kenneth Merrman at CDC. Both strains were passaged 9 times in FS-9 fetal foreskin and passaged three times in Vero continous line of African Green Monkey Kidney cells.

The Vero continous line of African green monkey kidney cell monolayers were grown to confluency in Falcon plaque flasks. Equal portions of cerebrospinal fluid or serum dilutions and known virus dose (50-70PFU per dose) were incubated at 37°C for one hour; 0.2 ml of the virus test mixtures were inoculated onto the confluent monolayers of Vero cells. The virus test mixture was incubated at 37° for 60 minutes. Next, the monolayers were overlain with a simplified Agar overlay composed of 1% Difco purified Agar with 40% medium 199 (Microbiological Associate) and 40% double distilled water 0.36M bicarbonate, antibiotics and 20% FBS. The flasks were overlain with neutral red strain overlay at 3 or 4 days and examined for plaques for 24 hours. Neutralization was considered to occur if dilutions of CSF reduced plaques formed by 50% compared to control virus.

Cerebrospinal fluid from patient 1, shown to contain high titer neutralizing activity, was centrifuged at 35,000 RPM for 20 hours over a 10-40% sucrose gradient in a Spinco (Type 35) rotor. Fractions were collected from below and tested for neutralizing activity and for IgG and IgM concentrations in immunodiffusion plates (Hyland Laboratories).

Serum and spinal fluid samples from 17 patients were studied. (Table 51). Five patients had encephalitis, 4 terminating fatally, 1 terminating with Korsakoff's syndrome. Seven patients had lymphocytic meningitis. Three were suffering from myelitis; one with simultaneous severe herpes virus hominis Type 1 stomatitis. One patient developed idiopathic encephalopathy and coincident oral mucocutaneous herpes virus hominis Type 1 post renal transplantation and a final patient had nonreactive tuberculosis with a cerebrospinal fluid sample taken as part of a routine FUO workup.

Neutralizing activity to herpes virus hominis Type 1 was demonstrated in the cerebrospinal fluid from the four patients with fatal encephalitis, one of whom (patient #2) had Type 1 herpes virus hominis isolated from spinal fluid in the 1st week of the illness and from brain at postmortem exam, eight days after onset. The isolate from cerebrospinal fluid was recovered in the presence of CSF neutralizing titer of 1:10. Patient #6 was experiencing meningoencephalitis and had Type 2 herpes virus hominis isolated from cerebrospinal fluid. No neutralizing antibody was found.

Another patient (patient #7) had transverse myelitis and profuse stomatitis; herpes virus hominis Type 1 was isolated from the oral mucocutaneous lesion. No neutralizing activity was demonstrated in cerebrospinal fluid from this patient. Patient 14, renal transplant patient, also had concomitant herpes virus Type 1 isolated from a lip

lesion and had a negative spinal fluid titer.

In order to characterize the neutralizing activity in the spinal fluid, cerebrospinal fluid from patient #1 which had high titer of neutralizing activity was centrifuged through sucrose gradient. The neutralizing activity was found to migrate exclusively with IgG. Indeed, the peak of neutralizing activity was coincident with the peak of IgG. Further, incubation of the cerebrospinal fluid with rabbit antihuman IgG heavy chain serum removed all neutralizing activity. The neutralizing activity represented 7S IgG immunoglobulin in the spinal fluid. No neutralizing activity was seem in the IgM 19S region of the sucrose gradient. Indeed, IgM was not detected in the spinal fluid of this patient.

This report demonstrates, contrary to the findings of other workers, that antiherpes neutralizing activity is found within the cerebrospinal fluid in patients with encephalitis. Furthermore, it has been shown that the neutralizing activity from one of our patients behaved as 7S IgG immunoglobulin.

The virologic diagnosis of herpes virus hominis central nervous system disease is difficult. A specific diagnosis often depends on brain biopsy. Diagnosis by serum antibody rise is always questionable because of the possibility of coincident mucocutaneous herpes virus infection.

Normal levels of IgG in the CSF range from 15-40 ug/ml whereas normal plasma levels of IgG are 12,000 to 18,000 ug/ml. The expected ratio of IgG concentration, CSF to serum, would be 1:300 to 1:2000. However, in other viral CNS infections HAI or CF antibodies have been found to reach CSF to serum ratios as high as 1:8 (Winchester, 1972).

Simultaneous serum and CSF specimens may be examined and diagnosis may be presumed by noting a relative CSF to serum ratio of 1:8 or greater. MacCallum has stated that CSF antiherpes virus titer of 1:4 is diagnostic of central nervous system herpes virus infection. We are studying additional cases prospectively in order to understand the kinetics of antibody within the CSF.

Herpesvirus hominis Type 1 may result in fatal encephalitis in the presence of high titer of antiherpes virus antibody. This may be accounted for by direct extension by neuronal bridging, and/or by the above mentioned phenomenon of antibody bound but infectious virus (sensitized virus). Further efforts will be made to understand the pathogenetic significance of CSF antiherpes virus antibodies.

Table 51 - Herpes virus neutralizing antibodies in selected clinical specimens

Patient	Disease			
1. Warren	Fatal encephalitis	512	280	CSF brain isolate
2. Bishop	Type 1 fatal encephalitis	80	10	
3. Harris	Fatal encephalitis	> 4	2	
4. Parker	Fatal encephalitis	> 4	< 4	
5. Bordeaux	Postencephalitic Korslkoff's syndrome	> 4	< 2	
6. McGlaughlin	Type 2 meningoencephalitis		< 4	CSF isolate
7. Bonner	Lymphocytic meningitis		< 4	
8. Bryant	Lymphocytic meningitis		< 4	
9. Gaster	Lymphocytic meningitis		< 4	
10. Hammer	Lymphocytic meningitis		< 4	
11. Quinn	Lymphocytic meningitis		< 4	
12. Robinson	Lymphocytic meningitis		< 4	
13. Soto	Myelitis, herpes labialis	> 4	< 4	Lip isolate
14. Jaworski	Postinfection myelopathy		< 4	
15. Corley	Myelitis		< 4	
16. Smith	Herpes labialis, transplant		< 4	Lip isolate
17. Hollis	Nonreactive tuberculosis		< 4	

Summary

I. Antigenic analysis of the structural proteins of Sindbis virus was carried out. The two envelope glycoproteins of Sindbis virus were isolated by detergent disruption and isoelectric focusing. The separated glycoproteins exhibited isoelectric points at pH 6 and pH 9, and they differed in amino acid composition and biological activity. The pI 6 (E_1) glycoprotein appeared to be the virus hemagglutinin and it antigenically cross reacted with closely related western equine encephalitis virus antiserum. In contrast, the pI 9 (E_2) glycoprotein appeared antigenically specific, and antiserum prepared against this protein neutralized infectious Sindbis virus.

Investigations on the dengue virus non-structural soluble complement-fixing (SCF) antigens were concerned with search for antibody to dengue-2 SCF in sera of children with dengue hemorrhagic fever and further purification of the SCF antigen by isoelectric focusing. Antibodies to dengue-2 SCF were found in about half of the convalescent sera from dengue hemorrhagic fever cases drawn 2 to 3 weeks after onset of symptoms. No SCF antibodies were found in sera drawn up to the time of hospital discharge (generally by 8 days after onset of illness), or in primary dengue fever cases. Due to the possibility of a dengue SCF serotype-specific response, and to exclude all other material in the SCF antigen preparations, further purification of all 4 SCF serotypes was carried out. Isoelectric focusing resulted in extensive purification of the SCF antigens. The isoelectric points are characteristic of each type and correlate well with their relative free solution electrophoretic mobility. The results thus supported the proposal that these antigens are "charge-isomers". Antisera have been prepared to these purified antigens and are currently being characterized.

A naturally occurring small plaque clone of dengue-2 virus shown to be greatly restricted in replication above 37°C was also shown to be slightly more heat stable that the parent virus (PR-159, Puerto Rico) from which it was derived. Preliminary temperature-shift experiments indicated that the temperature sensitive step in viral replication was somewhere between 8 and 12 hours after infection. Preparation of radioactive small-plaque-clone virions in LLC-MK_2 cells indicated that the minor envelope protein V-1 was slightly larger than the V-1 of the New Guinea C prototype dengue-2 virions when they were coelectrophoresed in polyacrylamide gels. Neither the small plaque clone, the parent virus, or the New Guinea C prototype virus would replicate to any extent in continuous lines of human lymphoblastoid cells. The dengue-2 strain that replicated well had been passaged through a monkey and Aedes albopictus mosquitoes. The effect of certain passage histories to create host-dependent mutants is being studied.

Monolayers of chick embryo cells were enucleated by centrifugation in the presence of cytochalasin B. Infection of enucleated cultures with Sindbis virus, a group A arbovirus, resulted in productive infection, suggesting that the nucleus of the cell is not necessary for replication of this virus. In contrast, enucleated cultures infected with Japanese encephalitis (JE) virus, a group B arbovirus, did not produce infectious virus and showed virus-specific fluorescence in only a low percentage of cells. These observations indicate that either the nucleus itself or some closely associated perinuclear structure is essential for replication of Japanese encephalitis virus.

Methods of labeling and purification that were adequate for producing radioactive JE virions from vertebrate cell cultures were not successful with cultures of Culex triaeniorhynchus in spite of good production of hemagglutinin in the Culex cells. The ability of Culex cells to take up and incorporate radioactive amino acids was compared with that of LLC-MK_2 cells. Total uptake over a 2-hour period was only 20% of that of LLC-MK_2 cells. Furthermore, LLC-MK_2 cells incorporated 40% of what they took up, whereas Culex cells incorporated only 30%. Overall it appears that poor uptake is the major reason for inefficient labeling of mosquito cells. In another attempt to explain the failure to obtain labeled virus from mosquito cells the percentage of infected cells was determined by the indirect fluorescent antibody technique, using a high MOI of JE. By this method, it was found that only 3-10% of the cells showed signs of virus replication. To see if a more susceptible population of cells could be obtained, the Culex cells were cloned. Only one successful clone was derived. In preliminary studies it appears to be culturally and morphologically different from the parent line, but similar with respect to supporting virus replication.

The effects of metabolic inhibitors were studied to obtain methods for more efficient labeling of Japanese encephalitis virus-specified intracellular polypeptides from chick embryo cells. Using a concentration of 1 ug/ml we tested the effects of a 2-hour pulse of actinomycin-D on protein synthesis in chick embryo cells. Simple washing of untreated cells caused a four-fold rise in specific activity forty hours after washing as opposed to unwashed cells. The specific activity of the actinomycin-D treated cells was < 1% of the washed controls by forty hours after the drug treatment. The effect of the time of addition of actinomycin-D on the synthesis of proteins in JE-infected chick embryo cells was studied by polyacrylamide gel electrophoresis of extracts that had been treated with 1 ug/ml of actinomycin-D for 2 hours before infection of from 4-6 hours, 6-8 hours, or 16-18 hours after infection. All cellular extracts were labeled for a 2-hour period prior to harvest at 26 hours post infection. In the first three conditions the amount of incorporated label was low and the high molecular weight region of the gel was obscured by the host background. However, the cells that received the 16-18 hour treatment gave a gel pattern that was virtually identical to the actinomycin-D, cycloheximide treated marker proteins,

with a level of radioactivity that was about five-fold higher than that of the cells that had been treated from 6-8 hours post infection. These results may make pulse-chase studies of the virus-specified polypeptides possible.

Detergent disruption and isoelectric focusing of Japanese encephalitis (JE) virus did not reveal an increase in the number of structural polypeptides in contrast to the resolution of an additional envelope glycoprotein of Sindbis virus. The largest envelope glycoprotein (V-3) was routinely separated at an isoelectric point of pH 7.7 and appeared free of contamination; however, the other two structural proteins of JE were not as easily resolved. A peak consisting of V-3 and V-1 was found at an isoelectric point of pH 4.4; however, this complex could be dissociated with higher detergent concentrations. The structural proteins V-2 and V-1 have not yet been separated completely but preliminary evidence indicates that they may possess isoelectric points in the range of pH 10-pH 11.

Preliminary isoelectric focusing studies using JE-infected cellular extracts were also performed. Extracts were prepared by homogenization in the presence of 1% Triton-X 100 followed by dialysis and clarification by low speed centrifugation. PAGE of the extract prior to focusing revealed an aberrant pattern with reduced amounts of the three largest virus-specified polypeptides and an unusually large amount of radioactivity in the low molecular weight region of the gel. Focusing of the extract yielded prominent peaks at pH's 5.60, 6.89, 8.30, 9.33 and 11.37, as well as several minor peaks. Most of the focusing peaks gave multiple protein peaks on gels. Several gave a series of unresolvable proteins while others qualitatively resembled the ^{14}C-JE intracellular protein marker suggesting that these were pieces of virus-modified cellular membranes that were not completely dissociated. The most significant finding was that PAGE of pH 6.89 peak produced a single protein that co-migrated with NV-3.

Some biologic and immunologic properties of mouse normal peritoneal lymphocytes (NPLs) were described and compared with splenic lymphocytes (SpLs) and where appropriate, peritoneal exudate lymphocytes (PELs). NPls represented a metabolically active population of lymphocytes. By immunofluorescent criteria, about 50% of NPLs were surface immunoglobulin (Ig^+) compared to approximately 40% Ig^+ cells among SpLs. Most interesting was the fact that PELs contained fewer than 5% Ig^+ cells among SpLs. Most interesting was the fact that PELs contained fewer than 5% Iq^+ cells, indicating that normal and exudate peritoneal lymphocytes represented functionally distinct populations. An attempt was made to assess the relative thymus dependent (T-cell) functional capacity of NPLs vs. SpLs. Both with respect to reactivity to concanavalin A (CON A) and allogeneic cells, NPLs were twofold less reactive than SpLs. Thus, while possessing almost equivalent numbers of B cells, NPLs, at least functionally, contained far fewer T cells. The above suggested that many NPLs were neither T nor B-cells by the usual criteria. The possible nature of these cells was discussed.

The ultrastructural morphology of mouse peritoneal lymphocytes (PELs) and normal peritoneal lymphocytes (NPLs) was described and compared. Both populations contained morphologically indistinguishable inactive and intermediate lymphocytes. However, lymphoblasts were found only among PELs. Further, uropod bearing lymphocytes were prominent among PELs but rarely seen among NPLs. Since such cells were indicative of a thymus derived (T-cell) population, this constituted morphologic confirmation to the functional data in the preceding section which suggested that NPLs represented a T-cell poor population and PELs a T cell-rich population. Finally, a unique cell was found among NPLs, unusual in that it was an inactive lymphocyte with a large segregated macronucleolus. These cells resembled Ig^-, θ^- cells ("null" cells) described by others among SpLs. The possibility that such "null" cells exist among NPLs was discussed in light of previously presented functional data.

The transfer of lymphocytes intradermally into histo-incompatible hosts results in a local graft vs. host (GVH) reaction. Mouse lymphoid populations differed in their ability to effect this reaction in a xenogeneic transfer into guinea pig skin. Thus while peritoneal exudate and peripheral blood lymphocytes mounted a strong response, normal peritoneal lymphocytes and spleen cells were relatively ineffective. Prior immunization of donor mice resulted in a terminal "flare" of mitotic-dependent activity with transferred peritoneal exudate and peripheral blood lymphocytes. The effects of manipulations on the host were also of interest. Thus non X-irradiated guinea pig hosts gave a better initial response to transferred cells. However, the response was short lived and in the appropriate cases, there was no terminal "flare" reaction. When the hosts were X-irradiated, the immediate transfer reactions were somewhat diminished but the reactions persisted for a long period of time. Decomplementation of host animals by cobra venom cofactor resulted in additional diminution of the transfer reaction. These results suggested that cells capable of initiating a GVH response are compartmentalized *in vivo* and suggests that factor affecting the location of a lymphocyte at a particular point in time may have profound effects on host defenses *in vivo*.

Experiments with dengue strains suitable as vaccine candidates continued. A dengue-2 small plaque virus pool (S-1) at the 19th passage level was prepared and tested for suitability as a pre-vaccine seed. Markers, including small plaque size, temperature sensitivity, temperature stability, and suckling mouse and monkey virulence were used to characterize and differentiate this seed from the parent virus. The reversion frequency for S-1 was 2×10^{-3} and reversion upon passage was dependent on multiplicity of infection. The S-1 seed inoculated in certified FRhL cells replicated poorly and reverted to virulence upon passage in these cells. A low passage pool of D-2 PR-159 virus in FRhL cells with small plaque and ts^+ markers was found to have low virulence and high immunogenicity in monkeys. Rhesus monkeys inoculated with small

plaque clones showed marked reduction in virulence and stimulation of high levels of antibody. Protection was demonstrated in small-plaque-clone-inoculated monkeys later challenged with the parent virus. Neurovirulence in monkeys inoculated with the S-1, p 19a seed pool, was also lower when compared with the parent virus. Chimpanzees also demonstrated a desirable lack of viremia along with high levels of antibody after inoculation with the small plaque clones. A dengue-3 human isolate was passaged in certified PGMK cells and a parent virus pool was prepared at the 4th passage level. The parent virus pool contained mixed, large and small virus plaque populations and will be used for plaque isolation of of ts^+ mutants. A 5-azacytidine-mutagenized pool of the D-3 parent will also be used for mutant clone isolation.

Japanese encephalitis SCF antigen, derived from suckling mouse brain and free of hemagglutinins, had a molecular weight of 55×10^3 and an isoelectric point of 5.3. The SCF antigen was stable to SLS and 2-ME treatment, while JE virion CF antigens were not. A final purification of the SCF antigen by isoelectric focusing increased the type specificity of this antigen in CF tests. In immunodiffusion tests, the highly purified SCF exhibited no increased type specificity over cruder preparations and produced two precipitin bands against separate classes of anti-JE immunoglobulins.

The polypeptides of BFS-283 strain of California encephalitis virus have been analyzed by polyacrylamide gel electrophoresis. Six radioactive peaks were detected in purified virions grown in two different cell types. Five of these radioactive peaks contain glucosamine, two of which appear to be glycoproteins. When degraded virions grown in two different cell types were compared by coelectrophoresis, host dependent differences were detected in the electrophoretic mobility of some glycoproteins but not with the nonglycoprotein. Treatment of the virions with the nonionic detergent NP-40 resulted in a dense nucleic acid-protein complex containing the smallest of the structural proteins, its molecular weight being about 17,500.

The plaque reduction neutralization test (PRNT) has been tested and found to accurately differentiate the various subtype of the California encephalitis (CE) complex. This test was used to identify to subtype both mosquito isolates from the Pocomoke Cypress Swamp (PCS), Maryland, and to identify to subtype neutralizing (N) antibody found in wild vertebrate sera. Turtle and deer sera were tested for N antibody to local strains of Keystone and Jamestown Canyon subtypes of CE. Turtle sera testing is incomplete, but results to date suggest few N antibody positive individuals. Deer were frequently found N antibody positive. Hamsters, raccoons and squirrels were experimentally infected with swamp strains of Keystone or Jamestown Canyon CE. Hamsters proved to be a good laboratory model. Raccoon exposures were inconclusive, but suggested only low level viremias were produced. Certain individual squirrels produced viremia probably capable of infecting feeding mosquitoes.

Sentinel rabbits were exposed in the PCS from May through October 1973, and bled periodically to detect acquisition of N antibody to CE. A total of 20 rabbits converted against Keystone subtype CE, 17 of which converted immediately after the appearance of Aedes atlanticus adults. Aedes atlanticus mosquitoes have previously been shown to emerge infected with the Keystone subtype of CE, and these seroconversions suggest the ability of transovarial infected A. atlanticus to transmit Keystone virus at their first blood feeding.

Influenza A strains isolated from WRGH patients in Jan-Feb 1974 were similar to A/Port Chalmers/1/73; in addition, one strain of Influenza B/Hong Kong/5/72 was isolated. Strains from an outbreak of influenza in Karen Hilltribe villages near Mae Sariang, Thailand were similar to A/Port Chalmers/1/73.

Availability of stable and potent adenovirus vaccines led to the lowest ARD rates experienced in basic combat trainees at the 6 CONUS training posts since adenovirus surveillance was instituted in 1966. During periods of vaccine administration, less than 5% of trainees hospitalized with ARD had adenovirus infections established by viral isolation.

A high incidence of cytomegalovirus infections was documented by serology among patients undergoing hemodialysis and in hemodialysis technicians at WRGH. Although nurses in the dialysis ward had a high prevalence of CMV antibody, while only 2 of 9 physicians had CMV antibody.

Herpes virus neutralizing antibody was demonstrated in the cerebrospinal fluid of one patient with Herpesvirus encephalitis. Neutralizing activity was found in an additional 3 patients with encephalitis. The diagnostic significance of this antibody is being evaluated.

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Task 00 Communicable Disease and Immunology

Work Unit 166, Viral Infections of Man

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6H12	74 07 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D.CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62759A	3A762759A831	00	075
b. CONTRIBUTING	61102A	3A161102B71Q	00	CH FR 167
c. [redacted]	CARDS 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Rickettsial Diseases of Military Personnel

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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17. CONTRACT/GRANT

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e. KIND OF AWARD: f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	3	135
	CURRENT 75	2	135

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Rickettsial Infections; (U) Laboratory Diagnosis; (U) Vaccines; (U)Epidemiology; (U) Ecology; (U) Vectors and Reservoirs

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) 1. Detection of rickettsial diseases. 2. Improvement of methods for clinical serologic diagnosis and retrospective seroepidemiology. 3. Immunological studies on host response, both humoral and cellular, to infection with wild type and attenuated rickettsial agents, which represent a serious hazard to troops operating in the field.

24. (U) 1. Development of quantitative immunofluorescent assay. 2. Development of a hemagglutination assay for use in field studies. 3. Assay of protective effects afforded by passive transfer of humoral antibodies and cellular components.

25. (U) 73 07-74 06 - Previously reported under Project 3A161102B71Q, Task 00, Work Unit 167. 1. Development of a quantitative immunofluorescent (SAFA) assay has recently begun. Preliminary results indicate the technique is technically feasible, but continued efforts are necessary to reduce non-specific background fluorescence. 2. Development of a rickettsial hemagglutination assay for field studies has recently been initiated to provide a relatively temperature stable antigen attached to stabilized red cells or latex particles that will evidence group-specific reactivity. At present, the test seems to be technically feasible in that antigen can be attached to stabilized red cells, but continued efforts are necessary to reduce cross-reactivity and to develop antigens which are stable during extended storage at ambient temperature. 3. Studies on cellular immunity show that transfer of spleen cells from an immune donor to a normal recipient is accompanied by substantial resistance of the recipient to challenge with an otherwise lethal inoculation of viable rickettsiae. Additional work is required to determine if this resistance is rickettsial specific and to elucidate interaction of humoral and cellular responses in conferring protection against subsequent infection. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

* Published upon or after director's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 FOR ARMY USE! ARE OBSOLETE

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 167 Rickettsial diseases of military personnel

Investigators.
Principal: Joseph V. Osterman; Aki Shirai
Associates: CPT Stanley Oaks, Jr., MSC; Janis Campbell; Christine Simon; Maureen Guild; PFC John Dietel; PFC Thomas Ropchak

Introduction.

The major research efforts in this work unit during the period covered were to attempt improvements in the cultivation and purification of rickettsia; in the techniques currently used for diagnostic serology; and to develop other promising methods for the detection of rickettsial antigens and antibodies. In addition, new laboratory investigations of host defense mechanisms against rickettsial infection were initiated. Diagnostic tests on clinical material were performed as required. The numbers of clinical specimens received for testing increased over previous years, partly as a result of the resurgence of Rocky Mountain Spotted Fever on the east coast.

I. Purification of rickettsial organisms for diagnostic tests and investigations of antigenic structure.

Large quantities of rickettsial suspensions have been produced in embryonated eggs for use in the various investigations. Sucrose gradient centrifugation and CsCl equilibrium centrifugation have been utilized to purify the rickettsial suspensions. Such techniques provide suspensions of organisms that are relatively free of contaminating egg protein and, therefore, more suitable for immunological analysis or use in serological tests. A project to identify specific antigenic fractions in purified rickettsial suspensions utilizing polyacrylamide gel electrophoresis has been initiated.

The growth of rickettsia in cell cultures has also been a subject of active investigation. Rickettsial growth in suspension cultures of L929 and Hela cells was quantified, and cell cultures in roller bottles have proved to be a method whereby large numbers of rickettsiae can be harvested as relatively pure suspensions.

II. Application of soluble antigen fluorescent antibody technique (SAFA) as a tool for the detection of rickettsial antibodies.

An attempt was made to adapt the SAFA technique (Toussaint, A.J., Expt. Parasit. 19:71-76, 1966) for use in detecting rickettsial antibodies

to soluble rickettsial antigen. Advantages of the SAFA technique would be: (1) elimination of subjective readings; (2) correction for non-specific fluorescence; and (3) elimination of reaction fade-out during test reading. Preliminary results indicated that the SAFA test detects rickettsial antibody, showing greater reactivity with homologous than with heterologous sera. Further studies have been initiated to determine the specificity and sensitivity of the test system.

III. Development of indirect hemagglutination tests (IHA) for the detection of rickettsial antibody.

Investigations were initiated during this reporting period to ascertain if IHA antigens of the requisite specificity and sensitivity could be developed for the detection and measurement of rickettsial antibody. Sheep (SRBC) or human O (HRBC) were treated with glutaraldehyde prior to sensitization with soluble antigens prepared from R. mooseri or Proteus OXK. R. mooseri were obtained from the yolk sacs of embryonated eggs and purified by treatment with Amberlite resin and potassium acetate. This preparation was put through a French press at 20,000 psi to disrupt the organisms and then centrifuged at 10,000 g for 1 hr to yield a supernatant fraction. SRBC sensitized with the supernatant fraction were agglutinated by antisera to both R. prowazeki and R. mooseri.

A similar approach was taken utilizing soluble Proteus OXK antigen and HRBC (Schneider, H. et al., Indirect hemagglutination test for Proteus OXK antibodies using sensitized, preserved human erythrocytes. 406 MGL Special Report, USA Medical Command, Japan, 1/3/68). Proteus OXK were grown in a suspension culture. The organisms, in the log phase, were harvested, treated with 0.05N NaOH and placed in a boiling water bath for 2 hours. The NaOH extract was then centrifuged at 7,000 g for 30 min, and the supernatant brought to neutrality with 0.1N HCl. The neutralized extract, dialyzed overnight at 4°C against saline, was the soluble antigen used to sensitize glutaraldehyde treated HRBC. The IHA reaction with this antigen was specific against standard Proteus antisera (i.e., reacting only with OXK and not with OX2 and OX19). However, only one of three sera from [illegible] typhus patients reacted. Current studies include the testing of more clinical sera to determine the specificity and sensitivity of these tests and the identification of specific antigenic fractions that might be used in the preparation of IHA antigens.

IV. Host defense mechanisms in experimental rickettsial infections.

Experiments to establish a test system for investigations of host defense mechanisms in rickettsial infections were conducted in collaboration with the Department of Virus Diseases. BALB/C mice and

2 strains of Rickettsia tsutsugamushi (the less virulent Gilliam strain, and the virulent Karp strain), were utilized. Mice infected by an intraperitoneal injection of 100 mouse ID_{50} (50% infective dose) of the Gilliam strain on day 0 developed resistance to a subsequent challenge infection with > 1,000 mouse LD_{50} (50% lethal dose) of the Karp strain. On day 3 following inoculation of the Gilliam strain, challenge with the Karp strain produced some evidence of disease (ruffled fur, etc.), but no mortality. Complete protection against disease had developed by day seven and continued through day 28, when the study was terminated.

An attempt was made to determine if cell mediated immunity (CMI) functioned in this system. In evaluating CMI, animals were sacrificed and their spleens removed on days 3, 7, 14, and 28 after vaccination with Gilliam strain. Cell suspensions of these spleens were prepared, and approximately 1×10^8 nucleated cells were inoculated into individual animals, which were then challenged with the Karp strain. Cells prepared from donors on day three afforded no protection. Cells from day seven animals, while not preventing disease, conferred some protection, since 80% of challenged animals survived. Spleen cells collected from animals on days 14 to 28 transferred 100% protection against both infection and death. A slight decrease in protection to seven-day levels was observed with transferred spleen cells from animals vaccinated 42 days previously. The protection probably was due to the lymphoid cells of the spleen. The spleen cells that afforded complete protection in recipient mice yielded > 98% lymphocytes when fractionated on a Ficoll-Hypaque gradient.

Experiments are now in progress to identify relevant subpopulations (i.e., T cell vs. B cell); compartmentalization of the protective cells with time (e.g., spleen vs. lymph nodes, etc.); and the possible cooperative interactions of lymphocytes and macrophages.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. CHG	U	U	NA	NL	☑ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71Q	00	168
b. CONTRIBUTING				
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11. TITLE (Procede with Security Classification Code)

(U) Bacterial Diseases of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
58 05	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
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c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	5	180
	CURRENT 75	5	180

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Pseudomonas aeruginosa; (U) Mycoplasmas; (U) Staphylococcus aureus; (U) Immunology; (U) Antibiotics; (U) Infectious Diseases

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) - Studies on the etiology, ecology, epidemiology, pathogenesis, physiological, immunological and diagnostic aspects of diseases of microbial origin which are current or potential problems to military forces. Current emphasis on meningococcal, gonococcal, pseudomonas, mycoplasma and staphylococcal infections in military forces.

24 (U) - Development of bacteriologic techniques for study of various infectious diseases. Field studies or prophyiactic regimens, spread and persistence of organisms in various military populations.

25 (U) - 73 07 - 74 06 The isolation and preliminary characterization of cell surface antigens of Pseudomonas aeruginosa strains has made it possible to begin to systematically examine the functions and immunology of the individual components. Attachment of type 1 gonococci to buccal epithelial cells can be inhibited by a crude pili antigen. Rabbit antiserum inhibited attachment with some evidence of specificity. Human vaginal secretions from infected patients inhibited homologous gonococcal attachment but not heterologous. Studies of a tissue culture model of gonococcal infection have shown that organisms which have penetrated into cells can be differentiated from surface attached bacteria by means of peroxidase conjugated antiserum. Chronicity of the tissue culture infection has been demonstrated. Normal human serum in the medium prevented penetration by colony type 4 (nonvirulent) gonococci, but type 1 forms (virulent) were not inhibited. Clinical laboratory support of infectious diseases problems has consisted of identification of bacterial strains, antibiotic sensitivity tests and serum antibiotic assays. Patients undergoing esophageal dilatation developed transient bacteremias with organisms thought to be introduced during the procedure. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 168 Bacterial diseases of military importance

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I. Pseudomonas aeruginosa Infections

Studies on the outer cell wall membrane of Pseudomonas aeruginosa.

Pseudomonas aeruginosa like most other gram negative organisms has a cell envelope structure consisting of an outer membrane, a peptidoglycan layer and an inner cytoplasmic membrane [1]. Eagon has shown that ethylenediaminetetraacetic acid (EDTA) liberates from isolated cell walls a protein-lipopolysaccharide complex that contains proteins characteristic of those found in outer membrane preparations [2]. Studies have been directed toward the isolation and characterization of the outer membrane complex (native complex) and separation of its components. The purified protein and lipopolysaccharide (LPS) antigens, along with the whole native complex (NC), were used for investigations of their immunologic specificity and their function as pyocin receptor sites.

Methods and Results:

Isolation and fractionation of the native complex (NC) was performed by the method of Zollinger [3]. Briefly, organisms in suspension were heated, subjected to mild shear in an omnimixer and the NC separated by differential centrifugation. Fractionation was carried out with Sephadex G-100 column after solubilization of NC with deoxycholate.

Protein and lipopolysaccharide (LPS) components were analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) as well as by chemical monitoring of fractions. Protein and LPS eluted from the column in two well defined peaks.

Radiolabeled LPS obtained in a similar manner when analyzed by SDS-PAGE yielded a single well defined band just behind the marker dye front with a characteristic R_f of 0.85-0.87. In this system the R_f's of LPS have shown some concentration dependence but R_f's of 0.86-0.89 have been consistently found for LPS from all of the Pseudomonas strains thus far examined.

The isolated protein fraction obtained from the Sephadex G-100 separation of NC of Pseudomonas type 2 when analyzed by SDS-PAGE showed peptidoglycan and polysaccharide as well as the ^3H-labeled proteins. The molecular weights of these proteins were very similar to those found in the NC. Differences, however, between purified protein preparations and NC proteins were found. A nonradiolabeled preparation of NC from P. aeruginosa immunotype 4 showed major protein bands at 60,000, 55,000 and 25,000 daltons. After isolation and purification of the protein from the NC the major [illegible]ere at 60,000 and 41,000 daltons respectively. These differ[illegible]y result from conformational changes caused by exposure of the pr[illegible]o denaturing solvents during purification. The desoxycholate buffe[illegible]as caused no shift in R_f but washing with ethanol has.

In order to compare membrane proteins of the seven Pseudomonas immunotypes, NC prepared from a representative strain of each of the immunotypes was subjected to SDS-PAGE and stained for protein and carbohydrate. Major protein staining bands were seen at 60,000, 55,000 and 24,000 daltons in all the immunotypes except type 3 where there appeared to be only two major bands at 57,500 and 24,000 daltons. Native complex from each of the seven immunotype strains contained minor protein bands corresponding to the 100,000 and 18,000 dalton proteins found in the radiolabeled preparations. Carbohydrate stains of duplicate gels indicated that these 100,000 and 18,000 dalton proteins were probably glycoproteins and that for immunotypes 2 and 6 the 68,000, 60,000 and 55,000 dalton proteins were probably glycoproteins as well.

Reactions of purified antigens with antisera. To investigate the immunologic specificity of cell surface reactions a solid phase radioimmunoassay was employed. The method consisted of drying antigens in the wells of flexible polyvinyl microtiter plates (Cook Engineering Co., Alexandria, Va.) overnight, adding dilutions of antisera to be assayed and incubating 2 hr at 37°C. After rinsing the wells 10 times with tap water, goat anti-rabbit globulin (Nutritional Biochemical Corp., Cleveland, Ohio) radiolabeled (I^{125}) by the lactoperoxidase method was added in a concentration previously determined by block titration.

Incubation at 37°C was again following by rinsing. The wells were then cut out and assayed for radioactivity in a gamma counter (Nuclear Chicago, Chicago, Ill.). The serum titer was defined as the highest dilution of serum which still yielded three times the amount of radioactivity found as background in antigen coated wells to which no antisera were added.

Native complex preparations from each of the seven immunotypes were tested for their ability to react with rabbit antisera made against each of the agglutination types. The results of assays performed with rabbit antisera raised against boiled organisms are shown in Table 1.

Table 1. Cross-reactions among Pseudomonas aeruginosa immunotypes by radioimmunoassay.

Immunotype of native complex	Reciprocal titer of indicated antiserum						
	1	2	3	4	5	6	7
1	2048	32	64	64	32	32	64
2	1024	1024	64	128	64	64	32
3	256	1024	2048	64	64	64	64
4	256	1024	1024	1024	32	32	64
5	256	256	512	1024	1024	32	16
6	256	256	256	256	512	1024	64
7	128	128	256	128	128	256	512

As expected, each antiserum reacted to the highest titer with the NC antigen prepared from the homologous organism, eg. antiserum 1 reacted to the highest titer with NC 1. The low level cross-reactivity (1:32-1:64) between all the antigens was partially accounted for on the basis of prevaccination titers and nonspecific binding (albumin control). An unexpected finding, however, was that all the major cross-reactions were one-way cross-reactions. For example, in Table 1 note that while antiserum to P. aeruginosa immunotype 1 reacted in high titer with NC from immunotype 2, antiserum raised against P. aeruginosa immunotype 2 organisms did not react to any significant degree with NC extracted from immunotype 1. When the experiments were performed using rabbit

antiserum raised against live organisms essentially the same results were obtained.

Pyocin receptors. A series of experiments were carried out to identify the antigen(s) of the cell wall which serve as pyocin receptors. Pyocins were extracted by the method of Farmer [4]. Assays for pyocin activity used a replicator apparatus as described by Baltimore [5]. Pyocin absorptions were performed by mixing an equal volume of absorbing material with the pyocin to be tested, followed by incubation at 37°C for 30 min and overnight in the cold. Serial two-fold dilutions were made in Gey's solution (Microbiological Associates, Bethesda, Md.) and assayed for pyocin activity. LPS and protein concentrations in the absorbing solutions were 5 mg/ml.

Absorption of three different pyocin extracts prepared from standard pyocin producer strains A, D and E with purified cell membrane components from P. aeruginosa agglutination type 4 removed pyocin activity against type 4 P. aeruginosa (Table 2).

Table 2. Pyocin activity following absorption of pyocin extracts with Pseudomonas antigens.

Absorbing material	Pyocin A	Pyocin D	Pyocin E
Nothing	1024*	1024	1024
Native complex from Pseudomonas type 4	0	4	0
Protein from Pseudomonas type 4	1024	512	1024
LPS (phenol extract) Pseudomonas type 4	0	1024	0
LPS (Parke-Davis) Pseudomonas type 4	0	4	0

*Pyocin activity expressed as reciprocal of highest dilution of pyocin able to kill Pseudomonas type 4.

Native complex and LPS absorbed the pyocin activity from the extracts while the purified protein did not. The different activities of LPS (phenol extract) and LPS (Parke-Davis) against pyocin D remains unexplained. Pyocins A, D and E had no activity against P. aeruginosa agglutination type 2. Native complex, protein, LPS (phenol) and LPS

(Parke-Davis) from P. aeruginosa agglutination 2 were generally unable to absorb the activity of pyocins A, D and E against agglutination type 4, implying specificity of the absorptions. There was one exception: Pyocin A, but not D or E, was absorbed by Parke-Davis immunotype 2 LPS indicating an available receptor for a pyocin from producer strain A in this antigen or nonspecific adherence with this particular preparation.

Discussion:

The function of the outer membrane proteins is currently unknown. They undoubtedly play a structural role as suggested by Eaton [1] and may also be important in specific immunity. In an attempt to study the immunologic specificity of cell surface reactions, NC was prepared from each of the seven P. aeruginosa immunotype strains which had previously been identified by a series of cross protection studies in mice by Fisher, Devlin and Gnabasik [6]. These NC preparations were then tested for their ability to specifically react with rabbit antisera prepared against each of the serotype organisms in a solid phase radioimmunoassay. The results are consistent with the hypothesis that each of the seven serotype organisms has a major type specific antigenic determinant on its surface available for interaction with antibody and two or three other antigenic determinants which are not available for antibody binding presumably due to steric hindrance. For example, immunotype 2 organisms are postulated to have antigen 2 on their surface available for interaction with antibody and, in addition, antigens 3 and 4 available to stimulate antibody synthesis in a rabbit but rather inaccessible for antibody binding reactions. These data are consistent with the finding that serum from patients convalescing from P. aeruginosa infections will frequently have significant levels of hemagglutinating and gel precipitating antibodies against multiple LPS antigens, even though the patients' bacteriological isolates agglutinated strongly with only one type specific antiserum [7].

Besides immunologic specificity at the cell surface there is also pyocin receptor specificity which can be used as an epidemiologic tool. The results presented here using uncharacterized pyocins tend to confirm that LPS may serve as a receptor site for pyocin while the protein containing fraction does not appear to have receptor activity. This is in contrast to certain colicins (E_3, E_2 but not K) where the receptor appears to be a 60,000 molecular weight protein extracted from the envelope fraction of colicin sensitive Escherichia coli cells [8].

The isolation and characterization of cell envelope components from P. aeruginosa has enabled us to begin investigating interesting relationships between pyocins and their receptors, serological cross-reactions between immunotype groups and the role of proteins and other antigens in opsonization.

II. Gonococcal Infections

A. Serologic typing of gonococci.

Despite the fact that Neisseria gonorrhoeae has been known to be associated with venereal diseases for more than 50 years little more than the broad general aspects of its epidemiology are known, in part because an acceptable means of strain differentiation remains to be devised. With this in mind, a bactericidal test and a hemagglutination system using endotoxin antigens were examined.

1. Bactericidal typing system. A bactericidal reaction assay has proven meaningful as a classification scheme and an epidemiologic tool for studying meningococcal diseases [9,10,11]. Recently, a similar assay for examining gonococcal antigens was devised [12]. In the present study we have adapted this test using meningococcal antisera to examine certain circumscribed aspects of gonococcal epidemiology.

Methods:

Gonococcal strains, rabbit antisera, complement, bactericidal test. The gonococcal strains, antisera, complement and the bactericidal test have all been previously described in the 1973 Annual Report.

The minimal inhibitory concentrations (MIC) for penicillin, tetracycline, erythromycin, gentamicin and clindamycin were determined using an agar dilution technique. Briefly, increasing concentrations of the antibiotics were incorporated into GC media with defined supplement and the gonococcal strains were inoculated with a Lidwell replicator apparatus. The lowest concentration which inhibited the growth of the organisms was recorded as the MIC.

Results:

Rabbit antisera raised to meningococci were capable of killing most N. gonorrhoeae in the presence of excess complement. By absorbing these antisera with various heterologous meningococcal strains, further differences between gonococcal strains could be discerned (Table 3).

When strains from three pairs of consorts were examined, two pairs were found to be the same (Table 3, strains 198 and 199, strains 152 and 154) and one pair (strains 129 and 130) appeared to be different. In order to test whether the latter were indeed different antigenically, antiserum was raised to strain 129, and then absorbed with strain 130. All of the bactericidal activity

against strain 130 was removed but the absorbed serum was still capable of killing strain 129.

Since the original results were obtained on organisms which had been transferred a number of times, the test was repeated using organisms lyophilized after the second laboratory passage. This would minimize any antigenic alterations related to passage on artificial media. Also, the same lots of GC media and complement were used in order to eliminate these factors as possible variables.

The MIC's of all paired strains were also simultaneously determined for five different antibiotics (Table 4). The MIC of penicillin of strain 129 differed significantly from the MIC of strain 130.

Two pairs of strains were isolated at different times from the same patients. One patient had been treated with 4.8 million units of aqueous procaine penicillin at the time of the first culture and was negative for N. gonorrhoeae on follow-up cultures of the cervix and rectum. She returned six weeks later and her cultures were again positive for N. gonorrhoeae. When these strains (187 and 197) were examined in the bactericidal system they were found to be different. Their MIC's to tetracycline and gentamicin also differed.

The second patient was treated similarly but an intrauterine device (IUD) was left in place. Although she became afebrile and felt well, she continued to have a vaginal discharge. She returned five weeks later when her husband developed acute gonorrhea. Her strains (170 and 180) were the same as determined by the bactericidal assay (Table 3) and antibiotic sensitivities (Table 4).

Discussion:

Antigenic differences between strains of N. gonorrhoeae can be distinguished using a bactericidal assay employing meningococcal antisera [9]. A similar bactericidal system using gonococcal antisera was described by Ward and Glynn [13]. They were able to divide 60 gonococcal strains into four broad but overlappings groups. By absorbing antisera raised to one gonococcal strain by another strain, antigenic differences could be further defined [12].

Using this system five pairs of strains were examined which on epidemiological grounds raised questions as to whether or not they might be the same. Three pairs were identical and two were not. These results were verified by antibiotic sensitivity patterns and bacterial absorption studies on one pair.

Table 3. Bactericidal killing of gonococci with meningococcal antisera.

GC strain number	Absorbed sera												Unabsorbed sera							
	32/60-118	35/60	60/35	60/138	89/118	138/60	118/60	118/89	118/126-138	126/60	126/18	6557/6516	32	35	60	89	138	118	126	6557
129	-	±	-	-	-	-	-	-	+	+	±	+	+	+	+	+	+	+	+	+
130	-	±	±	±	-	-	±	-	+	+	+	±	+	+	+	+	+	±	±	+
198	-	-	±	+	-	±	+	ND	-	+	±	-	-	+	+	+	ND	-	+	+
199	-	-	±	+	-	±	+	ND	-	+	±	-	-	+	+	+	ND	-	+	+
152	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+	+
154	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+	+
170	-	-	±	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+
180	-	-	±	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+
187	-	+	±	+	-	+	-	+	-	-	+	+	-	+	+	+	+	-	+	+
197	-	-	-	-	±	-	-	-	-	-	-	-	-	+	-	±	-	-	+	+

- No bactericidal killing at dilution ≤ 1:5. ± 80% bactericidal killing at dilution 1:5 or 1:10. + 80% bactericidal killing at dilution ≥ 1:20.

Table 4. Antibiotic sensitivity patterns of paired gonococcal strains.

GC strain	MIC of antibiotic				
	Penicillin[1]	Erythromycin[2]	Tetracycline[2]	Gentamicin[2]	Clindamycin[2]
129	0.031	0.50	1.6	3.2	0.8
130	0.5	0.25	1.6	3.2	0.8
198	1.0	1.0	0.8	3.2	0.8
199	1.0	1.0	0.8	3.2	0.8
152	0.50	0.50	0.8	3.2	1.6
154	0.50	0.50	0.8	3.2	1.6
170	0.031	0.50	1.6	1.6	6.4
180	0.031	0.50	1.6	1.6	3.2
187	0.25	0.0625	1.6	0.8	0.8
197	0.25	0.0625	0.1	5.2	0.8

1 unit/ml

2 µg/ml

The use of a bactericidal assay as a typing system, however, has several limitations. First, it is unlikely that all of the antigens involved in the bactericidal test are represented by the test sera. For example, in previous studies [12] it was shown that absorbing anti F-62 with 104 organisms did not remove all of the bactericidal activity against F-62 suggesting that an antigen not shared with 104 was present on F-62. All meningococcal sera killed strain 104 and all but one of the meningococcal sera killed strain F-62. If the unshared antigen was represented by a meningococcal antiserum, then that serum would have killed F-62 but would have had no effect on strain 104. Since this situation did not prevail, that antigen was not represented in this typing scheme. Just how many sera would be necessary to uncover all of the possible relevant antigens is only conjectural at this time.

Also, rabbits naturally acquire antibodies against cross-reacting antigens shared by other Neisseria species as well as non-Neisseria species. This raises the liklihood that each antiserum may be unique in terms of its reactions and may not be readily duplicated when different lots of antisera are employed, even though they may be made to the same antigens. Therefore, strains tested with the same lots of antisera may be compared, while those tested with different lots can not.

Finally, the bactericidal test is a complicated and cumbersome system. The source of complement as well as the media, environmental conditions, and growth rates can each influence the bactericidal titers. An elaborate system of controls is, therefore, necessary and strains to be compared should be tested at the same time.

Despite these limitations this system lends itself very well to studying certain situations, such as limited epidemics and recurrent infections in an individual or consorts.

2. Endotoxin-hemagglutination. Maeland [14], using various endotoxin preparations as antigens, absorbed antisera and an hemagglutination assay, was able to classify gonococci with six distinct groups. An attempt to expand upon this phenomenon as a potential gonococcal typing system was undertaken.

Methods:

Rabbit antisera were prepared as described previously (seven injections of live organisms) or by injecting $\approx 1 \times 10^7$ washed, boiled organisms by the same schedule.

Alkali treated endotoxin, phenol water endotoxin and endotoxin from boiled organisms were prepared by methods previously described [14,15].

Hemagglutination tests were performed by methods previously described [14,15].

Antisera were absorbed with boiled organisms or intact organisms (approximately 1 gm wet weight/cc) at 37° for 1 hr, then in the cold for 1 hr. x 3.

Results:

The HA titers for the various endotoxin preparations are shown in Table 5. The highest titers were obtained using the boiled endotoxin as antigen and this preparation was used thereafter.

In table 6 are listed the titers of antisera vs. various gonococcal endotoxins. Not only were significant cross reactions observed but homologous titers were not always greater than heterologous. These antisera were then absorbed with boiled heterologous strains and retested. Following absorption with a single strain, HA titers of each serum fell to <1:8 against the homologous and all other endotoxins shown in Table 6 with only occasional exceptions. This was true when each strain shown in the table was used for absorption.

Discussion:

These results show a great deal of antigenic cross reactivity among the endotoxins of the five gonococcal strains tested and the differences are not dissimilar enough to be readily discerned by HA test. Thus, our results are at variance with those of Maeland [14,15]. It should be pointed out that these strains have been shown to react differently in the bactericidal test. The results may be interpreted as suggesting that the antigens responsible for the bactericidal specificity are not endotoxins alone. Indeed, other studies [12] have shown that purified protein and endotoxin together were required to completely inhibit serum bactericidal activity. Neither purified antigen used alone was completely inhibitory.

B. Attachment of gonococci to epithelial cells.

Organisms which possess fimbrae or pili have an increased capability of sticking to mammalian cells and causing agglutination [16]. Punsalang and Sawyer [17] demonstrated that piliated gonococci.

Table 5. Hemagglutination titers of rabbit antigonococcal antisera against erythrocytes sensitized with various homologous endotoxin preparations.

Rabbit antisera		Alkali endotoxin	Boiled[1] endotoxin	Phenol water endotoxin
		Titer	Titer	Titer
101	boiled	4[2]	512	8
101	WB[3]	4	128	128
104	boiled	2	64	8
104	WB	<2	16	ND
108	boiled	8	64	32
108	WB	4	32	ND
120	boiled	2	1024	8
120	WB	4	1024	ND
125	boiled	16	1024	32
125	WB	4	1024	ND

[1] Lot H-1

[2] Reciprocal titer

[3] Whole organism

Table 6. Reciprocal HA titers of antisera vs. erythrocytes sensitized with endotoxin from various organisms.

Antisera (unabsorbed)	Gonococcal endotoxin[1]				
	101	104	108	120	125
101[2]	512	128	16	256	2048
104	2048	128	32	256	2048
108	1024	256	128	512	2048
120	256	128	32	128	1024
125	2048	2048	512	2048	2048

[1] Lot H-3

[2] Antisera to whole organisms

(colony types T_1 and T_2) adhered to human buccal epithelial cells in greater numbers than nonpiliated organisms (colony types T_3 and T_4). This attachment could be inhibited by rabbit antisera raised to piliated organisms and to crude pili preparations.

We have examined a) inhibition of gonococcal adherence by human genital secretions (local antibody), b) the specificity of the adherence using rabbit and human serum antibody and c) the antigen(s) involved.

Methods:

Epithelial adhesion. Human buccal epithelial cells were washed five times. Equal volumes of epithelial cells, 1:40 dilution normal human serum, antiserum or secretion and test organisms were mixed and incubated at 37° x 20 min in a rotary shaker. Smears were prepared and examined unstained by phase microscopy. Adherence was considered positive when >80% of buccal cells had 20 or more attached organisms.

Inhibition of adhesion (EAI): EAI was carried out by mixing equal volumes of inhibiting material with antiserum and incubating at 37° x 20 min before serially diluting the mixture and adding organisms as above. Smears were read as positive for attachment when >50% buccal cells had bacteria on them (controls were required to have >80% as above). Preparation of native complex from gonococci was previously discussed (Annual Report 1973). Crude gonococcal pili were isolated by suspending gonococci in saline, blending at top speed in an omnimixer, centrifuging and adjusting the supernatant first to pH 4.0 with HCl x 36-96 hrs, then to pH 7 with NaOH and reprecipitating with $MgCl_2$ 0.1 M repeated four times. Collection of genital secretions was described before (Annual Report 1973).

Results:

Rabbit studies. Antisera raised to piliated organisms and then absorbed with nonpiliated organisms inhibited the attachment of piliated gonococci to epithelial cells (Table 7). The highest dilution of antiserum which inhibited the attachment was greatest in every instance against its homologous organism with the exception of anti-101. These results were highly reproducible.

In order to identify the antigen responsible for the epithelial attachment attempts to block the EAI were carried cut using gonococcal native complex and a crude pili preparation (Table 8). The native complex had no significant effect on gonococcal attachment. The crude

Table 7. Inhibition of epithelial cell adhesion (EAI).

Rabbit antisera	Organisms					
	9	101	104	108	120	125
Anti 9	16[1]	2	2	<2	<2	2
Anti 101	<4	512	64	16	512	256
Anti 104	<4	<4	64	<4	<4	<4
Anti 108	4	8	4	32	4	2
Anti 120		4	8	2	32	8
Anti 125		4	8	8	<4	1024

[1] Reciprocal titer

Table 8. Blocking of EAI with various antigen preparations*.

Rabbit antisera	Inhibiting antigen preparation		
	None	NC[1]	Crude pili
Anti 104	32[1]	16	<1:4
Anti 120	32 - 64	32	8
Anti 125	512	256	<1:4
Anti 108	32	ND[3]	4
Anti 101	≥256	ND	16

* Each antiserum was tested against homologous antigens only.

[1] NC = native complex

[2] Reciprocal titer

[3] ND = not done

pili preparation consistently lowered the inhibiting titer.

Antisera raised to boiled organisms were incapable of blocking the EAI of the homologous organisms.

Human studies. Vaginal secretions were tested for their ability to inhibit epithelial adhesions (Table 9). Four of five secretions inhibited adhesion of the homologous organism. The highest titers were in those secretions concentrated 10-15 fold (134 and 136). Inhibition of heterologous organisms was significantly less.

Normal vaginal secretions not only failed to inhibit but had an additive effect on epithelial sticking, increasing the number of adherent organisms by as much as 1.5-2 fold, ie. 30-40 cells vs. 50-60 cells.

Discussion:

These preliminary data suggest that pili from various gonococci are antigenically different if, indeed, pili are responsible for the adhesion. These studies are a variance with those of Buchanan [18] who found that fluorescein conjugated antisera made to pili were capable of staining homologous and heterologous piliated strains but not their nonpiliated counterparts.

Studies to further define the antigens involved as well as the class of antibody are underway.

C. Gonococcal antigens: immunological studies.

Gonococcal infection elicits human humoral antibody responses [18,19,20,21]. The antigens to which antibodies are directed are not well characterized. Using an indirect immunofluorescent antibody (IFA) assay gonococcal native complex (NC) and cell membrane protein antigens were examined for their reactions with serum antibodies.

Methods:

IFA was performed by standard methods (Annual Report 1973), whole gonococcal organisms serving as the test antigen. Inhibition studies were carried out by incubating an equal volume of the NC or protein with the human antiserum for 30 minutes at 37°C, followed by serial dilutions of the serum in the usual manner.

Table 9. Inhibition of epithelial cell adhesion (EAI) by human vaginal secretions.

Patient	Vaginal secretions	Homologous organism	Heterologous organisms						
			101	104	108	134	149	N. cat.	N. subflava
134	2566	≥64*	8	8	8				
	2581	256							
	2594	1024	8	8	8				
	2600	8						<4	<4
136	2590	128	<4						
	2598	128	<4						
	2639	128	<4						
129	2541	8							
	2557	8							
149	2646	8		<8	<8				
	2653	8		<8	<8				
	2670	8		<8	<8				
152	2659	<4							
	2671	<4							
	2678	<4							
Normal	7120		<8		<8	<8	<8		
	7121		<8		<8	<8	<8		
	7123		<8		<8	<8	<8		

* Reciprocal of highest dilution causing inhibition of gonococcal adhesion.

Results and Conclusions:

The results are shown in Table 10. In infected patients both the protein and NC reduced serum IFA titers. They had no effect when tested with a normal human serum pool. However, one laboratory volunteer (CP) had a significant fall in titer after absorption with the protein antigen. NC reduced the titer in vaginal secretions of the two patients studied but the protein antigen had no effect against the one secretion which was tested with this material. Although few specimens were tested the results suggest that NC and cell membrane proteins appear to be important in inducing a human serum antibody response. Gonococcal native complex appears to be an important antigen against which IgG in vaginal secretions is directed. Further studies are planned to determine the extent to which infected and noninfected persons show antibodies to these antigens and whether other immunoglobulins are directed against the same antigen in local secretions.

D. Studies with chick embryos.

Introduction:

A major obstacle in studying the pathogenesis and immunology of Neisseria gonorrhoeae has been the lack of a suitable animal model. Many investigators, however, have shown that gonococci are capable of infecting chick embryos [22,23,24,25,26]. We have studied this model as a potential system for studying protective antigens.

Methods:

Seven, eight, nine and ten day old chick embryos (CE) were used. Dropped allantoic membranes were inoculated with organisms harvested from solid medium after 16-18 hrs growth and suspended to appropriate concentration of live organisms in buffer or NaCl.

A pool of rabbit antisera, heated to 50°C x 30 min, was used for neutralization studies.

Results:

Effect of serial passages in CE: Gonococcal strain $101T_1$ was passed 12 times and strain $104T_1$ was passed 23 times (CE to agar, to CE, to agar) with no increase in virulence, ie. 10^3 organisms killed 30-50% of inoculated embryos after four days incubation after few or many passages.

Table 10. Inhibition of IFA[1] with GS9 native complex and protein.

Specimen	Case No.	Inhibitors		
		None	Protein	NC[5]
Patient serum	153[2]	256[4]	64	16
Patient serum	201[2]	512	64	64
Normal serum	CP[2]	256	65	ND[6]
Normal serum	Pool[2]	128	128	128
Vaginal secretion	2689[3]	4	4	<2
Vaginal secretion	2632[3]	4	ND	<2

1 Against GS9 as antigen

2 Antihuman globulin conjugate used.

3 Vaginal wash - antihuman IgG conjugate used

4 Reciprocal of IFA titer

5 NC = Native complex

6 ND = Not tested

Antibody protection: The effect of pooled rabbit antisera in protecting chick embryos is shown in Table 11.

Table 11. Antibody protection of chick embryos.

Gonococcal strain	Inoculum size	Experimental group	No. dead/No. injected	% dead
104	10^6	Serum + org.	22/53	41
		NaCl + org.	29/52	58
		NaCl + serum	10/41	19
101	10^5	Serum + org.	8/32	25
		PBS + org.	12/26	46
		PBS + serum	2/26	7.5
108	10^2	Serum + org.	21/26	80
		PBS + org.	24/29	83

Against the less virulent strains 101 and 104, slight protection was afforded. Against strain 108 no protection was observed. No differences were seen when 7, 8, 9 and 10 day old embryos were used.

Discussion:

Bang [24], in 1941, reported an increased virulence for chick embryos after 31-35 passages and Walsh [25] reported similar results after 15 passages, using inducement of urethritis in human volunteers as a determination of virulence. We could document no increased virulence for chick embryos in two strains after 12 and 23 passages in the manner described.

Strain 108 appeared to be particularly virulent for the chick embryos. It is interesting that the embryos receiving the less virulent organisms were protected.

E. Inhibitory Lipids.

Bacteriocin production has been described in both Neisseria meningitidis and Neisseria gonorrhoeae. Typing schema utilizing sensitivity to these neisserial bacteriocins as a basis for the intra-specific classification of these species have been described but have gained little application. Production of bacteriocin-like substances was observed among strains of N. gonorrhoeae which were being studied to detect bacteriophages and for sensitivity to rough specific bacteriophages. Using classical bacteriocin cross-streak methods, a number of strains were studied for both production of and sensitivity to gonococcal bacteriocins.

The results of these studies were disappointing, sensitivity to bacteriocins was highly variable and all strains appeared to produce substances inhibitory to at least a few gonococcal strains. Attempts to optimize bacteriocin production by modification of the growth medium's buffering capacity and varying glucose or amino acid concentration resulted in a slight improvement. Attempts to detect bacteriocin activity in the supernates of broth cultures of bacteriocin producing strains were negative even after such cultures were induced with mitomycin C.

A few salient observations could be made from these different experiments. Strains having bacteriocin-like activity were for the most part sensitive to their products; a situation markedly different from that seen in other genera. Bacteriocin activity was observed only in cultures grown on solid media and could be best demonstrated when such cultures were killed with chloroform prior to their removal from the agar surface. In addition, all strains demonstrated inhibitory activity after chloroform treatment. Our inability to demonstrate bacteriocin activity in the supernates of induced or uninduced broth cultures and lack of immunity of strains to their own products suggested that inhibition was not the result of bacteriocins. The marked enhancement of activity produced by chloroform treatment suggested that toxic cellular components, possibly lipids, were released either by solubilization or by cell lysis.

The role of chloroform in liberating bacteriocin-like inhibitory substances was tested by extracting 20 different strains of N. gonorrhoeae using the Folch chloroform-methanol lipid extraction procedure. These extracts were tested for activity by preparing antibiotic filter paper discs soaked with the extracts or thin-layer chromatograms of the extracts and overlaying them with semi-solid agar suspensions of the same strains. Thin-layer chromatograms

showed inhibition over areas tentatively identified with lipid and amino acid detecting reagents. Using disc preparations, the extracts demonstrated a spectrum of activity against gonococcal strains as summarized in Table 12.

Table 12. Activity spectrum of Folch gonococcal extracts on selected gonococcal indicator strains.

Indicator strain	Extract of strain				
	7	5	17	20	16
3	+	+	+	+	-
13	+	+	+	-	-
10	+	+	-	-	-
12	+	-	+	-	-
11	+	-	-	-	-

+ = Zone of inhibition

- = No inhibition

The differential sensitivity of indicator strains to chloroform-methanol extracts indicate that patterns of sensitivity to such extracts could be useful as a method of intraspecific classification of N. gonorrhoeae strains.

At present work is underway to confirm and expand the potential of chloroform-methanol extracts to distinguish strains of N. gonorrhoeae and to determine their activity against other species of Neisseria and other bacterial genera. Work is also underway to separate and characterize the active component(s) in the extracts, the manner in which they act on live gonococcal organisms so as to provide a rational basis for selecting compounds of potential value in the treatment or prophylaxis of gonorrhoea.

F. A tissue culture model for Neisseria gonorrhoeae infection.

In spite of the prevalence of clinical gonorrhea too little is known about the pathogenicity of the gonococcus (GC) for the development of an effective control. A major research problem has been the lack of a suitable animal model in which the immunologic aspects of this disease

might be studied. GC infected tissue cultures (TC) were, therefore, investigated in an attempt to duplicate known parameters of GC infection in vitro. Of particular interest to this laboratory would be the use of this model to study the factors allowing GC to infect hosts which have been previously infected and have high levels of GC serum antibodies and immune cellular hypersensitivity [27].

Two features of GC infection in humans which have been reproduced in vitro are 1) its propensity for developing into the chronic carrier state [28] and 2) its ability to penetrate otherwise normal appearing epithelial cells of the infected organ [29].

Long term survival of GC in tissue cultures has been established [30] and penetration of tissue culture cells by the bacteria has been demonstrated by electron microscopy [31]. Because this latter technique is too cumbersome for any large scale study a method was developed for differentiating intracellular from extracellular bacteria by light microscopy. The penetration of the TC by GC was then studied as an indication of actual infection of the TC cell.

Methods:

General procedures: GC were grown on GC solid medium. Suspensions containing about 10^9 organisms per ml of medium 199 in Hanks salt solution, plus 2% fetal calf serum, were made for infecting TC cells. These suspensions were added to the TC and incubated for the indicated length of time at 37°C in 5% CO_2 atmosphere. At the end of the incubation period peroxidase-conjugated anti GC gamma globulin (P-anti-GC) was added. After 5 min further incubation, the TC monolayer was washed, fixed and reacted with diaminobenzidine which specifically stains the P-anti-GC brown. The TC was then counterstained with Giemsa. Monolayers were examined by light microscopy. Bacteria which were peroxidase negative (GC protected from reacting with the P-anti-GC) were considered to be intracellular. This interpretation has been confirmed by electron microscopy.

At least 200 TC cells were examined and the number containing intracellular GC (positive TC cells) was rated by the following scale:

less than 1% positive TC cells:	0
1 to 5% positive TC cells:	+
6 to 25% positive TC cells:	++
26 or greater % positive TC cells:	+++

Tissue culture cell lines: Except for human amnion (HA) cells, the cell lines used were kept in continuous growth in this laboratory using standard tissue culture techniques except that no antibiotics were used in the media. Primary cultures of HA cells were prepared here or were obtained from HEM Corp., Bethesda, Md.

Conjugated gamma globulin: Rabbits were immunized with GC (grown in medium 199 plus 10% normal rabbit serum) plus Freund's complete adjuvants. IgG was separated from the rabbit serum by ion exchange chromatography and conjugated to horseradish peroxidase by the method of Avrameas and Ternynck [32].

Results:

As indicated in Table 13, several TC lines were infected with GC. TCs were processed at regular intervals between one and 72 hrs of incubation with bacteria in order to determine the time of first penetration. All TC lines showed eventual penetration and no difference was detected in TC cell penetration among the three strains of GC tested on BHK and HA cells. Although not indicated in this table, both T_1 and T_4 colony types were tested against each TC line with no reproducible difference in ability to penetrate TC cells being detected. In addition to the time periods indicated in Table 13 infected BHK TCs were maintained for up to one month without complete destruction of the TC or loss of viability of the bacteria.

In addition to GC both Neisseria cararrhalis and Neisseria meningitidis were tested and found to penetrate the TC cells.

Negative controls are shown in Table 14. Killing the bacteria by heating them to 56°C or cooling to 4°C destroyed their ability to penetrate TC cells. Inactivating the TC cells phagocytic mechanism with cytochalasin also prevented penetration of the TC cells.

The results of our initial investigation into the protection from antibody afforded a bacteria once it has penetrated a TC cell are indicated in Table 15. The bacteria were allowed four hours to penetrate the TC cells and then P-anti-GC was added to the TC media. The TCs were incubated for one-half to four hours longer and then processed. Small peroxidase positive vacuoles were found in the cells beginning at one hr and a sharp reduction in the number of peroxidase negative or "protected" intracellular bacteria occurred at two hrs and four hrs.

In order to more closely approximate a human infection with GC human serum was substituted for fetal calf serum and a line of human

Table 13. Penetration of tissue culture cells by gonococci[1].

Tissue culture line	GC strain	Hour of first penetration	Relative degree of penetration at 4-8 hrs.
BHK (hamster kidney)	230	1 hr	+++[2]
	236	ND	+++
	265	ND	+++
HA (human amion)	230	1 hr	++
	236	ND	++
	265	ND	++
HR-6 (human lung)	230	ND	++
KB (human carcinoma)	230	ND	+++
HELA (human carcinoma)	230	72 hr	+ (at 72 hr)
LLC-MK_2 (monkey kidney)	230	8 hr	++
	Other Neisseria species		
BHK	N. catarrhalis	ND	++
BHK	N. meningitidis	ND	++

1 Colony type 1 used throughout.
2 "+" indicates relative numbers of TC cells penetrated by bacteria as explained in the test.
3 ND - time sequence studies were not done.

Table 14. Inhibitors of tissue culture penetration by gonococci[1].

Bacteria heated 56°C for 30 min.	No penetration
Bacteria refrigerated 4°C for 18 hrs.	No penetration
Tissue culture treated with Cylochalasin B	No penetration

[1] GC strain 230 T_1 and BHK cell cultures used.

Table 15. Time course of reaction of intracellular GC with peroxidase conjugated anti GC γ-globulin[1].

Time of incubation with antibody (hr)	Peroxidase detected in TC cells	Peroxidase neg. intracellular bacteria
1/2	None	+++[2]
1	Small amount	+++
2	Moderate amount	+
4	Moderate amount	0

[1] GC 230 T_1 infection of BHK cells used.

[2] "+" indicate relative numbers of TC cells containing bacteria not reacted with peroxidase.

cells, HR-6, was used. The results are summarized in Table 16.

Table 16. The role of human serum on the penetration of HR-6 cells by GC[1] colony types T_1 and T_4.

Serum donor	Colony type	1:10	1:20	1:80	1:320	1:1280
WR	T_1	++[2]				
fresh	T_4	0	0	0	+	++
WR	T_1	++				
heated[3]	T_4	0	0	0	++	++
JS	T_1	++				
fresh	T_4	0	0	0	+	++
BB	T_1	++				
fresh	T_4	0	0	+	++	++

[1] Strain 230 used.

[2] "+" indicates relative numbers of TC cells penetrated by bacteria as explained in the text.

[3] Serum heated to 56°C for 30 min.

The pathogenic colony type of GC, T_1, was found to penetrate the TC as before, whereas the nonpathogenic colony type, T_4, did not.

Discussion:

These experiments have demonstrated that GC penetrates tissue culture cells and that this process requires an active role on the part of both the bacteria and the TC cells. Also, the GC-TC parasitism can persist for at least one month. Although anti-GC antibody does not rapidly diffuse across the TC cell membrane to combine with the intracellular bacteria, P-anti-GC immunoglobulins are taken into the TC cell and eventually combine with the intracellular bacteria. This conclusion is supported both by the observations presented here and by data from electron microscopy performed on the same materials. Finally, when human serum was

used in the incubation media only GC colony type 1 penetrated the TC cells.

This model, therefore, reflects three known aspects of human infection with GC. First, epithelial cells of the infected organ are invaded by the GC [31]. Second, GC infections can exist in a chronic "subclinical" state [30]. Third, GC of colony type 1 but not of type 4 are infective [33].

Acknowledgement:

This work was done in conjunction with personnel in the Department of Experimental Pathology, WRAIR.

III. Clinical activities.

The Department of Bacterial Diseases supports clinical infectious disease activities in three areas: (1) Staff members make rounds as attendings and consultants to the Infectious Disease Service, WRAMC, being "on service" an average of two months per year. In addition, telephone and visiting consultations are available at all military hospitals (local and distant) and civilian hospitals and physicians for exceptional problems. (2) Teaching (both bedside and lecture) of house staff and medical studies. (3) Laboratory investigations related to special clinical infectious disease problems encountered.

The number and variety of clinical laboratory tests performed are given in tables 17 and 18. These two tables emphasize the scope of the support activities.

Occurrence of bacteremia after esophageal dilatation.

The objectives of the study were threefold: 1) To determine whether bacteremia occurs as a result of esophageal dilatation; 2) if bacteremia does occur, is it associated with particular esophageal diseases; and 3) is there any common causative bacterium.

When the three objectives are achieved a determination will be made as to the desirability of prophylactic antibiotic treatment prior to a patient undergoing esophageal dilatation, especially those patients with impaired health or on a course of immunosuppressive drugs.

At first it was planned to evaluate 50 to 100 patients of the Gastroenterology Service, WRAMC undergoing dilatation for bacteremia. However, all of the first 18 patients studied have had bacteremia

Table 17. Summary of specimens processed by the Diagnostic Section, Department of Bacterial Diseases - 1973-74.

Meningococci	32
Subacute bacterial endocarditis strains	5
Serum for gentamicin level	298
Serum for penicillin level	9
Serum for clindamycin level	8
Organisms tested for antibiotic minimum inhibitory concentration (MIC)	46
Serum for bactericidal testing	36
S. aureus bacteriophage typing	434
Throat cultures	80
Mouse blood cultures	14
Miscellaneous	77
Mycoplasma and/or bacterial variants	6

Table 18. Subacute bacterial endocarditis isolates.

Specimen No.	Patient	Causative Agent
529	Galloghy	Pseudomonas spp.
190	DeQuoy	Alpha streptococcus
886	Clark	Alpha streptococcus Neisseria sicca Bacteriodes spp. Unknown anaerobe
544	Fitz	Streptococcus bovis
587	Bishop	Staphylococcus aureus

immediately following the procedure.

Methodology:

Oral temperatures and throat cultures were obtained initially. Blood samples were obtained through a 19 gauge pediatric intravenous injection set which was inserted with sterile conditions - operator wore surgical gloves; skin site was prepared with Betadine scrub; the catheter was protected by a barrier drape. Samples were taken before the esophageal dilatation, immediately after and 5, 10, 15 and 30 min. post-dilatation.

Bacteriologic methods: 0.3 ml of each sample was placed immediately on three blood agar plates, spread with sterile glass spreaders and one plate each was incubated aerobically under CO_2 and anaerobically. All plates were observed for at least one week. For all samples, beginning with five min. sample, a blood culture bottle was inoculated with approximately 0.3 ml blood. Each bottle which appeared to contain growth was subcultured to MacConkey, blood agar and mannitol salt agar plates. Results of blood cultures on the 18 patients are given in Table 19.

When blood cultures were found to contain mainly Bacillus subtilis, Staphylococcus aureus and S. epidermidis, examination of the dilator tubes was made. The first observation was that the rubber, mercury-filled dilators of various diameters were carefully washed after use but were then hung on the wall until needed. The second observation was that although sterile jelly and towels were used during the procedure, neither the dilator nor the physician's hands were rendered sterile before the procedure. Therefore, six dilators, chosen at random, were each placed in one liter of trypticase soy broth and transported to the laboratory for incubation at 37°C for four hrs. The tubes were removed and the broths were further incubated overnight. All flasks exhibited very heavy foul smelling growth.

Subcultures of all six flasks yielded B. subtilis, S. epidermidis, Pseudomonas spp., typical mannitol positive, coagulase producing S. aureus and an atypical mannitol negative, coagulase positive, pigmented S. aureus.

Thus, the bacteremias associated with esophageal dilatation appear to be caused by organisms on the dilator itself. Studies are now in progress to reassess the occurrence of bacteremia when sterile dilators and sterile techniques are used in the procedure.

Table 19. Time course of bacteremia following esophageal dilatation.

Patient No.	Pre	Minutes				
		0-1	5	10	15	30
1	-	SE	B	B	SE	B
2	-	SE	-	-	-	-
3	-	SE,B	SE	-	-	-
4	-	SE,B	B	SE	-	-
5	-	SA	SA	-	-	-
6	-	SA	SA	SE	-	-
7	-	-	SA	-	-	-
8	-	SA	OT	-	-	-
9	-	SE	SE	-	-	-
10	-	-	-	SE	-	SA
11	-	-	OT	-	-	OT
12	-	SE	-	SE,B	-	-
13	-	SE	SA	OT	SA	-
14	-	SA	-	SA	-	SA
15	-	-	SE	SE	SA,SE	-
16	-	-	SE	SE	SE	-
17	-	SE	-	SA	SA,SE	-
18	-	SE	SE	SA,SE	B	-

SA = Staphylococcus aureus

SE = Staphylococcus epidermidis

B = Bacillus subtilis

OT = Other

Summary and recommendations.

The isolation and preliminary characterization of cell surface antigens of Pseudomonas aeruginosa strains has made it possible to begin to systematically examine the functions and immunology of the individual components. Gonococcal research directed towards development of methods for serotyping strains has provided data which suggest that a bactericidal typing scheme is feasible. Attachment of type 1 gonococci to buccal epithelial cells can be inhibited by a crude pili antigen. Rabbit antiserum inhibited attachment with some evidence of specificity. Human vaginal secretions from infected patients inhibited homologous but not heterologous gonococcal attachment to buccal cells in vitro. Purified gonococcal cell wall antigens are being studied for their immunologic reactivity using an immunofluorescence assay. Studies of a tissue culture model of gonococcal infection have shown that organisms which have penetrated into mammalian cells can be differentiated from surface attached bacteria by means of peroxidase conjugated antiserum. Chronicity of the tissue culture infection has been demonstrated. Normal human serum in the medium prevented penetration by colony type 4 (nonvirulent) gonococci, but type 1 forms (virulent) were not inhibited. Clinical laboratory support of infectious diseases problems has consisted of identification of bacterial strains, antibiotic sensitivity tests and serum antibiotic assays. Patients undergoing esophageal dilatation developed transient bacteremias with organisms thought to be introduced during the procedure.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
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(U)Leishmaniasis; (U)Parasitic Disease; (U)Latin America; (U)Epidemiology; (U)Serology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) With emphasis on Leishmaniasis, the objective is the acquisition of data concerning prevalence and distribution of parasitic diseases in Latin America; recognition of infectious diseases of actual or potential military importance; increase of knowledge of reservoirs and vectors involved, and improvement of diagnosis, treatment and control of these diseases.
24. (U) Conventional parasitologic procedures and modifications were utilized.
25. (U) 73 07 - 74 06 Efforts to develop a system to screen drugs for anti-leishmanial action with a primary in vitro screen and a secondary animal model were pursued. Serum antibody response to chemotherapy of cutaneous leishmaniasis was studied. The pathogenesis of infection with Angiostrongylus costaricensis was studied in monkeys with both graded single doses and repeated exposure. Host susceptibility, life cycle, and pathogenesis studies were conducted on Isospora aretopitheci, a coccidian parasite which can cause mortality in captive monkeys. Improved culture methods developed here for mass production of both promastigote and amastigote forms Leishmania were evaluated. A survey of canine disease in the Canal Zone was conducted as a service to the local Army command prior to initiation of a guard dog program.
For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*Available to contractors upon originator's approval

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 169 Field Studies of Leishmaniasis and Other Tropical Diseases

Investigators:

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A. Leishmaniasis

1. In vitro production of Leishmania.

In attempts to produce large numbers of organisms for use in laboratory investigations, several liquid culture media have been tried. It was found that several tissue culture media with 30% fetal calf serum (FCS) added produce optimum numbers of organisms very rapidly. Trials with concentrations of FCS ranging from 5% to 80% confirmed that 30% is optimal in TC 199, a mammalian cell medium, as well as in Grace's insect cell medium and Schneider's modified Drosophilia medium. The insect cell media produce exceedingly rapid growth, and much greater numbers of organisms than possible in traditional blood agar media. In plastic disposable tissue culture flasks containing 5 ml. of this media incubated at 27 C, populations of 2×10^8 were produced in 5 days. Several strains of L. braziliensis isolated from humans in Panama, L. tropica, and L. donovani, and 2 species from reptiles, L. adleri and L. hoogstrali, all grew satisfactorily in this system. Promastigotes of L. braziliensis produced in this fashion appeared to have no changes in characteristics from those produced in other media. They were infective for tissue culture and rodents, and a laboratory accident demonstrated that they could produce a typical leishmanial infection in a human.

It has been shown by others[1-2] that higher temperatures can produce rounded-up forms in hemoflagellates which resemble the amastigote stage. In the blood agar medium used, this was achieved only by stepwise increases in temperature by small increments spread over several generations of parasites. In these liquid media, change from 27 C to 34 C resulted in conversion to amastigote-like form by the great majority of the promastigotes. In Schneider's medium, almost 50% had converted in 24 hours, but in TC 199, the big change did not occur until day 4. A typical conversion experiment is shown in Table 1.

If these organisms prove to be de facto amastigotes, with the biologic characteristics of the intra-cellular parasites, this will be a development of major significance. Since amastigotes have heretofore been available only as obligate intracellular parasites, the host cell has always been a complicating factor. Easily available extra-cellular parasites will permit studies of the parasite not previously possible. Two differences between the promastigotes and this amastigote-like form have been discovered which suggest a true amastigote character; the antigenic character of the cell wall, and differential susceptibility to chemicals. (The second characteristics will be discussed in section 3a (2) of this report.) The indirect fluorescent antibody test (IFA) is based upon staining of cell walls of amastigotes produced in tissue culture[3]. The promastigote form of the same strain has different staining characteristics which make it unsuitable for antigen in the IFAT. Amastigotes produced extracellularly in TC 199 medium were found to have IFA staining characteristics similar to those produced intra-cellularly in tissue culture when tested against known-positive human sera. In a trial of axenic amastigotes as antigen, agreement with results of tissue culture-produced amastigotes was excellent both qualitatively and quantitatively as indicated in Tables 2 and 3 and Figure 1. Electron microscope studies of L. donovani and L. tropica by others[4-5] have shown that the amastigote has a double membrane cell wall as opposed to a single membrane for the promastigote. It is hypothesized that the specific antigen/antibody binding site is the inter-membrane area. Specimens from liquid media are being examined by electron microscopy at the Department of Experimental Pathology, WRAIR, to determine if a double membrane is present in the axenic amastigotes.

2. Serum antibody response to chemotherapy.

In prior studies of the effect of treatment on serum antibodies (Annual Report 1972) it was shown that there is a reduction in titer after successful treatment in the great majority of cases, although reversion to seronegativity occurs in only approximately 10%. There is an inverse correlation between the degree of titer reduction and frequency of recurrence, so that serologic testing has some value for predicting the probability of treatment failure. In the current study, efforts are being made to monitor treated patients with specimens taken at regular intervals. During this year adequate follow-up through 7 months post treatment was achieved with three patients who were initially treated unsuccessfully with Metronidazole, followed by apparently successful administration of Pentostam. The titer curves are illustrated in Figs. 2-5. Among these patients, the titer drop tended to occur quite early, reaching the lowest level by 5 months. This is in basic agreement with earlier information, but some titer fall had been seen after 6 months post treatment in a few patients. A slight decrease in titer after unsuccessful treatment with Metronidazole was seen in one patient (Fig. 5), but in another who suffered an extreme exacerbation of a large lesion, it was accompanied by a rise, which then fell after successful antimony treatment. (Fig. 3)

3. Systems for screening drugs for anti-leishmanial activity.

a. In vitro systems.

(1) Vero cell model. The tissue culture model using a stable monkey-kidney cell line infected with L. braziliensis had been found adequately reliable to offer promise as a means of screening for anti-parasite activity. (Annual Report 1972) Techniques for administering the drug, and handling and staining the infected cell sheets were developed and improved. Two major difficulties were encountered: First, it was determined that the period of exposure to the drug necessary to exert a detectable effect was in the order of several days. Prior to drug exposure, one or two days is necessary to establish the intracellular infection, so the total period for a cell culture test is 8-10 days. In this time, significant overgrowth of the monolayer occurs and the parasites are obscured. Secondly, the extended period makes it difficult to schedule tests within the framework of a 5-day work schedule, and an appreciable work load for week-ends inevitably resulted. When it appeared that axenic amastigotes from liquid media might afford a simpler means of demonstrating drug action, further work on the Vero cell system was held in abeyance.

(2) Axenic amastigotes. The observation that amastigote-like forms produced in TC 199 medium (vide supra) had cell walls possessing the antigenic properties of intra-cellular parasites and not those of promastigotes, suggested they might also have the enzyme systems and metabolic characters of true amastigotes. This prompted trials against a clinically effective drug. Four concentrations of methylglucamine antimoniate (Glucantime) were tested against promastigotes and axenic amastigotes of a recent human isolate of L. brasiliensis from Panama. At daily intervals, 0.1 ml. aliquots were withdrawn from test vessels and inoculated into fresh liquid medium which was incubated at 27 C. The number of viable organisms after drug exposure was indicated by the growth of promastigotes in these back cultures. Results of these cultures after a typical 4-day exposure are illustrated by Table 4. None of these drug concentrations produced any lasting effect on the promastigotes. However, the amastigote forms were affected by the drug in all but the highest dilution. If this in vitro system proves to be a valid means of measuring effect of compounds against amastigotes of Leishmania it would have tremendous advantages over an animal model, or even a tissue culture model for a primary screening procedure, in terms of economy of materials, shorter test time, and ease of interpretation. Additionally, it would measure direct effect on the parasite, and avoid the complicating factor of the drug barrier presented by the host cell wall.

b. Rodent Model. Rodents have been shown to be important reservoir hosts for L. tropica in the Old World, and are likewise

important hosts in the New World. In the search for an animal model for a secondary drug testing system, rodents would appear to be likely candidates. The multimammate rat (Meriones) and the laboratory mouse have been used to test drugs in L. tropica infections, but only the hamster has been widely utilized for New World Leishmania. However, the slow evolution and lack of discrete lesions, make this an unsatisfactory model for extensive drug testing. Of wild rodents, Proechimys semispinosus, Sigmodon hispidus and Tylomys panamensis are the species which have been experimentally infected with L. braziliensis.[6-7] (Annual Report 1971-72) The following numbers of laboratory bred and raised animals were tested for susceptibility to recent human isolate of L. brasiliensis from Panama: Cavia porcellus (10), Mesocricetus auratus (20), Mus musculus (WRAIR strain-20), (MARU strain-20), Rattus norvegicus (WRAIR strain-30), (Fischer strain-30), Sigmodon hispidus (40), Tylomys panamensis (15), and Zygodontomys microtinus (40). Half the animals were inoculated with promastigotes produced on blood agar medium, and half with promastigotes from Schneider's liquid medium. Infection, as evidenced by development of swelling at the site of inoculation developed in 20/20 hamsters, 1/10 guinea pigs, 37/40 cotton rats and 12/15 Tylomys. However, lesions persisted more than a week only in the hamsters and a few cotton rats, and cultures were positive at 60 days only in the 20 hamsters and 5 cotton rats. Sigmodon still appears to be the best candidate host, although attempts to standardize method of administration, inoculum size, age of culture, and passage level of strain did not alter the previously observed pattern of sporadic susceptibility to persistent lesions. This is interpreted to be the result of genetic variation of the Sigmodon population, and a selective breeding program of the susceptibles has been instituted.

4. Immunity and host-parasite relationships.

Among the parameters of immunologic response of humans to American cutaneous leishmaniasis, the intra-dermal reaction is well known, and has long been used as a diagnostic test. We have reported earlier cell mediated immunity measured by in vitro lymphocyte blastogenesis in patients with current and recent infections,[8] as well as serum antibody detected by the indirect fluorescent antibody method.[3] However, the sequence of appearance of these events has not been defined. Serum antibody usually appears early, within 2 months of evolution of the lesion, as does the intradermal response. In our experience, over 90% of patients exhibit positive skin tests when diagnosis is established. No information is available concerning time of appearance of blast transforming ability.

An accidental laboratory infection where time of infection could be pinpointed has recently provided us the opportunity to

record these responses in a human subject. A papular cutaneous lesion developed about 8 weeks after a scratch from a needle being used to inoculate animals. At 77 days (11 weeks) post-infection, organisms were cultured from the lesion, and the serologic test was positive with a titer of 1:128, but there was no intradermal response to leishmanin. At 91 and 99 days, there was no blast transformation in the presence of leishmanial antigen.

Treatment with antimonials was administered, on day 93 through 99 after infection. When tests were readministered on day 114, positive results were obtained with both the intradermal and blast transformation tests.

5. Cryobank.

During the year a liquid nitrogen cryobank for the preservation of biological specimens has been established at this laboratory. The primary purpose was for the preservation of working quantities of low-passage level strains of Leishmania to insure standardized inocula for drug screening tests. Secondarily, it also facilitated the acquisition and maintenance of a variety of strains from other areas for comparative studies. As of 15 June 1974, the cryobank had 41 stabilates of 12 species and subspecies of Leishmania, representing Old and New World forms, 22 stabilates of Leptospira, and local isolates of Toxoplasma and trypanosomes.

B. Angiostrongylus costaricensis

Abdominal angiostrongylosis of man is a disease entity discovered in Costa Rica less than 10 years ago,[9] but awareness of its existence has prompted a wider search and it is now known from almost 200 cases in that country and in Honduras, and probably Venezuela. The infective cycle occurs naturally among rodents and slugs in Panama,[10] and it is quite likely a human disease here also. Studies in the pathologenesis of the infection in rodents and subhuman primates have been conducted, using the natural host, Sigmodon hispidus, the cane mouse Zygodontomys microtinus, and 6 capuchin monkeys (Cebus capucinus) and 12 spider monkeys (Ateles geoffroyi).

In the cotton rat, the cycle is complete and 1st stage larvae are passed at 24 days after infection. The infectious larvae evidently penetrate the gut immediately after ingestion and migration could be traced on microscopic examination because the passage is marked by linear tracts of bacteria and neutrophilic and eosinophilic bacteria. There was a relatively equal distribution of larvae from the duodenum to the cecum, and no larvae were found outside the intestinal tract. On days 3-5, there were eosinophilic

granulomas in submucosal and mesenteric tissues, some containing remnants of molted cuticle. On day 6, all rodents had serosal and mesenteric hemorrhages adjacent to branches of the cranial mesenteric artery, and young worms were present within the lumen of arteries adjacent to these areas. On day 14, there was a focal hemorrhage infarct of the tip of the cecum and several parasites within the branches of the cranial mesenteric artery. On the other hand, the pattern of infection in Zygodomys was that of an abnormal host. The migrating larvae were not confined to the intestinal tract, and on day 5 one animal had a hepatic abcess with focal fibrinopurulent peritonitis and adhesion of the liver to the serosal surface of the stomach. No parasites were found in the arteries, except for a single degenerating worm in a subserosal artery of the duodenum. This vessel was surrounded by an intense inflammatory reaction of mixed cell type which involved primarily the adventitial portion. No patent infection developed. The course of infection in both species of monkeys was found to parallel the disease produced in the cotton rat. At 21 days post-infection, as in the Sigmodon, both Ateles and Cebus had numerous adult worms in the branches of the cranial mesenteric artery, the majority located in the branches supplying the cecum and ileocecal junction. First stage larvae were present in the feces of Ateles at 30 days post-infection, and in the Cebus at 33 days, a finding presently never demonstrated in humans. Both species of monkeys developed a severe eosinophilic granulatomous typhlitis and enteritis similar to the ileitis and appendicitis found in man. One Ateles died from this cause at 33 days. One Ateles continued to pass larvae in low numbers until it was killed 465 days after infection. It is postulated that some humans might pass larvae during the infection and even if they were detected, a misdiagnosis of Strongyloides stercoralis would likely be made unless the examiner was familiar with Angiostrongylus.

To determine the effect of reinfection, 2 Ateles were treated with thiabendazole (100 mg/kg) cleared of the infection, and then reinfected with 2 doses of 25 infectious larvae given 14 days apart. An additional animal was given 3 doses of 25 larvae each at one week intervals. Surprisingly, the picture in these reinfected animals was not significantly different from the response seen in an initial infection.

The tissue reaction found in Sigmodon is very minimal, while that found in the monkey is greater than in the natural host, but not as severe as found in man.[11] The greatest damage caused by the parasite is the interruption of blood supply to vital areas of an organ due to mechanical blockage of the vessel by the worm or the vast quantities of eggs produced. Occlusion also occurs from fibrin clots formed around dead or dying worms.

C. Toxoplasma/Isospora

Judged by the paucity of literature reports, Isospora infections in non-human primates are quite rare. An incidental finding of oocysts in feces of a recently captured Cebus monkey and efforts to identify it, revealed that there are only 6 reports of Isospora infections in non-human primates, representing 4 species. This parasite, Isospora arctopitheci Rhodain, 1933, was redescribed from material obtained from natural infections found locally in 5 of 36 marmosets, Saguinus geoffroyi and 1 of 5 Cebus capucinus and from experimental infections.[12] When early experiments caused fatalities in marmosets, further investigation of this parasite was considered justified because of the current and future importance of New World primates in medical research. The short sporulation and pre-patent periods and direct mode of transmission could cause explosive fatal outbreaks if this parasite was introduced into a colony.

The complete life cycle stages are not known for any of the Isosporan parasites of primates; neither the 4 species from non-humans, nor the 3 species known from humans. A study of this infection has been initiated, with a total of 14 experimentally infected marmosets sacrificed at various times from 1 to 7 days. The endogenous life cycle occurs primarily in the duodenum and jejunum, and only occasionally are parasites encountered in the ileum. On days 1 through 5 the cycle is schizogonic, with the possibility of multiple generations of merozoites occurring within the same host cell. By day 7, most of the epithelial cells show stages of gametogeny, and many host cells had 2 developing gamonts within the same parasitophorous vacuole. No oocysts were observed. A pink proteinaceous hyalin globule appeared adjacent to the macrogamete, the nature of which has not yet been defined. The material from this series is under study to elucidate the complete life cycle.

The classical concept of early workers was that Isospora was characterized by a direct life cycle and extreme host-specificity. When Toxoplasma gondii was found to be an intestinal coccidian of felines with the morphological characteristics of Isospora in 1970,[13-16] it was thought to be an exceptional example. However, 2 other species from felines, I. rivolta and I. felis were recently shown to infect laboratory rodents in extra-intestinal sites. These rodents, when fed to clean cats transmitted the infection.[17] Our investigations have demonstrated that I. arctopitheci also has the ability to establish extra-intestinal infections from sporulated oocysts in laboratory mice and domestic chickens. Selected organs removed from these vector hosts 20-40 days post-inoculation produced normal infections in recipient coccidia-free marmosets which showed a pre-patent period of 7-9 days.

The concept of host-specificity of Isosporan parasites has never before been questioned. However, susceptibility trials with I. artcpitheci produced patent intestinal infections in several species of New World monkeys. This host range in primates is remarkable, but its ability to also infect several species of wild and domestic carnivores (Table 5) makes it unique among coccidian parasites. The ability of this parasite, and others of the genus, to produce extra-intestinal infections in widely divergent orders of vertebrates raises the possibility that they might also produce toxoplasmosis-like infections in man.

D. Miscellaneous studies

1. Leptospirosis.

Because leptospirosis has been known to occur in common-source outbreaks with high attack rates in military units in Panama, [18] and because cases have been diagnosed every year in spite of inadequate laboratory diagnostic support services available, it was considered worthwhile to maintain surveillance of selected FUO cases by the hemolytic leptospira (HL) test and by culture in appropriate media. During the period 128 clinically suspect cases were referred for work-up. Of these 16 (12.5%) had titers considered diagnostic (≥1:160) and from 6 (4%), organisms were isolated in culture. Attack rates were highest among military patients (10 of 47), but in all cases exposure was associated with recreational activities and not military duties. Of ancillary interest is the fact that adequate laboratory procedures ruled out leptospirosis in acutely ill patients who were considered to be most "typical" on clinical grounds. One of these was subsequently shown to be due to Venezuelan equine encephalitis.

2. Canine disease survey.

At the request of the Staff Veterinarian, US Army Forces Southern Command, a survey of canine diseases in the Canal Zone and adjacent areas in the Republic of Panama was conducted to assess risks prior to instituting a patrol dog program. A total of 250 dogs was examined by appropriate techniques to diagnose presence of infection by Ehrlichia canis, Babesia canis, various filariids, Toxoplasma gondii, and 86 of these were examined for intestinal parasites. No evidence of Ehrlichia or Babesia infection was found by direct blood smears, serologic test are pending. Canine filariasis was found to be widely distributed, in all localities, but with only a 5.2% overall prevalence rate. Dogs housed indoors at night in the Canal Zone had a very low infection rate. Over 40% had intestinal parasites, but only hookworm, with a prevalence of 38.4%, constituted a serious problem. Serologic tests for antibodies to Toxoplasma are in progress.

3. Paragonimus.

Dr. Ichiro Mujazaki, Professor Emeritus, Kyushu University Medical School, Fukuoka Japan, was a guest investigator for a month to study Paragonimus infections in a study supported by USAMRDC, through USA R&D group (Far East).

Table 1

Growth and Conversion Rates of Leishmania Promastigotes
in Two Types of Liquid Media When Changed From 24 C to 34 C Incubation Temperature

DAYS at 34 C	Schneider's + 30% FCS			TC 199 + 30% FCS		
	No. of Organisms/ml.	% of Types*		No. of Organisms/ml.	% of Types*	
0	3.75×10^7	100	Promastigotes	1.05×10^7	100	Promastigotes
1	7.40×10^7	53	Promastigotes	1.90×10^7	92	Promastigotes
		47	Amastigotes		8	Amastigotes
2	7.80×10^7	76	Amastigotes	2.10×10^7	95	Promastigotes
		24	Promastigotes		5	Amastigotes
3	7.26×10^7	79	Amastigotes	1.97×10^7	97	Promastigotes
		21	Promastigotes		3	Amastigotes
4	6.10×10^7	87	Amastigotes	3.60×10^7	78	Amastigotes
		13	Promastigotes		22	Promastigotes
5	7.25×10^7	90	Amastigotes	2.85×10^7	80	Amastigotes
		10	Promastigotes		20	Promastigotes

*Percentages of promastigotes or amastigotes based on the count of at least 100 organisms.

Table 2

QUALITATIVE AGREEMENT BETWEEN TEST RESULTS
WITH EXTRA- AND INTRA-CELLULAR AMASTIGOTES
IN THE LEISHMANIA IFA

RESULTS	NO. SERA
NEGATIVE (<1:8) -- BOTH TESTS	10
POSITIVE -- BOTH TESTS	149
POSITIVE -- VERO-NEGATIVE 199	1
POSITIVE -- 199-NEGATIVE VERO	0
TOTAL	160

Table 3

QUANTITATIVE AGREEMENT BETWEEN
TEST RESULTS WITH EXTRA- AND INTRA-CELLULAR
AMASTIGOTES IN THE LEISHMANIA IFA

RESULTS	NO.	AGREEMENT
SAME TITER WITH BOTH ANTIGENS	108	88.0%
2-FOLD VARIATION BETWEEN ANTIGENS	24	
4-FOLD VARIATION BETWEEN ANTIGENS	14	97.3%
>4-FOLD VARIATION BETWEEN ANTIGENS	4	
TOTAL	150	

Table 4

Comparative Effect of 96 Hours Exposure to Methylglucamine Antimoniate on Promastigotes and Axenic Amastigotes of L. braziliensis

A. PROMASTIGOTES

Drug Concentration	Days Incubation at 27 C 2	3	4	5	6	7
0.1 µg/ml	9.8×10^6*	1.7×10^7	5.9×10^7	6.2×10^7	4.3×10^7	5.3×10^7
1.0 µg/ml	2.5×10^6	2.0×10^7	6.1×10^7	5.0×10^7	3.5×10^7	6.3×10^7
10.0 µg/ml	5.3×10^6	1.5×10^7	4.2×10^7	3.9×10^7	3.9×10^7	5.3×10^7
100.0 µg/ml	2.0×10^6	1.1×10^7	2.8×10^7	4.4×10^7	1.0×10^8	5.7×10^7
0.0 µg/ml	5.0×10^6	3.2×10^7	5.2×10^7	4.9×10^7	5.4×10^7	6.7×10^7

B. AMASTIGOTES

Drug Concentration	Days Incubation at 27 C 2	3	4	5	6	7
0.1 µg/ml	4.3×10^5*	7.0×10^6	1.8×10^7	6.0×10^7	4.8×10^7	3.6×10^7
1.0 µg/ml	0	4.0×10^5	1.8×10^5	2.5×10^6	5.9×10^6	1.3×10^7
10.0 µg/ml	0	0	0	3.0×10^5	4.5×10^5	9.0×10^5
100.0 µg/ml	0	0	0	1.5×10^5	0	0
0.0 µg/ml	5.0×10^5	1.9×10^6	2.2×10^7	1.3×10^7	3.2×10^7	3.0×10^7

*No. organisms/ml culture

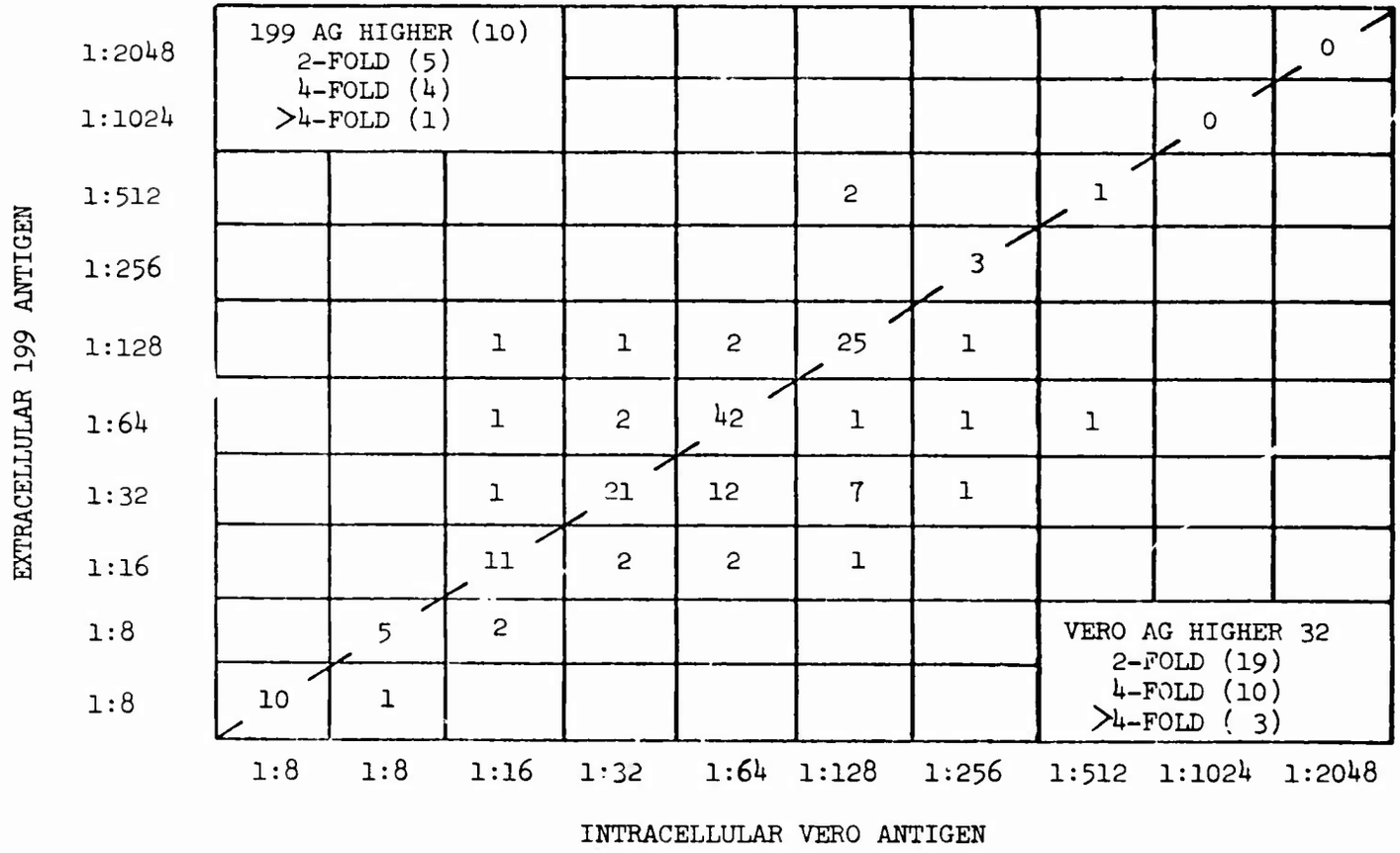
Table 5

Susceptibility of Experimentally Exposed Animals to Infection with Isospora arctopitheci

Scientific Name	Common Name	Susceptible	Prepatent Period (Days)	Patent Period (Days)
PRIMATES				
Aotus trivirgatus	Night monkey	2/2*	6-10	3-5
Ateles geoffroyi	Spider monkey	1/2	8	3
Cebus capucinus	White face monkey	2/2	8	23
Saguinus geoffroyi	Marmoset	18/20	7-9	55
Macaca mulatta	Rhesus monkey	0/2	-	-
CARNIVORES				
Bassuricyon gabbii	Olingo	0/1	-	-
Felis catus	Cat	3/4	7-11	1-4
Nasua nasua	Coatimundi	2/2	9	1$^{\phi}$-7
Potos flavus	Kinkajou	2/2	8-9	2†-7

*No. oocyst positive/no. tested
$^{\phi}$Died first day of patency
†Escaped second day of patency

COMPARISON OF IFA LEISHMANIA TEST RESULTS WITH AMASTIGOTES
PRODUCED INTRA- AND EXTRA-CELLULARLY IN VITRO



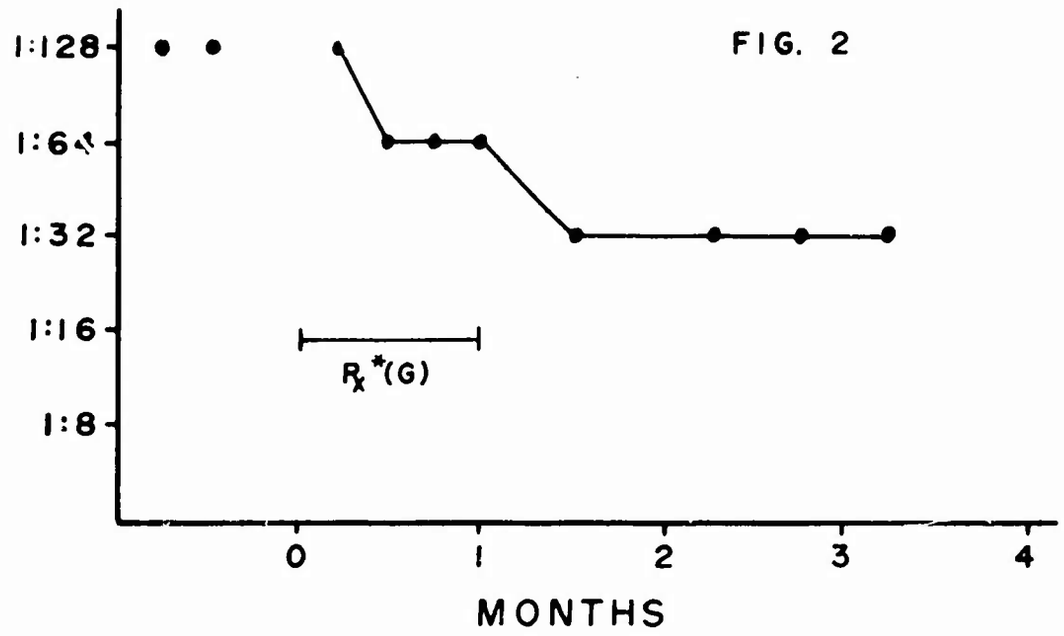


FIG. 2

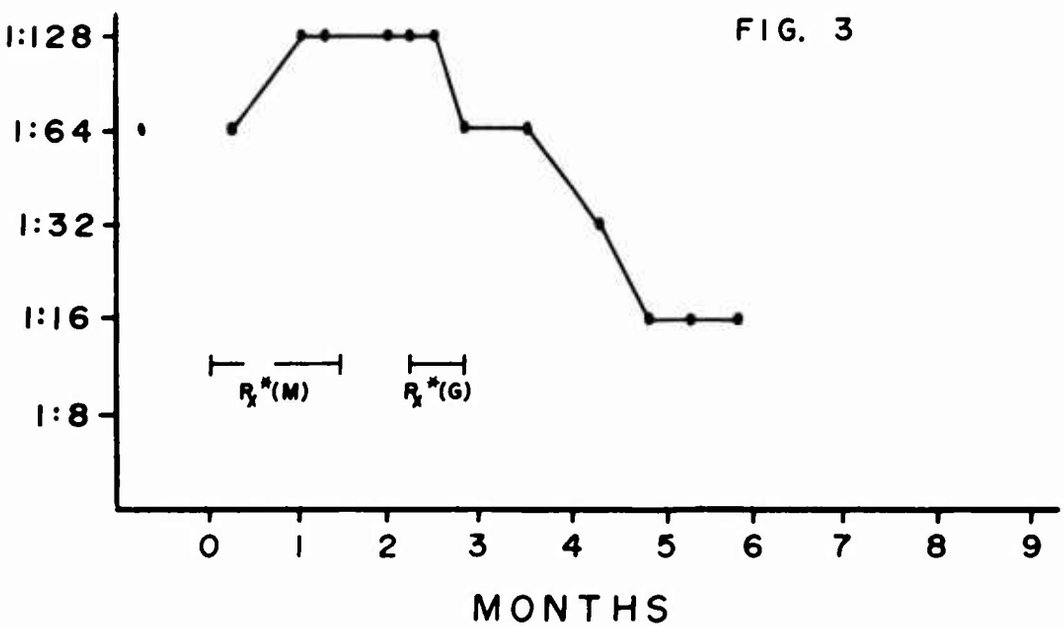


FIG. 3

*(M)= METRONIDAZOLE *(G)= GLUCANTIME

FIG. 4

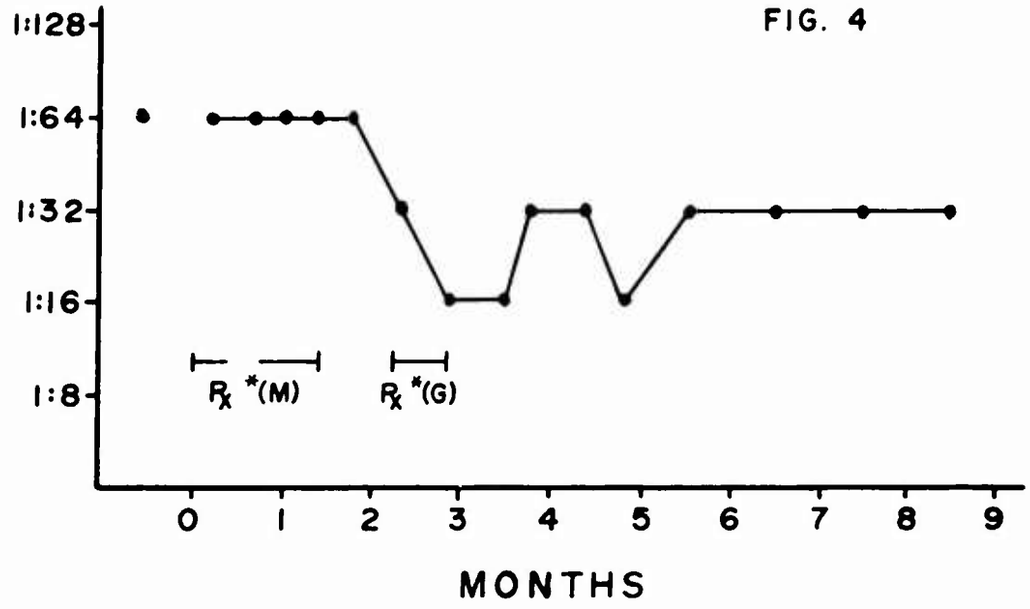
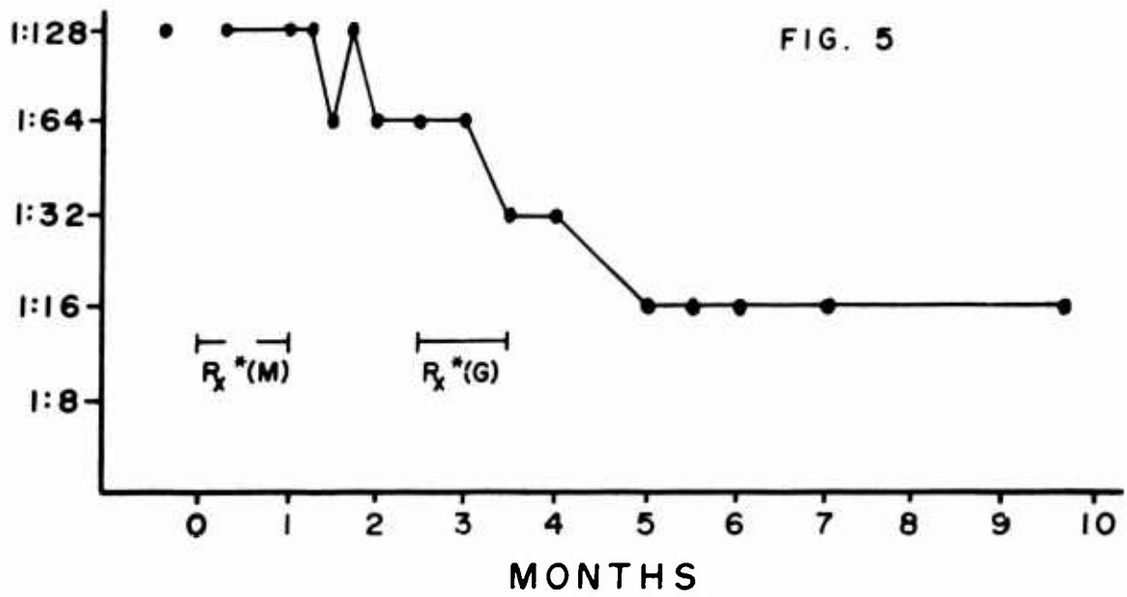


FIG. 5



*(M)= METRONIDAZOLE

*(G)= GLUCANTIME

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6447	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	☑ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71Q	00	170
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with security Classification Code)

(U) Zoonotic Diseases of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 06	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	3.5	187
	CURRENT 75	3.5	187

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Rogul, M., Ph.D.

NAME: Keenan, K. P., CPT DA

PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Zoonoses; (U) Military Dogs; (U) Canine Zoonoses; (U) Leptospirosis; (U) Melioidosis; (U) Pseudomonas

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To study zoonoses of real or potential military significance, to determine the natural history, biological characteristics of agents, to develop suitable diagnostic procedures, treatment and control measures.

24. (U) Conventional microbiological and chemical technics are used. New procedures are developed as needed.

25. (U) 73 07 - 74 06 The basic lesion in experimental bataviae leptospirosis in dogs was primarily of damaged mitochondria and cell membranes. Hamsters vaccinated with a leptospiral ribosomal vaccine were protected against serologically homologous but not heterologous serovars. The vaccine prevented development of leptospiruria. Spirochetal contaminants appearing in bacteriologic and tissue culture media were identified as Leptospira biflexa and traced to use of deionized water and filtration sterilization technics. Approximately 1424 pathogenic leptospiras isolated from Malaysian waters comprised 29 serovars including a new type designated evansi. A genetically distinct leptospira was also found to be phenotypically distinct and designated serovar illini. Case of icterohaemorrhagiae leptospirosis in a zoo monkey was epidemiologically related to Norway rats. S and R strains of Pseudomonas pseudomallei similarly incorporate NH_4^+ via pyruvate but may differ in regulatory mechanisms for N_2 assimilation. An unusual pseudomonad associated with wound infection in an Oklahoma farmer was indistinguishable from P. pseudomallei in cultural and biochemical characteristic but differed in antigenic, serologic and virulence attributes. Concept of iridescent plaquing in P. aeruginosa has been refined on basis of interaction of a quinolinol and pyocyanine. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 73 - 30 June 74.

*Available to contractors upon ord/dinator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 170 Zoonotic Diseases of Military Importance

Investigators.
Principal: A. D. Alexander, Ph.D.
Associates: M. Rogul, Ph.D., M. G. Groves, MAJ, VC, K. P. Keenan, CPT, VC, J. J. Brendle, B.S., C. A. Montgomery, MAJ, VC, Helen Jervis, Dr. Nat. Sc., and L. Evans

Description.

Studies are conducted on zoonotic diseases of real or potential military importance and entail epidemiological investigations to determine natural history and occurrence of diseases, basic and applied research which bear on development of suitable diagnostic, treatment, and control measures. Principal efforts are on agents of leptospirosis, melioidosis and related pseudomonads.

1. Pathogenesis of experimental bataviae leptospirosis in dogs.

A. Background. Strains of the serogroup Bataviae are among the most frequently occurring leptospiras in Asia. They are also important causes of human and animal diseases in Africa and Europe, and occur in the United States, Middle and South America (1). Aside from their epidemiological importance, bataviae serovar (syn. serotype) strains are particularly interesting because of their high virulence for dogs. The bataviae infectivity system in dogs provides a model that simulates natural infections in man and animals, and provides a unique opportunity to resolve questions on pathogenesis. Findings to date on this model system are reported.

B. Methods.

Animals. Beagle dogs 4 to 15 months of age, of both sexes, were used. These animals were not vaccinated with any leptospiral serovar. No detectable leptospiral agglutinins were present at time of inoculation. Weanling hamsters were utilized for several microbiological procedures.

Experimental Infection. Leptospira interrogans, serovar bataviae strain 1415, an isolate from Malaysia, was used to infect dogs. Its virulence was maintained by sequential hamster passage. Inocula for dogs were prepared from dilutions of a 10% suspension of triturated liver obtained from an infected moribund hamster. The concentration of organisms in the liver suspension was determined by counts of an appropriate dilution of liver suspension in a Petroff-Hauser counting chamber. The virulence of the inoculum was titrated by inoculating groups of

weanling hamsters intraperitonally (i.p.) with serial 10-fold dilutions of liver suspension using a 0.5 ml dose. Dogs were infected by i.p. injection of 5 ml of a suspension containing 3.4×10^6 to 6.9×10^7 organism per ml. The LD_{50} hamster infectivity units ranged from 10^5 to 10^8. Controls were inoculated with sterile diluent (either phosphate buffered, pH 7.2, physiological salt solution, or Stuart's basal medium).

Microbiological examinations. Following infection of dogs, blood was periodically obtained for isolation of leptospiras to determine the occurrance, duration and magnitude of leptospiremic period and to determine the serological response to infection. Urine was obtained at appropriate intervals to determine the occurrance of leptospiruria. Attempts were also made to isolate leptospiras from aqueous humor, brain, liver and kidney on specific dogs. Direct cultural technics were used for blood and tissues. Urine was cultured, either directly or by use of hamster inoculation technics (2), or by both methods.

Clinical Pathology. All the animals were examined daily for 2 weeks following inoculation. Venous blood was collected daily or every other day in EDTA vacutainers for hematological examinations. White blood cell (WBC) counts and red blood cell (RBC) counts were performed on an electronic cell counter (Model B, Coulter Electronics, Inc., Hialeah, Florida). Platelet counts were also performed electronically (MK-4 Platelet Counter, General Sciences Corp., Bridgeport, Conn.). Packed cell volumes (HCT) were determined by standard microhematocrit methods. Wintrobe tubes were used to determine 1 hour erythrocyte sedimentation rates (ESR). Hemaglobin (Hgb.) was determined by the cyanmethemoglobin method. Blood films on slides for differential WBC counts were stained by the Wright-Leishman method. Every second or third day, samples of venous blood were collected in both citrated and ordinary vacutainers for plasma and serum collection, respectively. Fibrinogen, prothrombin times (Protime) and partial thromboplastin (PTT) times were determined on plasma samples by standard methods. Sera were examined by standard methods for levels of serum alkaline phosphatase (Alk'p'tase), amylase, creatinine, blood urea nitrogen (BUN), creatine phosphokinase (CPK), glucose, lipase, serum glutamic oxalacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), total direct and indirect serum bilirubin, magnesium, calcium, chloride, cholesterol, carbon dioxide content, potassium, sodium, inorganic phosphate, total protein, protein components separated by electrophoresis, lactic dehydrogenase (LDH), and lactic dehydrogenase isoenzymes. Routine urinalysis was performed; also, plasma and urine osmolality was determined. On several series of animals endogenous clearance of creatinine and phenolsulfonphthalein excretion were determined by standard methods.

Pathological observations. Complete necropsies were performed on all dogs that died or were sacrificed. Routine and special samples were collected for histopathology, histoenzymology, histochemistry, and electron microscope studies.

C. Results.

Microbiological Findings. Leptospiremia and leptospiruria were demonstrated in all inoculated dogs regardless of clinical or pathological evidence of disease. Leptospiras were detectable in blood as early as 24 hours post intraperitoneal inoculation, and persisted for as long as 5 days post inoculation (P.I.). Using quantitative technics, the greatest number of organisms per unit of whole blood was evident on days 2 to 4 P.I. Following inoculation, leptospiruria was detected as early as day 3 P.I., and when examined 2 to 4 weeks P.I. Two dogs surviving 1 month and four dogs surviving 5 months after inoculation were euthanized and samples of various organs were taken aseptically and cultured. No leptospiras were isolated from kidney, urine, liver, cerebral cortex, cerebellum, midbrain, and aqueous humor of the eye obtained from four dogs euthanized at 5 months P.I. Leptospiras were demonstrated only in urine and kidney of the remaining two dogs similarly examined. In surviving dogs, detectable antibody was demonstrated by day 10 P.I., reaching optimum levels of 1:400 to 1:3200 by days 21 to 30.

Clinical Observations. The clinical manifestations of bataviae infection in dogs fell into three general classifications by the following criteria: (1) an acute fatal icteric syndrome that included spectacular hematological, renal (BUN>80 mg/dl), and hepatic (bilirubin>15 mg/dl) changes; (2) an acute, non-fatal icteric syndrome with signs and clinical pathological changes similar to the fatal group; and (3) a mild asymptomatic non-icteric response with only minimal clinical signs. Of 30 dogs inoculated, 11 developed the acute fatal icteric disease, 7 developed the acute non-fatal icteric course, and the remainder had mild, non-icteric infection. In general, the severity of response to infection depended on the virulence of the inoculum and/or the age of the dogs.

All the dogs developed fevers 2 to 3 days P.I. Depending on the severity of the case, depression, anorexia, scleral and conjunctival infection, and generalized lymphadenopathy were the next most common signs. The following are manifestations seen in the acute, fatal, and non-fatal icteric cases.

Integumentary. Dehydration developed rapidly with loss of skin pliability, drying of the mucus membranes and recession of the ocular orbits. Icterus was first evident the 3rd day P.I., and was generalized.

Musculo-skeletal. Inflammatory changes in the skeletal muscles and other organs were evident by a stiff, tucked up walk, reluctance to move and evidence of pain on palpation and manipulation of limbs and neck.

Cardiovascular. The hemorrhagic signs were clinically evident in the severe group by the development of petechiae and ecchymoses on the oral, conjuntival, scleral and genital mucus membranes, as well as on the abdomen and flanks. Epistaxis, rectal bleeding and melena were inconsistant signs in this group. Moribund animals showed auscultable arrhythmias, weak and irregular pulse and other evidence of cardiovascular collapse prior to death.

Respiratory. All the severe and moderately sick dogs developed a dry spontaneous or palpable cough. Tonsillitis was a common finding. A number developed acute broncho-pneumonias with muco-purlent to purlent respiratory discharges, rhinitis, conjuntivitis, and auscultable wet rales. Pulmonary hemorrhage was seen at necropsy. Broncho-pneumonia was usually associated with secondary bacterial infections. Several animals developed acute pulmonary edema terminally.

Digestive signs were associated with hemorrhagic and renal manifestations (uremia), i.e., vomiting, hemorrhagic, diarrhea, melena, stomatitis and glossitis. Palpable intussusceptions appeared in several dogs.

Renal signs included all those associated with acute uremia. Oliguria was common to all the severely ill animals, and anuria preceeded death by 24 to 36 hours.

Hepatic signs included severe generalized jaundice. In some cases the edge of the liver was palpable at the costal border.

Ocular signs included the above-mentioned scleral injection, conjunctivitis with or without exudate, and hemorrhages in the ocular mucus membranes. Aside from icterus, the retina was not grossly altered, and physical evidence of iridocyclitis was not seen in these animals. Evidence of meningeal or central nervous system involvement was difficult to evaluate in the severely affected dogs, but pathological findings indicative of such involvement was found at necropsy.

In general, the severely sick dogs became moribund and died between 5 to 8 days P.I. The moderately sick dogs improved and began recovering at this time.

Clinical Pathological Findings. This data is being analysed statistically and will be presented in detail at a later date. Presented in this report are data from two dogs classified in the acute fatal icteric group (Table 1). Dog L-2 (13.5 mos. old, beagle, male) was inoculated with 5×10^7 organisms/ml. i.p. Dog L-18 (5.5 mos. old,

Table 1: Clinical pathology findings on two acute icteric fatal cases of bataviae Leptospirosis

	Dog L-2				Dog L-18				
Day P.I.	0	2	4	5	0	2	4	6	7
Temperature °F A.M.	101^{9}	103^{6}	104^{4}	99^{0}	101^{3}	103^{0}	103^{9}	101^{8}	101^{0}
Hematocrit volume %	51	45	56	58	40	42	34	38	33
WBC/cmm	9100	9600	22400	27900	12300	15000	7850	15400	31200
Bands/cmm				558			78	770	
Segs./cmm	5733	7488	17696	22878	7134	11250	5495	13090	28650
Lymph./cmm	2639	1632	2912	837	4674	3300	2119	924	2190
Eosin./cmm	273	288	448	279	492	150	78		
Mono./cmm	455	192	1344	2790		300		616	362
E.S.R. mm/hour	0	1	5	3	0	6	30	17	10
Platelets x 10^{3}/cmm	244.	242.	300.	104.	375.0	185.0	15.5	24.5	59.0
Prothrombin time (sec.)	7.0	6.5	7.5	6.5	7.0	6.5	5.5	6.5	6.0
P.T.T. (sec.)	15.0	14.0	14.5	14.1	13.5	10.5	13.0	11.0	12.0
Fibrinogen (mg/dl)	135	132	138	128	145	502	400	645	555
Alk'p'tase (IU/l)	52	86	1710	2690	104	113	357	1789	1281
Amylase (smogyi Units)	1078	1606	638	1378	626	609	913	1038	1645
Creatinine (mg/dl)	.6	.7	1.0	5.6	.6	.6	.6	3.1	4.0
B.U.N. (mg/dl)	26	11	18	84	15	10	10	108	122
C.P.K. (IU/l)	56	43	176	198	66	47	69	94	60
Glucose (mg/dl)	89	81	104	147	107	98	60	98	101
Lipase (Cherry-Crondall units)	2.0	2.0	.3	.6	2.4	.2	1.3	0	.7
S.G.O.T. (IU/l)	46	52	263	379	38	35	63	59	48
SGPT (IU/l)	40	39	160	390	40	49	65	166	130
Total Bilirubin (mg/dl)	.1	.3	14.8	19.8	.2	.3	5.9	21.0	25.6
Direct Bilirubin (mg/dl)	0	.1	5.7	6.9	0	0	3.8	9.4	13.7
Ca (mg/dl)	9.7	9.8	9.1	9.6	11.3	11.0	10.7	10.0	9.3
Chloride (meq/l)	107	112	106	107	107	113	115	92	100
Cholesterol (mg/dl)	122	163	N.D.	N.D.	128	152	209	191	364
CO_2 Contest (meq/l)	25	25	19	22	26	24	20	19	15
K (meq/l)	4.1	4.0	3.0	4.0	4.2	4.2	4.1	3.1	3.5
Na (meq/l)	141	147	147	148	146	149	154	139	135
Inorganic P (mg/dl)	4.2	4.3	7.2	20.0	6.7	6.7	5.5	13.2	13.2
Total Protein (gm/dl)	5.9	5.4	6.2	7.5	5.3	5.3	5.4	6.5	6.4
Total L.D.H. (IU/l)	31	37	74	64	30	36	72	87	52

beagle, female) was inoculated with 6.9 x 10^6 organisms/ml. i.p. Both animals had a typically acute course and were euthanized when moribund. Baseline and Day 0 data was within normal limits for dogs of these respective ages. Control dogs handled and sampled in like fashion showed none of these changes.

The hematological and chemical changes were similar in all clinically ill animals, varying only in severity. These data reflect the polysystemic nature of leptospirosis, although renal, hepatic and hemorrhagic manifestations dominate the clinical presentation.

Hematology findings frequently indicated blood loss, with low HCT, Hbg and RBC counts; however, this was often masked by the severe dehydration that accompanies the renal involvement. Clinical evidence of hemorrhage was always accompanied by thrombocytopenia, with elevations in fibrinogen levels, and protimes and P.T.T. values remaining normal compared to controls. Frequently a leucopenia was seen early in the disease, but later a marked leucocytosis with a left shift developed.

Hepatocellular damage and intrahepatic cholestasis was evident by demonstrable increases of serum alkaline phosphatase, serum glutamic pyruvic transaminase, total conjugated and free bilirubin, and cholesterol. Increases in serum lactic dehydrogenase and serum glutamic oxalacetic transaminase may in part reflect liver disease, but also indicates other organ involvement. For example, increases in serum creatine phosphokinase were indicative of skeletal muscle pathology. The technic for demonstration of lactic dehydrogenase isoenzymes were of limited use in differentiating specific organ involvement. Other methods have since been shown to be more useful for this analysis when working with canine sera. Changes in protein electrophoresis patterns were indicative of hepatic involvement with a lowering of the albumen fraction (which also explains in part the fall in serum calcium). Also consistant with acute hepatitis and icterus was an increase in the alpha-2 and beta globulins. The gamma globulins increased slightly, but were not conspicious. Changes in the serum proteins must be viewed in light of the severe dehydration that occurs.

Renal disease was evident by elevations of blood urea nitrogen and plasma creatinine. The ratios of urine osmolality to plasma creatinine were useful measures during the oliguric period. Ratios of 1.2 to 1 in the former and less than 10 to 1 in the latter were considered indicative of acute nephritis. Proteinuria, bilirubinuria, increased numbers of casts, WBCs and RBCs in the urine were demonstrated in the severe and moderately ill dogs. Changes in serum electropytes were also typical of acute nephritis. Sodium and chloride deficits paralleled each other in a progressive fashion. Potassium levels fell initially, but became elevated with the onset of oliguria and anuria. Progressive hyperphosphatemia was very evident in the severe dogs. Fall in plasma CO_2 content reflected the development of metabolic acidosis in these animals.

One-half of all the inoculated animals were studied with endogenous creatinine clearance and phenolsulfonphthalein excretion tests. These data indicate the renal involvement is acute and generalized, and parallels other clinical pathology evidence of nephritis.

All the above data indicated that acute development of renal disease was the major factor in mortality. The acute non-fatal icteric group was less effected and made a rapid recovery to normal function.

Serum amylase, while being dependent on renal excretion for removal from the body, was elevated most markedly in those dogs with severe gastroenteritis. Since serum lipase levels remained normal or were depressed, we feel elevations in serum amylase were evidence of gut pathology rather than pancreatic or renal disease particularly in view of the fact that amylase is in high concentration in the canine gut.

Histoenzyme and Histochemistry Findings. Histochemistry studies to date were performed on 11 dogs. Two were classed in the acute fatal icteric group, 3 in the acute non-fatal icteric group, 3 in the mild non-icteric group, and 3 control dogs. The tissues studied were small bowel, heart, lung, kidney, and liver.

A slight increase in the activity of myocardial and macrophage acid phosphatase and a possible decrease in DPNH diaphorase was seen in the heart of the more seriously affected animals. No consistant changes which could be attributed to the leptospiral infection were seen in bowel and lung tissue in this study group.

Fat inclusions were found in the cortical kidney tubules of infected dogs but not in control dogs. In kidneys of the infected dogs, an irregular decrease of the alkaline and glucose-6-phosphatases, decreased leucine aminopeptidase activity of the cortical tubules, and a generalized loss of the mitochondrial enzymes (succinic delydrogenase, glutamic dehydrogenase, beta-hydroxy butyric dehydrogenase and alpha-glycerophosphate dehydrogenase) could be demonstrated. The severity of these changes in the experimental dogs correlated closely with the severity of the illness. The activity of the acid phosphatase appeared increased in the kidneys of all experimental dogs irrespective of the severity of disease.

In the liver, the amount of glycogen demonstrated histochemically decreased only in the most severely ill animals, possibly as a result of anorexia. Fat inclusions in the hepatocytes increased in most infected dogs, but only moderately. The activity of the peribiliary acid phosphatase also seemed increased and appeared more diffuse than in the controls, probably due to increased fragility of the lyosomes. Glucose-6-phosphatase also decreased but only in the

most severely affected dogs. The oxidative enzymes, alpha-glycerophosphate dehydrogenase and beta-hydroxy-butyric dehydrogenase, seemed to show the most consistant decrease in activity. In the controls alkaline phosphatase could be demonstrated only in the endothelium of capillaries and in a few periportal sinusoids; however, in all experimental dogs, it could be demonstrated in the bile canaliculi and in the cell membranes of the hepatocytes in amounts increasing with the severity of the disease. These changes in alkaline phosphatase, although not specific for leptospirosis, seem to be a very sensitive index of liver damage.

Histopathology and electron microscopic studies are presently underway.

D. Discussion.

Experimental bataviae leptospirosis in the dog provides a model that encompasses manifestations ranging from inapparent to fatal renal-hepatic disease forms parelleling the spectrum of natural disease seen in man and animals and could serve as a model to define pathogenetic disease mechanism. As in the case of fatal human leptospirosis, acute renal failure was the one feature most commonly associated with mortality (3,4,5,6,7).

The basic mechanism of leptospiral pathogenicity is postulated to be a "toxin" (8,9,10). Such a toxin, acting on capillaries, would provide a mechanism for many of the morphological and functional injury seen in the disease. Wide spread capillary damage would explain the thrombocytopenia and hemorrhage. The cholestasis appears to be due to damage to the hepatocyte and its bile secretory apparatus via a basic subcellular lesion to cell membranes and mitochondria as our histochemical findings indicate. This same type of finding was seen in the kidney tubules. The subcellular pathology demonstrated in the kidney and liver have been postulated to be caused by either direct action of the "toxin" on the cell itself or due to faulty oxygen supply to the cell as a result of widespread capillary damage (10). Both mechanisms are probably operative in the severe cases.

2. Ribosomal vaccine for leptospirosis.

Vaccines currently used to immunize animals against leptospirosis are suspensions of killed leptospira. Protection afforded by these bacterins for anyone of the 150 or more serovars depends on the presence of homologous or antigenically closely related serovars in the vaccine. They do not provide protection against serologically unrelated strains and do not insure protection from establishment of a renal carrier state.

In recent years, a new class of subcellular immunogens rich in ribonucleic acid and protein has been reported to protect mice against a number

of bacterial infections (11). They are isolated from lysed bacterial cells by differential centrifugation and are composed primarily of ribosomes. Presented data indicates that ribosomal preparations may have the distinct advantage over bacterins or other subcellular vaccines of providing a greater cross-protection spectrum against infection and disease (12).

In view of the reported success of ribosomal immunogens in protecting animals against bacterial infections, we prepared several ribosomal vaccines and studied their immunogenic properties.

Detailed experimental procedures have been previously reported (13). Briefly, ribosomes were collected by differential centrifugation from cells lysed in a French pressure cell. Ribosome suspensions were standardized by either ribonucleic acid or protein content and inoculated into young, adult hamsters. Vaccinated hamsters were challenged 3 weeks post vaccination and then observed for 2 weeks. One kidney from each surviving vaccinated and control hamster was cultured for leptospira to determine if a renal carrier state was present. Leptospira serovars used in experiments were bataviae strain 1415, canicola strain Moulton, and grippotyphosa strain 1540.

Results of experiments to determine the effect various enzymes had on the immunogenicity of vaccines, the effect of various routes of vaccine administration, and the duration of immunity produced by the vaccines were reported last year (13).

Table 2 shows the relative chemical composition of all the vaccines prepared from bataviae organisms. The ribonucleic acid to protein ratio for the sodium dodecyl sulfate (SDS) treated vaccines (vaccines B through I) ranges from 1.6:1 to 2.5:1 with most approximating the expected 2:1 ratio. The presence of cell membranes in vaccine preparations probably accounts for the large hexose concentrations found. Numerous membrane-like structures were noted on electron photomicrographs taken of a single vaccine (vaccine C). The main objective of this study was to assess the ability of the ribosomal preparations to protect hamsters from infection with unrelated serovars and not to determine the exact immunogenic moity present. Therefore, no attempt was made to eliminate the cellular membranes from the ribosomes other than what was accomplished by differential centrifugation and treatment with SDS.

The effect of RNA and protein concentrations on survival of vaccinated hamsters is summarized in Table 3. Animals vaccinated with as little as 12 µg of RNA and 6 µg of protein were protected against a challenge dose of 10^3 LD_{50}s, and hamsters administered 31 µg RNA and 12 µg protein of vaccine G survive a massive homologous challenge of $10^{5.4}$ LD_{50}s.

A sham vaccine was prepared from Brucella canis ribosomes. Hamsters vaccinated with this preparation and ones given a bataviae

Table 2: Chemical composition of bataviae-1415 ribosomal preparations

Vaccine	Composition in mg/100mg Protein		
	RNA	Hexose	DNA
A[a]	90	12	6
B	200	19	ND[b]
C	163	50	<5
D	200	41	<2
E	246	50	ND
F	238	53	2
G	250	52	ND
H	195	44	4
I	180	37	<1

[a]Sodium dodecyl sulfate not used in preparation of vaccine
[b]Not done

Table 3: Effect of RNA and protein concentrations on survival of hamsters vaccinated with bataviae-1415 ribosomal preparations

Vaccine	Antigen/Hamster		Challenge Dose		Survivors/ Total
	RNA	Protein	Lepto/ Hamsters	LD_{50}s	
C	100µg	61µg	2×10^4	$10^{3.8}$	5/5
	50µg	31µg			5/5
	25µg	15µg			5/5
	12µg	8µg			2/5
	100µg	61µg	2×10^5	$10^{4.8}$	5/5
	50µg	31µg			5/5
	25µg	15µg			5/5
	12µg	8µg			3/5
E	123µg	50µg	1×10^4	$10^{4.3}$	5/5
			1×10^5	$10^{5.3}$	5/5
			1×10^6	$10^{6.3}$	2/5
G	62µg	25µg	4×10^5	$10^{5.4}$	5/5
	31µg	12µg			5/5
H	25µg	13µg	3×10^4	10^3	5/5
	12µg	6µg			5/5

ribosomal vaccine (vaccine E) were challenged with virulent bataviae organism to determine if ribosomes stimulated a non-specific protective response. No difference between the survival of the B. canis vaccinates and the non-vaccinated controls was observed (Table 4). Animals were protected by the homologous vaccine.

Hamsters vaccinated with vaccine E were also challenged with leptospira serovar canicola. Intertype immunity appeared to occur with low challenge (7×10^2) dose but was unremarkable with greater challenges. On the other hand, vaccinates were well protected against homologous bataviae challenge containing 10^5 organisms (Table 5).

A group of hamsters given either a bataviae ribosomal vaccine (vaccine F) or a bataviae bacterin (organism inactivated with 0.3% v/v formalin) were tested extensively for intertype immunity. Vaccinates were challenged with either the homologous bataviae serovar or the heterologous canicola or grippotyphosa serovars (Tables 6 and 7). Excellent homologous protection was afforded by both the bataviae ribosomal and bacterin preparations. Neither immunogen protected against the grippotyphosa challenge. Bacterin vaccinated hamsters challenged with canicola organisms had a higher survival rate than did controls (Table 6) but were not protected against infection (Table 7). Ribosomal vaccinates were not apparently protected against canicola challenge.

A canicola ribosomal vaccine was prepared and evaluated for its ability to produce intertype immunity. Results are summarized in Table 8. Good homologous immunity was observed in vaccinated hamsters. No apparent protection against bataviae or grippotyphosa challenge was seen.

With the exception of the first vaccine prepared (vaccine A), bataviae ribosomal preparations effectively prevented the development of renal leptospirosis in vaccinated animals (Table 9). Only one hamster out of 66 which received a minimum of 10^3 LD_{50}s bataviae organisms had leptospira cultured from its kidney. Fifty of 52 hamsters challenged with 10^4 LD_{50}s had negative kidney cultures. The two hamsters in this group with infected kidneys had received a relatively small, 25 µg RNA and 50 µg protein, dose of a bataviae ribosomal preparation. In the vaccinates surviving a massive challenge of 10^5 LD_{50}s or greater, only two of 26 animals developed renal leptospirosis.

Passive immunization studies are summarized in Table 10. Sera and spleen cells were collected from inbred, LSH hamsters vaccinated 3 weeks previously with either a bataviae bacterin or ribosomal preparation. Recepients were challenged 24 hours after transfer of either sera or cells. Good protection was afforded recipients of the sera from either type of vaccinated donor. Greater cellular immunity appeared to be provoked in those hamsters receiving cells from the bacterin vaccinates.

Table 4: Comparison of immunity produced in hamsters by bataviae-1415 (vaccine E) and Brucella canis ribosomal preparations against bataviae-1415 challenge

Vaccine	Antigen/Hamster		Challenge Dose		Survivors/ Total	Mean Day of Death
	RNA	Protein	Lepto/ Hamster	LD_{50}s		
Controls	-	-	1×10^3	$10^{3.3}$	0/5	7.6
	-	-	1×10^4	$10^{4.3}$	0/5	7.1
	-	-	1×10^5	$10^{5.3}$	0/5	6.0
	-	-	1×10^6	$10^{6.3}$	0/5	5.2
bataviae-1415	123µg	50µg	1×10^4	$10^{4.3}$	5/5	-
			1×10^5	$10^{5.3}$	5/5	-
			1×10^6	$10^{6.3}$	2/5	12.2
Brucella canis	49µg	50µg	1×10^3	$10^{3.3}$	0/5	8
			1×10^4	$10^{4.3}$	0/5	7
			1×10^5	$10^{5.3}$	0/5	6.6
			1×10^6	$10^{6.3}$	0/5	5.6

Table 5: Comparison of immunity produced by a bataviae-1415 ribosomal preparation (vaccine E) against bataviae-1415 and canicola-Moulton challenges.

Challenge Organism	Challenge Dose		Controls				bataviae-1415 vaccinates[a]			
	Lepto/ Hamster	LD_{50}s	Survivors/ Total	Negative Culture/ Survivors[b]	% Protected[c]	Mean Day of Death	Survivors/ Total	Negative Culture/ Survivors[b]	% Protected[c]	Mean Day of Death
bataviae-1415	1×10^2	$10^{2.3}$	0/5	-	0	8.5	ND	ND	ND	ND
	1×10^3	$10^{3.3}$	0/5	-	0	7.6	ND	ND	ND	ND
	1×10^4	$10^{4.3}$	0/5	-	0	7.1	5/5	5/5	100	-
	1×10^5	$10^{5.3}$	0/5	-	0	6.0	5/5	5/5	100	-
	1×10^6	$10^{6.3}$	0/5	-	0	5.2	2/5	2/2	40	12.2
canicola-Moulton	7×10^1	-[d]	0/5	-	0	9.9	ND	ND	ND	ND
	7×10^2	-	0/5	-	0	9.0	4/5	1/4	20	9.5
	7×10^3	-	1/5	0/1	0	8.1	1/5	0/1	0	9.2
	7×10^4	-	3/5	0/3	0	11.2	1/5	0/1	0	7.8
	7×10^5	-	4/5	0/4	0	11.5	3/5	0/3	0	8.0

[a] 50µg protein and 123µg RNA per hamster.
[b] Number of hamsters with negative leptospira kidney cultures per survivor.
[c] Percentage of hamsters surviving challenge and having a negative kidney culture.
[d] Inadequate data to calculate a valid LD_{50}.

Table 6: Comparison of immunity produced by bataviae-1415 ribosomal (vaccine F) and bacterin preparations against bataviae-1415, canicola-Moulton, and grippotyphosa-1540 challenges.

Challenge Organism	Challenge Dose		Controls		Ribosomes[a]		Bacterin[b]	
	Lepto/ Hamster	LS_{50}s	Survivors/ Total	Mean Day of Death	Survivors/ Total	Mean Day of Death	Survivors/ Total	Mean Day of Death
bataviae-1415	6×10^1	$10^{1.8}$	0/5	8.9	5/5	-	5/5	-
	6×10^2	$10^{2.8}$	0/5	8.2	5/5	-	5/5	-
	6×10^3	$10^{3.8}$	0/5	7.3	5/5	-	5/5	-
	6×10^4	$10^{4.8}$	0/5	6.4	5/5	-	5/5	-
	6×10^5	$10^{5.8}$	0/5	5.1	5/5	-	5/5	-
canicola-Moulton	2×10^1	-[c]	0/5	10.6	0/5	10.3	5/5	-
	2×10^2	-	1/5	9.0	0/5	10.0	5/5	-
	2×10^3	-	1/5	8.4	3/5	9.5	5/5	-
	2×10^4	-	2/5	11.0	4/5	8.0	4/5	9.0
	2×10^5	-	2/5	8.6	1/5	7.0	5/5	-
	2×10^6	-	2/5	5.8	4/5	6.5	2/5	6.3
grippotyphosa-1540	5×10^2	$10^{0.2}$	2/5	11.7	0/5	9.6	2/5	11.7
	5×10^3	$10^{1.2}$	0/5	9.5	0/5	8.4	0/5	9.7
	5×10^4	$10^{2.2}$	0/5	8.0	0/5	7.6	0/5	8.1
	5×10^5	$10^{3.2}$	0/5	6.0	0/5	5.3	0/5	7.2
	5×10^6	$10^{4.2}$	0/5	5.0	0/5	5.6	0/5	5.0

[a] 75µg RNA and 31µg protein per hamster.
[b] 75µg RNA and 103µg protein per hamster.
[c] Inadequate data to calculate a valid LS_{50}.

Table 7: Comparison of protection produced by bataviae-1415 ribosomal (vaccine F) and bacterin preparations against infection from bataviae-1415, canicola-Moulton, and grippotyphosa-1540 challenges

Challenge Organism	Challenge Dose		Number of Hamsters Protected[a]/Total		
	Lepto./ Hamster	LD_{50}s	Control	Ribosomes[b]	Bacterin[c]
bataviae-1415	6×10^1	$10^{1.8}$	0/5	5/5	5/5
	6×10^2	$10^{2.8}$	0/5	5/5	5/5
	6×10^3	$10^{3.8}$	0/5	4/5	5/5
	6×10^4	$10^{4.8}$	0/5	5/5	5/5
	6×10^5	$10^{5.8}$	0/5	5/5	5/5
canicola-Moulton	2×10^1	-[d]	0/5	0/5	0/5
	2×10^2	-	0/5	0/5	0/5
	2×10^3	-	1/5	0/5	0/5
	2×10^4	-	0/5	0/5	0/5
	2×10^5	-	1/5	0/5	0/5
	2×10^6	-	0/5	0/5	1/5
grippotyphosa-1540	5×10^2	$10^{0.2}$	1/5	0/5	0/5
	5×10^3	$10^{1.2}$	0/5	0/5	0/5
	5×10^4	$10^{2.2}$	0/5	0/5	0/5
	5×10^5	$10^{3.2}$	0/5	0/5	0/5
	5×10^6	$10^{4.2}$	0/5	0/5	0/5

[a]Number of hamsters surviving challenge and having a negative kidney culture.
[b]75µg RNA and 31µg protein per hamster.
[c]75µg RNA and 103µg protein per hamster.
[d]Inadequate data to calculate a valid LD_{50}.

Table 8 : Comparison of immunity produced by a canicola-Moulton ribosomal preparation against canicola-Moulton, bataviae-1415, and grippotyphosa-1540 challenges.

Challenge Organism	Challenge Dose		Controls				canicola-Moulton vaccinates[a]			
	Lepto/ Hamster	LD_{50}s	Survivors/ Total	Negative Culture/ Survivors[b]	% Protected[c]	Mean Day of Death	Survivors/ Total	Negative Culture/ Survivors[b]	% Protected[c]	Mean Day of Death
canicola-Moulton	9×10^2	-	2/5	2/2	40	11.5	5/5	5/5	100	-
	9×10^3	$10^{0.8}$	1/5	0/1	0	9.3	5/5	4/5	80	-
	9×10^4	$10^{1.8}$	1/5	0/1	0	7.0	5/5	5/5	100	-
bataviae-1415	1×10^0	-	4/5	2/4	40	9.5	5/5	4/5	80	-
	1×10^1	$10^{0.7}$	0/5	-	0	9.1	2/5	0/2	0	11.0
	1×10^2	$10^{1.7}$	0/5	-	0	8.6	0/5	-	0	9.5
	1×10^3	$10^{2.7}$	0/5	-	0	7.5	0/5	-	0	8.2
	1×10^4	$10^{3.7}$	0/5	-	0	6.5	0/5	-	0	6.6
	1×10^5	$10^{4.7}$	0/5	-	0	5.4	0/5	-	0	5.7
grippo-typhosa-1540	4×10^0	$10^{0.0}$	2/5	1/2	20	10.5	0/5	-	0	11.4
	4×10^1	$10^{1.0}$	0/5	-	0	11.3	2/5	0/2	0	13.2
	4×10^2	$10^{2.0}$	0/5	-	0	11.4	2/5	0/2	0	10.7
	4×10^3	$10^{3.0}$	1/5	0/1	0	10.3	3/5	0/3	0	8.5
	4×10^4	$10^{4.0}$	0/5	-	0	10.2	1/5	0/1	0	10.0
	4×10^5	$10^{5.0}$	1/5	0/1	0	7.9	1/5	0/1	0	9.0

[a] 75µg protein and 110 RNA per hamster.
[b] Number of hamsters with negative leptospira kidney cultures per survivor.
[c] Percentage of hamsters surviving challenge and having a negative kidney culture.

Table 9: Results of kidney cultures on bataviae-1415, ribosome-vaccinated hamsters surviving challenge with homologous organisms.

Vaccine	Antigen/Hamster		Number of survivors having positive kidney cultures per total number of survivors[a]			
	RNA	Protein	10^2LD_{50}	10^3LD_{50}	10^4LD_{50}	10^5LD_{50}
A	344 µg	370 µg	1/11	-	-	-
	172 µg	185 µg	3/11	-	-	-
C	100 µg	61 µg	-	0/5	0/5	-
	50 µg	31 µg	-	0/5	0/10	0/5
	25 µg	15 µg	-	0/5	0/5	-
	12 µg	8 µg	-	0/5	0/5	-
D	50 µg	25 µg	-	-	0/3	1/3
	25 µg	12 µg	-	-	2/4	1/1
E	123 µg	50 µg	-	0/5	0/5	0/2
F	75 µg	31 µg	0/5	1/3[b]	0/5	0/5
G	62 µg	25 µg	-	0/5	0/5	0/5
	31 µg	12 µg	-	0/5	0/5	0/5
H	25 µg	13 µg	-	0/5	-	-
	12 µg	6.4µg	-	0/5	-	-
I	100 µg	53 µg	0/5	-	-	-
	50 µg	27 µg	0/5	-	-	-
	25 µg	13 µg	0/5	0/5	-	-
	12 µg	6.6µg	-	0/5	-	-
	6.2µg	3.3µg	-	0/5	-	-
	3.1µg	1.7µg	-	0/3	-	-

[a]Results grouped by minimum LD_{50} with which hamsters were challenged.
[b]Two vaccinates died of causes other than leptospirosis.

Table 10: Comparison of the protective effect of passively transferred cells and sera from hamsters vaccinated with bataviae-1415 ribosomal (vaccine I) and bacterin preparations.

Group	Antigen: ug RNA		Survivors[a]/ Total	Mean Day of Death	Negative Culture/ Survivor	% Protected
	Vaccinates	Donors				
Controls	-	-	0/5	7.4	-	0
Bacterin	100	-	5/5	-	5/5	100
	50	-	5/5	-	5/5	100
	25	-	4/5	15	2/4	40
Ribosomes	100	-	5/5	-	5/5	100
	50	-	5/5	-	5/5	100
	25	-	5/5	-	5/5	100
Control: cells	-	-	0/5	7.4	-	0
serum	-	-	0/5	7.2	-	0
Bacterin: cells	-	100	5/5	-	5/5	100
	-	50	5/5	-	5/5	100
	-	25	2/5	10.3	1/2	20
serum	-	100	5/5	-	5/5	100
	-	50	5/5	-	5/5	100
	-	25	5/5	-	5/5	100
Ribosomes: cells	-	100	2/5	10	1/2	20
	-	50	4/5	10	3/4	60
	-	25	5/5	-	1/5	20
serum	-	100	5/5	-	5/5	100
	-	50	5/5	-	5/5	100
	-	25	5/5	-	5/5	100

[a]Hamster challenged with 10^2 LD_{50}s of bataviae-1415.

3. Leptospira biflexa contaminants in bacteriological and tissue culture media.

A strain of Leptospira biflexa appeared as a contaminant in leptospiral cultures and leptospiral culture media. The contaminant was traced to the deionized water supply which was used in the preparation of media. The media was sterilized by filtration through 0.22 µm pore size membrane filter. The strain was identified to be L. biflexa on the basis of serological tests and its relative resistance to growth inhibition by copper ions and 8-azaguanine.

Subsequently, a spirochetal contaminant was found in tissue cultures by Dr. J. Tumilowicz at Electro-Nucleonics Laboratories, Inc., Bethesda, Md. The contaminant was isolated and identified as a strain of L. biflexa in this laboratory. The tissue culture media containing the contaminant was derived from a commercial source. It was prepared with deionized water and sterilized by filtration. In retrospect, the findings were not surprizing since L. biflexa has frequently been found in tap water and can easily penetrate bacterial retaining filters.

Detailed reports of these two findings have been accepted for publication (31,32).

4. Characterization of leptospiral isolates from Malaysia.

Requisite serologic tests were carried out to complete the serological characterization of 1424 pathogenic leptospiras isolated from natural waters and wet soils in Malaysia (14). Strains were isolated during the period 1961 to 1966 from five different geographic or ecological sites. Approximately 1185 strains were isolated from jungle waters and soils within a radius of 20 miles from Kuala Lumpur in the State of Selangor. Fifty-six isolates from ricefields and 17 isolates from pools of water in mining areas were also obtained in the proximity of Kuala Lumpur. The 104 leptospiral strains from the State of Pahang were derived mostly from shore sand along a jungle stream and river located near Luala Lipis. Jungle streams in the western part of Sabah were the source of 62 leptospiral isolates. Nearly all of the isolates were recovered from hamsters dying from leptospirosis after their exposure to sample water or sample soil washings. The survey method (15) and culture typing technics are described elsewhere (16).

The 1,362 strains isolated from soil and water in West Malaysia (Selangor and Pahang) could be divided into 13 serogroups comprising a total of 29 serovars (Table 11). The wide variety of pathogenic serovars in jungle milieu was consistant with the broad array of serovar infections found in soldiers on jungle patrols (17). Within the State of Selangor, the relative distributions of serovars in the ricefield and jungle areas studied were similar. However, of 17 strains isolated in mining pools, 16 appeared to be related to serovar australis and one to serovar paidjan.

Table 11: Pathogenic leptospiras isolated from Malaysian waters and wet soils

Serogroup	Serovar	Percent of isolates within locations				
		Selangor jungle	Selangor rice-field	Selangor mining pool	Pahang jungle	Sabah jungle
No. of samples		1185	56	17	104	62
Icterohaem-orrhagiae	mankarso[1]	18.3	23.2		2.9	8.1
	smithie	2.0				
	birkin	1.1			1.0	
	Total	21.4	23.2		3.8	8.1
Australis	australis[1]	13.5	7.1	94.1	9.6	
Bataviae	paidjan[1]	11.6	7.1	5.9	1.9	58.1
	bataviae[1]	3.7				9.7
	Total	15.4	7.1	5.9	1.9	67.7
Canicola	jonsis	8.1			1.0	
	schuffneri	4.6	1.8		31.7	
	malaya[1]	0.5				
	sumneri	0.8	3.8		8.7	
	Total	14.1	5.4		41.3	
Autumnalis	bangkinang	4.1	14.3		3.8	17.7
	rachmat[1]	4.1	1.8		7.7	
	gurungi	0.6				3.2
	weerasingha[1]	1.0	1.8			1.6
	sentot[1]	0.6	1.8		3.8	1.6
	Total	10.4	19.6		15.4	24.2
Pyrogenes	zanoni	7.3	8.9		5.8	
	biggis	2.0	1.8		11.5	
	hamptoni	1.5				
	abramis	0.2				
	Total	11.0	10.7		17.3	
Hebdomadis	wolffi[1]	7.2	23.2		5.8	
	ricardi	1.5			1.0	
	worsfoldi	0.1				
	Total	8.8	23.2		6.7	
Grippo-typhosa	grippo-typhosa[1]	4.9	1.8		3.8	
Javanica	javanica[1]		1.8			
Celledoni	whitcombi	0.2				
Pomona	pomona	0.3				
Tarassovi	tarassovi[1]	0.1				
Ranarum	evansi[1]	0.1				

[1]The identity of representative strain was confirmed by agglutinin-adsorption tests.

The relative frequency of recovered serovar strains in the Pahang jungle superficially differed from that of the Selangor jungle. The variable findings may have reflected differences in size and types of samples and time of sampling. In the limited survey in Sabah mainly bataviae and autumnalis and a few icterohaemorrhagiae serovars were found.

All except four of the representative strains were related to Malaysian serovars previously studied in this laboratory (16,18). Three of the exceptional strains were identified to be serovars sentot, werrasingha, and tarassovi, respectively, on the basis of agglutinin-adsorption tests. Serovar sentot and a member of the Tarassovi serogroup have been reported from Malaysia previously (19). Serovar werrasingha was isolated in Ceylon and identified by Y. Chernukha, (C. Sulzer, Center for Disease Control, personal communication). It had not heretofore been found in Malaysia. It was found in waters of both East and West Malaysia.

The fourth strain (267-1348) cross-reacted to some but not all of the serovars in the Canicola, Icterohaemorrhagia, Javanica and Autumnalis serogroups and to homologous titer with a strain isolated from the kidneys of a frog in Iowa (20). The frog strain was classified by Babudieri (21) as a new pathogenic serovar ranarum in a separate serogroup Ranarum (22). In comparative cross agglutination tests with diverse serovar antisera, the reactions of ranarum and strain 267-1348 were essentially identical (Table 12). Reciprocal agglutinin adsorption tests were carried out between strain 267-1348 and ranarum as well as with various serovar representative strains with which it cross-reacted to relatively high titer. The results of tests are summarized in Table 13. The antibodies responsible for specificity of the test serovars were not removed after adsorption with strain 267-1348 nor was the anti-267-1348 serum significantly reduced after adsorption by any of the test antisera. Accordingly, it represents a new serovar which we designate evansi. A report of the culture typing findings has been prepared and will be submitted for publication.

5. Phenotypic characteristics of a genetically distinct leptospira, serovar illini, strain 3055.

Strain 3055 isolated from the urine of a normal bull in Illinois was previously found to be genetically different from other genetically characterized leptospiras (34). In additional studies conducted jointly with Dr. L. Hanson, College of Veterinary Medicine, University of Illinois, the strain was found to be antigentically unrelated to any of the known pathogenic and saprophytic serovars. Strain 3055 resembled L. biflexa strains in its ability to grow at 13 C and in its relative resistance to 2, 6-diaminopurine, 8-azaguanine and $CuSO_4$. It was designated serovar illini. A detailed report of the characteristic of serovar illini is being published (33).

Table 12: Cross-agglutination reactions of strains 267-1348 and ICF (serovar ranarum)

Antisera[1]		Reciprocal of titer[2] with	
Serogroup	Serovar	267-1348	ICF
Ictero-haemorrhagiae	birkini	400	-
	smithii	400	100
Canicola	jonsis	200	100
	broomi	800	200
	bindjei	400	100
	schuffneri	400	200
	benjamin	1600	800
	malaya	800	400
	galtoni	3200	800
Pyrogenes	robinsoni	1600	800
Javanica	javanica	400	-
	poi	1600	6400
	sorex	100	1600
	ceylonica	-	400
Autumnalis	djasiman	1600	1600
	gurungi	1600	1600
Ranarum	ranarum (ICF)	3200	3200
	evansi (267-1348)	25600	3200

[1]Homologous titers ranged from 1:6400 to 1:102400.
[2]Titers of 1:100 or less (-) with the following serovar antisera: sarmin, icterohaemorrhagiae, copenhageni, mankarso, naam, canicola, kabura, kashirski, portland-vere, zanoni, abramis, pyrogenes, manilae, hamptoni, biggis, sofia, anhoa, celledoni, panama, shermani, medanensis, bataviae, tarassovi, pomona, autumnalis, rachmati, fort-bragg, bangkinang, erinacei-auriti, mooris, sentot, lanka, australis, grippotyphosa, cynopteri, canalzonae.

Table 13: Cross-agglutinin adsorption studies on strain 267-1348

Antiserum Against	Adsorbed with	Reciprocal of titer[1] with Homologous strain after adsorption	Reciprocal of titer[1] with Adsorbing strain before adsorption
267-1348 (102400)[2]	ranarum	6400	12800
267-1348 (25600)	jonsis	25600	1600
267-1348 (25600)	broomi	25600	1600
267-1348 (25600)	benjamin	25600	1600
267-1348 (25600)	malaya	25600	6400
267-1348 (25600)	robinsoni	25600	1600
267-1348 (25600)	javanica	25600	1600
267-1348 (25600)	poi	25600	1600
267-1348 (25600)	coxus	25600	6400
267-1348 (25600)	djasiman	25600	1600
267-1348 (25600)	gurungi	25600	6400
ranarum (51200)	267-1348	12800	51200
jonsis (25600)	"	25600	400
broomi (102400)	"	102400	400
benjamin (25600)	"	6400	1600
malaya (102400)	"	102400	1600
robinsoni (25600)	"	25600	1600
javanica (25600)	"	25600	400
poi (102400)	"	102400	1600
coxus (102400)	"	102400	1600
djasiman (25600)	"	25600	6400
gurungi (25600)	"	25600	1600

[1] Cross agglutinins against heterologous (adsorbing strain) antigens were undetectible at 1:100 dilution following adsorption with respective heterologous antigens. Homologous titers followed adsorption with homologous antigen were 1:100 or less except for javanica, malaya, and poi which were 1:400.

[2] Reciprocals of homologous titers of unadsorbed sera shown in parenthesis.

6. Leptospiral jaundice in a spectacled bear at Baltimore Zoo.

Serum submitted from a jaundiced spectacled bear at the Baltimore Zoo contained high titer agglutinins for serovar icterohaemorrhagiae. The bear area was heavily infested with Norway rats. Subsequently, cultures of kidneys from 15 rats trapped in the area were submitted to WRAIR. Five of the rats were positive. All cultures were identified to be members of the Icterohaemorrhagiae serogroup.

7. Regulatory mechanisms in smooth and rough strains of Pseudomonas pseudomallei.

The investigation of the physiological differences between smooth and rough cultures of P. pseudomallei was continued (23). Recognition of different biochemical activities of smooth and rough strains have a bearing in laboratory diagnosis of isolated strains. Physiological, biochemical and nutritional studies of the "suicidal tendency" of P. pseudomallei cultures by Rogul and Carr (24) attributed this phenomenon to the accumulation of ammonia in the agar culture media. The so-called "suicidal" strains (smooth cultures) were unable to cope with this accumulation of ammonia, whereas "non-suicidal" strains (rough cultures) excreted oxalic acid which detoxified the ammonia. To determine the physiological basis of ammonia toxicity in Pseudomonas species, the intermediary metabolism of two genetically stable strains, rough strain 7815C (non-suicidal) and smooth strain 165L (suicidal), were studied. Surprisingly, the ammonia scavenger, glutamate dehydrogenase, was not detected in the soluble portion of whole cell extracts (23). The absence of this major pathway for the incorporation of ammonia into an organism led to further investigation of the ammonia metabolism of these two Pseudomonads.

After harvesting the organisms from chemically defined minimal media, sonicated extracts were made and the soluble and particle bound enzyme fractions were isolated. Assays were performed to determine the presence or absence of the enzymes of ammonia incorporation and elimination. Since both organisms contain a strong NADH and NADPH oxidase which interfers with the other enzymatic assays, both NADH and NADPH oxidase activities were determined and then inhibited in other enzymatic assays by the use of quinacrine HCl. The specific activities of the assayed enzymes are presented in Table 14.

As noted before, the enzymes for the incorporation of ammonia into the organism via glutamic dehydrogenase are virtually absent. Both the soluble and particulate fractions show very low or undetectable activity of both the NADH and NADPH dependent glutamate dehydrogenase. Similarly, there was no detectable aspartate ammonia lyase activity in the soluble fraction when two different methods for ammonia determination were used. As of this time, the particulate fraction has not been tested for aspartate ammonia lyase activity.

Table 14: Activities of soluble and particulate bound enzymes of rough (7815-C) and smooth (165C) strains of Pseudomonas pseudomallei

Enzyme	Cofactor	Sonicated cell fraction			
		soluble		particulate	
		7815C	165L	7815C	165L
glutamate dehydrogenase	NAD	-	-	-	-
	NADP	-	-	-	-
	NADH	0.07	0.05	-	-
	NADPH	0.07	0.05	0.3	-
alanine dehydrogenase	NAD	-	-	-	-
	NADP	-	-	-	-
	NADH	1.7	0.7	1.2	-
	NADPH	2.6	1.2	0.6	-
aspartate ammonia lyase		-	-	ND[b]	ND
NADH oxidase		1.04	3.9	8.3	362.3
NADPH oxidase		0.3	0.5	1.4	0.7

[a] Specific activity as n moles substrate oxidized or reduced or NH_3 formed or utilized per milligram of protein per minute.

[b] not done.

The only active ammonia incorporating enzyme appears to be alanine dehydrogenase which utilizes either NADH or NADPH as a cofactor. The enzyme of strain 165L does not appear to be membrane bound as all of the enzyme activity appears in the soluble extract; in contrast, the alanine dehydrogenase of strain 7815C appears to be both membrane bound and free in solution, although the activity of the NADPH dependent enzyme appears to be greater in the soluble fraction.

In no case was there any indication of the presence of the degradative enzymes of ammonia metabolism, the enzymes showed no activity when NAD or NADP were used as cofactors of suitable substrates. However, nonspectrophotometric methods will have to be used to confirm the absence of the deaminating alanine enzyme.

Both soluble and particulate fractions of each strain exhibited strong NADH and NADPH oxidases. Most of the activity appeared in the particulate fraction of both strains. The greatest activity occurred in the particulate fraction of strain 165L which had 100 times more activity than the soluble fraction of strain 165L or the particulate and soluble fractions of strain 7815C.

8. An unusual pseudomonad from the United States resembling Pseudomonas pseudomallei.

An organism (C6786) resembling Pseudomonas pseudomallei was submitted to the Division of Veterinary Medicine by the Center for Disease Control, Atlanta, for further study. The organism was isolated from the crush wounded leg of an Oklahoma farmer. It resembled P. pseudomallei in many respects except serologically. Because of the serious implications of an autochthonous P. pseudomallei infection, a great deal of effort was made to identify this organism.

Table 15 lists the most salient laboratory features of this organism. These features are indistinguishable from those of P. pseudomallei. The production of a nitrite reductase, poly B hydroxybutyrate granules, oxalic acid and high gelatinase activity correlated with the acid producing rough strains of P. pseudomallei which formed red colonies on MacConkey agar. It was further tested for utilization of single carbon compounds from different sources. These tests aided in distinguishing this organism from some readily identifiable denitrifying bacteria (Table 16). It was not like those denitrifiers described by Palleroni, et al (25). The pattern did resemble that of P. pseudomallei (26).

The antibiotic sensitivity patterns were also like that of P. pseudomallei (Table 17). Ten day old cultures did not fluoresce on trypticase soy, sheep veal infusion or 3% glycerol-brain heart infusion agars. There did not seem to be any fluorescence of young cultures on P or F media, but it was difficult to assess because the colonies were shiny.

Table 15: The reactions and properties of Pseudomonas sp. strain C6786 in different diagnostic tests

Test	Growth	Comment
Trypticase soy broth (TSB)	+	at 41 C
Crystal violet agar	+	37 C
VCN agar	+	
CTAB agar	-	
SS agar	+ weak	very inhibited
Sheep veal infusion agar (SVIA)	+	good growth
2% NaCl in SVIA	+ weak	very inhibited
4% NaCl in SVIA	-	
Blood Azide agar	-	
P agar	+	no pigment
F agar	+	no pigment
Sabroud's Dextrose agar	+	
Indole		-
Malonate	+	weak
Pseudomonas isolation agar (Difco)	+	yellow
Starch hydrolysis on Mueller Hinton Agar		+ not strong
3% glycerol in brain heart infusion agar	+	very rough
MacConkey agar	+	very red
TSB and nitrate broth (10 days)		oxalic acid
O-F basal media*	+	acid
Nitrate broth		reduced past nitrite
Gelatinase		+
Poly Beta hydroxy butyrate granules		+ microscope
Catalase activity		+
DNA ase activity		-
cytochrome oxidase activity		+
Citrate		alkaline
Phenylalanine deaminase		-
DNA % G+C (Bouyant density)		66.7%

*O-F - Forms an acid in basal broth without carbohydrates

Table 16: The utilization of single carbon sources by Pseudomonas sp. strain C6786

Single Carbon Sources	Growth
Agar	
Glucose	+
Mannitol	+
L malate	+
L+ alanine	+
Glutamate	+
Aspartate	+
alpha ketoglutarate	+
para hydroxybenzoate	-
maltose	+
L arginine	+
D+ xylose	-
ethylene glycol	-
acetamide	± (poor)
nicotinate	-
Broth	
succinate	+
B hydroxybutyrate	+
DL lactate	+
para hydroxybenzoate*	-
pellicles formed	

*para hydroxybenzoate: Second transfer showed growth after about a week, 3rd transfer showed growth in 4 days.

Table 17: Antibiotic sensitivities of *Pseudomonas sp.* C6786

Antibiotic	Concentration	
Ampicillin	10 mcg.	R
Aureomycin	30 mcg.	S
Bacitracin	10 units	R
Cephalothin	30 mcg.	R
Chloromycetin	30 mcg.	S
Coly-Mycin	10 mcg.	R
Dihydrostreptomycin	10 mcg.	R
Elkosin	1 mg.	S
Erythromycin	15 mcg.	R
Furacin	100 mcg.	R
Gantrisin	2 mg.	S
Kantrex (Kanomycin)	30 mcg.	S
Lincomycin	2 mcg.	R
Neomycin	30 mcg.	R
Oleandomycin	15 mcg.	R
Penicillin	10 units	R
Polymyxin	300 units	R
Sonilyn	1 mg.	S
Sulfathizole	1 mg.	S
Tetracycline	30 mcg.	SS
Nafcillin	1 mcg.	R
Gentamicin	100 mcg.	R
Furadantin/Macrodantin	100 mcg.	R

S - Sensitive SS - Slightly sensitive R - Resistant

The organism did not agglutinate in antisera to P. pseudomallei. It did agglutinate in antisera to some strains of P. cepacia, P. marginata and P. stutzeri at serum dilutions greater than 320 fold.

Hamsters were inoculated with 10 fold dilutions of an inoculum containing 13 X 10^9 organisms. Two of 3 of the hamsters receiving 13 X 10^9 organisms died in 24 hours. The rest of the test animals survived for a week.

In another series of virulence tests, a hamster which received 250 organisms died in 6 days. His liver and spleen contained C6786. His inoculated cagemates were sacrificed and cultured. They did not contain any organisms in their livers or spleens. This strain is so weakly virulent that we hesitate to even call it an opportunist in hamsters, since one hundred P. pseudomallei cells are generally enough to kill any hamster.

The moles % guanine + cytosine in C6786 DNA (66.7% in Table 15) was slightly lower than most P. pseudomallei strains (69.5%). Based on this, on the serology and on the lack of virulence for hamsters, we were convinced that the C6786 organism was not P. pseudomallei. On the basis of antibiotic sensitivities, serological, cultural and physiological characteristics, it is our belief that this organism belongs in a complex containing P. pseudomallei, P. mallei, P. cepacia, and P. marginata. This complex was grouped by Ballard et al. (26). When tested for DNA relatedness by the method of Deley et al. (27), strain C6786 appeared to have some DNA sequences in common with P. cepacia and P. marginata.

9. Iridescent lysis of Pseudomonas aeruginosa cultures.

Investigations on the iridescent lysis (IRL) of Pseudomonas aeruginosa strain 227 (23) were continued. We had previously found that pyocyanine was produced by this strain in the presence of glycerol and its production was inhibited in glucose media. This closely correlated with the IRL on agar lawns. At about the same time, we found a strain of P. aeruginosa (strain 1000) which did not exhibit IRL on 3% glycerol agar medium. However, in the presence of 3% glucose, it consistently produced non-lytic iridescent plaques (IR). With lesser amounts of glucose production of IR plaques was erratic.

Assay of pyocyanine excreted into the agar or maintained in the cell lawn. The lawns of agar cultures from strains 227 and 1000 were carefully scraped from the agar surface and assayed for pyocyanine. The remaining agar matrix was frozen and thawed. The resulting expressed fluids were also assayed for pyocyanine. The results indicated (Table 18) that strain 227 excreted more pyocyanine than strain 1000 and particularly more in the presence of glycerol. Strain 1000 excreted the same amount of pyocyanine whether grown on glucose or

Table 18: Pyocyanine content in different agar cultures of *P. aeruginosa* strains 227 and 1000.

Media	Pyocyanine in expressed fluids strain 227	strain 1000	Ratio of 227/1000
	ug/ml		
3% glycerol-casamino agar	81.62	9.83	8.3
3% glucose-casamino agar	17.95	8.76	2.1
Ratio of glycerol/glucose	4.6	1.1	
	Cellular content of pyocyanine strain 227	strain 1000	Ratio of 227/1000
	ug/mg cell protein		
3% glycerol-casamino agar	8.733	0.056	156.0
3% glucose-casamino agar	0.484	0.258	1.9
Ratio of glycerol/glucose	18.0	0.22	

glycerol agar. The lawns of strain 227 also contained more pyocyanine than lawns of strain 1000 and this was especially large in glycerol cultures. On the contrary, cells in the lawns of strain 1000 contained less pyocyanine when grown on glycerol compared to glucose. These results could almost be anticipated from our previous findings. The arguments for pyocyanine as the basic agent of iridescence and lysis were very compelling, but inconclusive, especially in light of the findings by Wensinck et al. (28) that the iridescent material was a quinolinol rather than pyocyanine. For this reason, we repeated our search for unique lipids in glycerol cultures by using different assay technics.

Reexamination of cell lipid extracts. We had previously examined all the lipids extracted by the Folch et al. method (29) on Silica gel thin layer chromatograms (TLC). This time we excluded all the water soluble lipids by using the extraction method of Wensinck et al. (28) and analyzed the alcoholic extract in the Cary 15 spectrophotometer. An UV absorption spectrum corresponding to 2-alkyl-4 quinolinols was obtained in all extracts except glycerol-grown P. aeruginosa 1000 cells. The amounts of quinolinols were assayed and presented in Table 19. In other experiments, we found that the quinolinol content varied with time, especially for P. aeruginosa 227 glucose grown cultures. However, the results presented are indicative of the relative amounts of quinolinols in agar cultures. It now remained to be seen if these lipids were somehow overlooked on TLC.

The extracts were examined on TLC developed in a tank containing $CHCl_3$:CH_3COOH:15N NH_4OH (65:30:5). At first, no spots indicative of the quinolinols were found. However, on closer scrutiny we discovered fluorescent spots which volatilized and disappeared from the TLC two minutes after removal from the developing tanks. When the developing front was allowed to migrate 8 cm, P. aeruginosa 227 glycerol grown extracts had one fluorescent spot which migrated with the front and a lower fluorescent spot about 2.5 cm lower. Both had UV spectrographs characteristic of 2-alkyl-4 quinolinols. P. aeruginosa 1000 glucose grown cells had one lipid spot 2.5 cm away from the front and P. aeruginosa 227 glucose had no spot that could be visualized by UV. Since the lower spots usually disappeared first, we assumed that this was the spot in P. aeruginosa 227 glucose which was assayed spectrophotometrically in the alcoholic extracts.

Premature introduction of pyocyanine and 2-alkyl-4 quinolinols into agar cultures. Pyocyanine and the quinolinols were extracted from cultures of strain 227. The lipid extracts were incorporated into sterile paper discs and allowed to dry. The impregnated discs were then placed on freshly inoculated lawns of strains 227 and 1000 on 3% glycerol and 3% glucose agar. The results are shown in Table 20. It was obvious the pyocyanine inhibited plaquing in both strains, but

Table 19: Spectrophotometric estimation of 2-alkyl-4 quinolinols in plaque material.[a]

P. aeruginosa strain culture source	Amount of plaque material			
	mg/mg protein	ratio[b]	mg/gm dry weight	ratio[b]
Strain				
227/glycerol agar	0.136	1.0	13.9	1.0
227/glucose agar	0.008	0.06	0.77	0.06
1000/glucose	0.008	0.06	0.72	0.05
1000/glycerol	0.0004	0.003	0.048	0.003

[a]Assay derived by Wells (30). Pyo Ib; $E^{1\%}_{1cm}$ at 328nm = 438.
[b]All ratios were based on the yield of quinolinols in cells of *P. aeruginosa* strain 227 grown on glycerol agar.

Table 20: The affect of pyocyanine or quinolinol extracts on P. aeruginosa agar cultures.

	3% glucose agar	Effect of pyocyanine	Effect of quinolinols	3% glycerol agar	Effect of pyocyanine	Effect of quinolinols
P. aeruginosa 227						
Pitting	none			many pits		
Plaques	none			many plaques	inhibits	inhibits
Growth	good	inhibits	no effect	good	inhibits	no effect
P. aeruginosa 1000						
Pitting	none			none		
Plaques	many	inhibits	slightly inhibits	none		
Growth	good	no effect	no effect	good	no effect	no effect

only inhibited the growth of strain 227. Whenever plaques were inhibited, so was any previously concomitant lysis. The quinolinol mixture was difficult to assess because of its propensity to volatilize and dissipate. Nonetheless, in the immediate vicinity of the impregnated discs, the IR (plaquing) was obviously reduced or obviated. Perhaps it was a matter of concentration, but no growth inhibition occurred around the quinolinol discs. One interesting sidelight was that the quinolinols on strain 227 glycerol agar seemed to stimulate the early production of a blue, green pigment, suggestive of a pyocyanine precursor. The most interesting conclusions to be drawn are that these compounds are the cause of IRL. Their influence when introduced prematurely into the cultures suggested a feedback inhibition. From the preliminary data, it seemed that the quinolinols were responsible for iridescence and maybe pyocyanine production, whereas, pyocyanine was more responsible for lysis in susceptible cultures.

Summary and Conclusions.

1. Pathogenesis of experimental bataviae leptospirosis in dogs.

Clinical, microbiological, clinical biochemistry, and histochemical studies have been completed. Histopathological and electron microscopic studies are underway. Data compiled to date indicates that the basic lesion of bataviae leptospirosis in a dog is a subcellular one, primarily of damaged mitochondria and cell membranes.

2. Ribosomal vaccine for leptospirosis.

Results of studies indicate that ribosomal preparations are good immunogens which can protect hamsters against both death and development of the renal carrier state when challenged with homologous serovars. However, on a per milligram of RNA basis, they offer no better protection than the standard leptospiral bacterins and suffer from the disadvantage of being expensive to prepare. Very little protection was provided by either the bacterin or ribosomal preparations against challenge with heterologous serovars.

3. Leptospira biflexa contaminants in bacteriological and tissue culture media.

Contamination of bacteriological and tissue culture media by Leptospira biflexa was traced to use of deionized water and filtration sterilization technics. Chances for leptospiral contamination of media sterilized by filtration may be eliminated by the use of distilled water, or boiled deionized water, or by heating media at 56 C for 1 h.

4. Characterization of leptospiral isolates from Malaysia.

A total of 1424 pathogenic leptospiras isolated from natural waters and wet soils in Malaysia comprised 29 different serovars (syn. serotypes). All except two of the serovars had been found previously in Malaysia. The exceptional serovars were weerasingha, an Autumnalis serogroup member originally isolated in Ceylon, and a new serovar designated evansi. Serovar evansi had serological affinities with serovar ranarum which was isolated from the kidney of a frog in Iowa. The large variety of serovars found in jungle areas was consistent with similar previous findings of diverse serovar infections in troops who had operated in Malaysian jungles.

5. Phenotypic characteristics of a genetically distinct leptospira, serovar illini, strain 3055.

A genetically distinct leptospiral strain (3055) was found to be serologically unrelated to all known serological types of pathogenic and saprophytic leptospiras. It was, therefore, designated illini. Phenotypically it could not be readily distinguished from strains of L. biflexa, although it probably represents a new species on the basis of its DNA base composition.

6. Leptospiral jaundice in a spectacled bear at Baltimore Zoo.

A case of leptospiral jaundice in a spectacled bear in the Baltimore Zoo was epidemiologically related to high infestation of infected rats in the area.

7. Regulatory mechanisms in smooth and rough strains of Pseudomonas pseudomallei.

Under balanced nutritional conditions with glycerol as a carbon source and ammonium sulfate as a nitrogen source, the major pathway of NH_4^+ assimilation in P. pseudomallei appeared to be via the enzymatic reaction of pyruvate with NH_4^+ to form alanine. This reaction is catalysed by alanine dehydrogenase with either NADH or NADPH as a cofactor. This knowledge will allow further investigation of the distribution of ammonia within the organism by determination of transaminase enzyme activity. Further experimentation to elucidate the factors regulating nitrogen metabolism in these organisms will be performed. First, to determine the effect of nutritional factors, such as NH_4^+ concentration, glucose and glycerol concentration on the activity of the ammonia scavaging enzymes; and, second, to determine the nitrite and nitrate reductase enzyme activity of these organisms since the rough strain is able to reduce nitrate to a gas while the smooth strain 165L is unable to reduce nitrate beyond nitrite.

8. An unusual pseudomonad from the U.S. resembling Pseudomonas pseudomallei.

An unusual pseudomonad (C6786) isolated from an infected wound in an Oklahoma farmer was indistinguishable biochemically from P. pseudomallei but differentiable on basis of serology. Unlike P. pseudomallei, it was avirulent for hamsters. Furthermore, it appeared to differ from P. pseudomallei in DNA composition. The findings underline the importance of use of confirmatory serological tests to identify P. pseudomallei strains.

9. Iridescent lysis of Pseudomonas aeruginosa cultures.

In addition to pyocyanine, a quinolinol appeared to be a significant factor in production of iridescent lysis on agar lawns of Pseudomonas aeruginosa. Quinolinols appeared to be responsible for iridescence and perhaps pyocyanine production, whereas, pyocyanine was more important for lysis in susceptible cultures.

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 170 Zoonotic Diseases of Military Importance

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DOD'K INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71Q	00	171
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11. TITLE (Precede with Security Classification Code)

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19. RESPONSIBLE DOD ORGANIZATION

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(U) Biological Products; (U) Dengue Virus Vaccine: (U) Freeze-Drying; (U) Meningococcus Vaccine; (U) Typhoid Fever Vaccine; (U) Vaccines

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) This work unit is concerned with the development of manufacturing methods and the production of new effective vaccines for military use, and with the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

24. (U) Increased effectiveness and reduced reactivity are pursued by use of new physical and chemical methods for processing. Improvement in stability and reduction of logistic requirements are achieved by application of modern freeze-drying and packaging techniques.

25. (U) 73 07 - 74 06 - Investigations have continued on the development of new and improved biological products for military use. - 1. New lots of three candidate protein antigens, suitable for human use, were prepared from the B-11 strain of Neisseria meningitidis, for evaluation of their immunogenicity in man. 2. Two experimental freeze-dried living typhoid fever vaccines for oral administration, one from a streptomycin dependent strain and the other from an epimerase-less strain of Salmonella typhi, were prepared for human trials. 3. Studies have continued on the adaptation of dengue viruses to cell culture systems which are suitable for the preparation of vaccines for human use. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 171 Development of biological products

Investigators.

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Description.

This work unit is concerned with the development of manufacturing methods for the production of new effective vaccines for military use, and with the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

Progress.

1. Meningococcal Vaccine.

During the past year studies have continued on the development of pilot scale methods for the preparation of protein antigens from Neisseria meningitidis group B, for use in the immunization of man against this type of meningococcal meningitis.

Of the six protein antigens previously prepared in this laboratory, three looked very promising as immunogens in a mouse protection assay for the evaluation of potential group B meningococcal protein vaccines (Annual Report 1973). In order to obtain preparations for evaluation of immunogenicity in man, new lots of these three protein antigens, B-59-AG (Antigen I), B-59-TCA (Antigen II) and B-60-Soap (Antigen III), were prepared suitable for human use.

Some modifications and additions to the original procedures were made to facilitate the processing and improve the products. In the first two preparations the phenol-inactivated filtrates were

centrifuged immediately after adjustment to pH 4.5 with glacial acetic acid. Other modifications to the processing were as follows:

B-59-AG - The ethyl alcohol precipitate was collected, dissolved in alkaline water (pH 11.0), and the resulting solution was dialyzed against distilled water at 5 C. The dialyzed solution was then adjusted to pH 7.4 from pH 4.5 with 1 N NaOH.

B-59-TCA - The final TCA precipitate was suspended in distilled water, adjusted to pH 9.0 with 10 N NaOH, and dialyzed against distilled water at 5 C. The pH of the dialyzed solution was adjusted with 1 N NaOH from pH 4.9 to pH 7.4.

B-60-Soap - The dialyzed material was adjusted to pH 4.5 with 10% acetic acid and held at 5 C for 18 hours. A very stable emulsion formed and ethyl alcohol was added to a final concentration of 80% alcohol. The heavy precipitate was collected after standing at 5 C for 3 hours and then dissolved in water. The solution was adjusted to pH 7.4 from pH 6.2 with 1 N NaOH and dialyzed against distilled water at 5 C.

Each of the final dialyzed preparations (pH 7.4) was centrifuged at 100,000 x g for 1 hour. The sediments were discarded. The supernatants were filtered through 0.22 micron Millipore filters supported on either side by Millipore clarifying pads. Each filtrate was dispensed into vials (so that each vial contained two milligrams of protein) and was freeze-dried.

The final freeze-dried products were assayed for total solids and for protein, nucleic acid, sialic acid, and carbohydrate content. In addition, antigenicities were measured by gel diffusion (Ouchterlony) against seven immune rabbit sera. The results are recorded in Table I. Furthermore, in all preparations, a large portion of the total protein content was excluded in the void volume of a G-200 Sephadex column, indicating a molecular weight of 100,000 daltons or greater.

TABLE I

Summary of Assays on B-11 Protein Antigens

Assay	Method	Lot: B-59-AG	B-59-TCA	B-60-Soap
Total Solids, mg		7.38	8.30	24.9
Protein, mg	Lowry	1.51	2.58	1.52
Nucleic Acid, mg	U. V. Spectroscopy	0.38	3.54	0.43
Sialic Acid, mg	Svennerholm	0.15	0.20	15.08
Carbohydrate, mg	Tryptophane Reaction	0.15	0.76	2.76
Gel Diffusion #Positive/#Sera	Ouchterlony	7/7	5/7	3/7

The three protein preparations were tested for safety in mice (100 µg protein/dose) and in guinea pigs (500 µg protein/dose). All animals survived, appeared healthy and showed a weight gain at the end of the observation period, with one exception. The three guinea pigs inoculated with the 500 µg protein level of Antigen B-59-AG showed a weight loss averaging 29 grams. However, no weight loss was observed with the 250 µg level of this antigen.

The three antigens were also tested for pyrogenicity in rabbits. The protein level for each antigen at which no significant rise in temperature was observed over the 3 hour test period is shown in Table II.

TABLE II

Rabbit Pyrogenicity Test

Antigen	Maximum Pyrogen-Free Level (Protein (µg))
B-59-AG	0.0025
B-59-TCA	0.025 (0.25 is borderline)
B-60-Soap	0.025 (0.25 is borderline)

Finally, the protein antigen preparations were tested for immunogenicity by the mouse protection assay system (previously described in Annual Report 1973). The results are summarized in Table III.

TABLE III

Mouse Protection Activity of B-11 Protein Antigens

Antigen	ED_{50} (µg protein)	95% C.L. (µg protein)
B-59-AG	11.54	4.58 - 31.30
B-59-TCA	3.92	1.56 - 9.56
B-60-Soap	2.30	0.92 - 5.60

On the basis of the results obtained, the B-59-TCA and B-60-Soap preparations were considered candidate immunogens suitable for evaluation in man. These preparations, along with two other protein antigens prepared by CAPT Wendell D. Zollinger of the Dept. of Bacterial Diseases, are included in a proposal to test new meningococcal

protein vaccines in human volunteers, submitted to the Army Investigative Drug Review Board for approval by Dr. M. S. Artenstein, Dept. of Bacterial Diseases, WRAIR.

2. Typhoid Fever Vaccine.

During this year two experimental freeze-dried, living typhoid fever vaccines for oral administration were prepared for human trials to be conducted by the Div. of Infectious Diseases, University of Maryland School of Medicine. One vaccine was prepared as previously described (Annual Report 1973) using the streptomycin-dependent vaccine strain (27V) of Salmonella typhi, and the second vaccine was prepared using an epimerase-less strain of S. typhi Ty21a. Studies were also continued on the method of production of the 27V living vaccine with the goal of shortening the production procedure to a more practical time schedule. In addition, investigations were initiated on methods for producing a soluble, purified typhoid vaccine which might be less reactive than the current commercially available acetone killed and dried vaccine.

a. Investigations were continued on the development of an improved procedure for the pilot scale production of a stable high-titered living typhoid vaccine (Salmonella typhi strain 27V, streptomycin-dependent) for oral administration to humans. The procedure as outlined by Dr. M. Snyder of the University of Maryland School of Medicine (Annual Report 1973) required extended periods of incubation (48 hours on agar and 16 hours in broth) resulting in a prolonged production schedule.

Previous experimental results (Annual Report 1973) indicated that the inclusion of 250 μg of streptomycin in trypticase soy agar permitted a reduction from 48 to 24 hours in the incubation time required for plate counts of viable organisms. Accordingly, the effects of varying concentrations of streptomycin in the trypticase soy seed broth, and of the volume of inoculum transferred to the seed broth, on the growth of the 27V strain were examined. The results of these studies are shown in Table IV.

TABLE IV

Effect of Size of Inoculum, Streptomycin Concentration and Incubation Time on Growth of S. typhi strain 27V in Seed Broth

Concentration of Streptomycin in the Seed Broth	Volume of Inoculum	Incubation (Hours)			
		4	6	8	16
(µg/ml)	(ml)	(Viable count x 10^8)			
0	0.1	0.32	0.7	0.6	
	0.5	1.7	2.6	3.6	4.6
	1.0	3.2	4.6	8.1	
	2.0	6.1	7.1	5.6	6.6
10	0.1	0.43	1.2	2.1	
	0.5	2.0	3.3	5.0	6.1
	1.0	2.8	5.0	6.4	
	2.0	5.8	8.8	9.1	7.8
50	0.1	0.36	1.2	2.9	
	0.5	2.6	4.9	8.1	8.2
	1.0	3.7	8.5	8.9	
	2.0	6.9	7.6	11.2	9.4
250	0.1	0.6	3.1	7.7	
	0.5	3.9	6.9	10.1	8.8
	1.0	4.9	8.3	11.6	
	2.0	7.4	15.5	11.8	8.5
500	0.1	0.61	3.4	8.8	
	0.5	4.5	8.7	10.4	8.9
	1.0	4.7	9.8	11.7	
	2.0	10.8	10.6	12.2	9.8
1000	0.1	0.65	2.8	8.2	
	0.5	4.5	9.6	11.0	10.1
	1.0	5.5	9.4	12.3	
	2.0	8.4	12.9	11.8	10.5

From the results above it appears that the previously used incubation period of 16 hours in trypticase soy broth can be shortened by altering the concentration of streptomycin in the broth and the size of the inoculum. Maximum viable titers are obtained after 6 hours incubation when 2 ml of inoculum are transferred to seed broth containing 250 µg of streptomycin per ml.

The effect of an increased concentration of streptomycin in the broth and agar, and the effect of shortened incubation times on the number of viable organisms and on their survival through the freeze-drying procedure were also determined. A frozen seed culture was quick-thawed and streaked on trypticase soy agar containing 250 µg of streptomycin per ml. After 18 hours incubation, the growth on the agar surface was harvested in 5 ml of saline, and 100 ml of seed broth containing 250 µg of streptomycin were inoculated with 2 ml of the harvest. After incubation for 5 - 6 hours, 2 ml of seed broth culture were then transferred and distributed over the surface of trypticase soy agar (in Kolle flasks) containing 250 µg of streptomycin per ml of agar and the flasks were incubated at 36 C. After 15, 18, 21, 24 and 48 hours incubation, a representative number of Kolle flasks was harvested at each time period, and the pools distributed 10 ml per 50 ml vial, frozen and freeze-dried. The results of viable counts on the fluid and freeze-dried preparations are shown in Table V.

TABLE V

Viable Counts and Percent Recovery of Salmonella typhi strain 27V, Harvested and Freeze-Dried after Varying Incubation Times

Incubation Time on Agar	Viable Count ($\times 10^{10}$) Fluid Harvest	Freeze-Dried Product	Recovery
(Hours)			(%)
15	6.9	1.7	25
18	3.3	2.6	79
21	3.8	2.6	67
24	3.3	2.6	79
48	11.0	2.4	22

Although the viable counts in the fluid harvests were higher at 15 and 48 hours than at the intermediate incubation times, survival rates over the freeze-drying procedure for the organisms

harvested at intermediate times were significantly higher, resulting in final dried products at approximately the same number of viable organisms. The results definitely show that 48 hours incubation does not offer any advantages and that 18 - 24 hours incubation is sufficient.

The results of these and previous studies indicate, therefore, that the production time of attenuated vaccines prepared with this streptomycin-dependent strain of S. typhi can be reduced from 5 days to 3 days with no reduction in the number of viable organisms in the final freeze-dried product.

b. An epimerase-less strain of S. typhi (Ty21a) on blood agar was provided by Dr. M. Snyder of the University of Maryland School of Medicine. The major defect in this strain is the lack of the enzyme uridine-diphosphate-galactose-4 epimerase which is responsible for the conversion of galactose to lipopolysaccharide cell wall components. The strain is therefore a "rough" strain and is avirulent in experimental animals.

Colonies were picked from the blood agar plates and seeded on Brain Heart Infusion Agar (BHI) plates. After incubation at 36 C for 24 hours the growth on the plates was harvested in a sucrose-phosphate-glutamate solution, pH 7.2 (SPG). Human serum albumin (HSA) was then added to a concentration of 2% and the suspension was distributed 1 ml per 6 ml serum vial, and frozen at -70°C. This served as seed material for the production run.

A vial of frozen seed material was quick-thawed and the contents were seeded on BHI agar. After incubation at 36 C for 18 hours, a loopful of growth was transferred to 350 ml of BHI broth in a 1 L flask. This was incubated at 36 C with frequent shaking. After 6 hours, growth was well established and 2 ml of this seed culture was distributed over the agar surface in each of 150 Kolle flasks (60 ml of BHI agar/flask). After 18 hours incubation at 36 C, 10 ml of SPG solution were added to each Kolle flask, and the growth was removed by scraping and suction. The resulting harvests were pooled and HSA was added to 2.5%. The pool was immediately distributed 10 ml per 50 ml vial, frozen at -60°C and freeze-dried. The resulting freeze-dried vaccine contained 76×10^8 viable organisms per ml. Approximately 130 vials of this vaccine were provided to the University of Maryland School of Medicine for evaluation.

c. During this period studies were initiated on the application of various inactivating and purification procedures to fluid and agar grown cultures of S. typhi, strain Ty2. The initial

experiments with the fluid grown preparations were designed to determine the effectiveness of phenol, formalin and cetavlon as inactivating agents for rendering the cultures safe for further processing. The inactivating agents were added to 18 hour broth cultures, and samples were removed at various time periods, during which the cultures were held at 22 to 24 C. The results of plating these samples are shown in Table VI.

TABLE VI

The Rate of Inactivation of S. typhi, Ty2 Treated with Formalin, Phenol and Cetavlon

Time of Inactivation	Inactivating Agent 0.5% Phenol	0.1% Formalin	0.1% Cetavlon
		(Growth + or -)	
15 minutes	4+	4+	-
30 minutes	4+	4+	-
60 minutes	3+	4+	-
120 minutes	2+	4+	-
240 minutes	1+	-	-
24 hours	-		

Cetavlon completely inactivated the organisms within 15 minutes, formalin within 4 hours, and phenol within 24 hours. Each culture was then centrifuged and both the supernatant and sediments were extracted by various procedures. The resulting extracts are currently being characterized and will be compared with the commercially available acetone killed and dried typhoid vaccine in terms of immunogenicity, safety and toxicity.

3. Dengue Virus Vaccine.

During this period studies have continued on the adaptation of dengue virus type 2 to cell culture systems which are suitable for the preparation of vaccines for human use.

a. Passage of dengue virus type 2 (strain PR159) in a continuous line of fetal rhesus monkey lung (FRhL) cells (Wallace 1973) was continued. Five additional passages were made using the procedures previously described (Annual Report 1973). A summary of the results of the recent passages is presented in Table VII.

TABLE VII

Passages of Dengue 2 Virus (PR159) in FRhL Cells

Passage	Inoculum (0.5 ml)	Day of Harvest	Titer (PFU/0.2 ml)
5	2.5×10^6 PFU	3*	1.6×10^6
	(Pass 4-Day 5)	5	2.4×10^6
6	8.7×10^6 PFU	3*	1.2×10^6
	(Pass 5-Day 5)	5	2.1×10^6
7	1.1×10^6 PFU	4*	3.1×10^6
	(Pass 6-Day 5)	6	5.3×10^6
8	1.8×10^7 PFU	3*	4.2×10^6
	(Pass 7-Day 6)	5	1.2×10^6
9	4.2×10^6 PFU**	3*	3.0×10^4
	(Pass 8-Day 3)	5	1.5×10^6

* Culture fluids harvested and cell sheets refed with MEM+2% FBS.
** Volume of inoculum for this passage was 0.2 ml.

A small lot of pre-seed material was made from the passage 8 harvests. At the passage 9 level a seed lot was prepared by the following method. Fluids from $25cm_2$ flasks were removed from confluent monolayers, the cell sheets were washed with HBSS and then each flask was inoculated with 0.2 ml of the passage 8 pre-seed material. After an adsorption period of approximately 2 hours the inocula were removed and each cell sheet was washed three times with 5 ml of HBSS. The flasks were refed with 5 ml of maintenance medium which consisted of the following:

Minimum Essential Medium (Eagle)
0.25% Human Serum Albumin
1.0% Glutamine
0.1% Streptomycin and Neomycin

The fluids were harvested on day 3 and the cells were refed with the same medium as above. On day 5 the fluids were harvested again and mixed with an equal volume of HSA which had been previously adjusted to pH 7.5 with 1 M NaOH. Aliquots were dispensed into glass sealed ampules and stored at -70 C. This seed virus will be used for studies on the development of a vaccine for human use.

It was also of interest to determine if adaptation and/or attenuation had taken place during the passage of the virus in FRhL cells. Therefore, passage 3 and passage 6 materials were compared in terms of replication in FRhL cells at a high and a low multiplicity of infection (MOI), and in terms of mouse neurovirulence. The results are presented in Tables VIII and IX.

TABLE VIII

Growth Curves of Dengue Virus (PR159) in FRhL Cells

		Passage 3		Passage 6	
Inoculum:		2.7×10^6 PFU	270 PFU	2.9×10^6 PFU	290 PFU
Day	1	6.1×10^3*	0	3.8×10^4	0
	2	1.4×10^5	3.5×10^1	4.5×10^3	3.0×10^1
	3	9.8×10^6	7.3×10^3	6.1×10^5	6.2×10^3
	4	7.7×10^6	2.8×10^4	9.2×10^6	4.5×10^4
	5	3.3×10^6	Acid-no titer	4.5×10^6	8.6×10^4
	6	1.8×10^6	1.4×10^5	3.6×10^6	9.4×10^4
	7	1.0×10^6	8.4×10^4	1.9×10^6	7.3×10^4
	8	5.9×10^6	5.7×10^4	9.6×10^5	4.6×10^4

* PFU/0.2 ml

TABLE IX

Inoculation of Suckling Mice with Dengue Virus (PR159)

Dilution	Passage 3	Passage 6
Undil.	7/7*	7/7
10^{-1}	7/7	7/7
10^{-2}	7/7	7/7
10^{-3}	7/7	7/7
10^{-4}	6/7	3/7
10^{-5}	5/7	4/7
10^{-6}	2/7	0/7
10^{-7}	0/7	0/7
	$LD_{50}=10^{-5.4}$/0.02 ml	$LD_{50}=10^{-4.5}$/0.02 ml

Each mouse was inoculated IC with 0.02 ml.
* No. of deaths/total inoculated.

From the above results, it does not appear that adaptation has taken place, since the passage 3 material is comparable to the passage 6 material at both high and low multiplicities of infection. The passage 6 material seems to be slightly less neurovirulent for mice, but more data will be necessary to confirm this observation.

b. Dengue virus type 2 (PR159) which had been previously passaged once in certified primary African green monkey cells was also passaged in FRhL cells. Table X shows the results of the passages.

TABLE X

Passages of Dengue 2 Virus (PR159) in FRhL Cells

INOCULUM: GM/1/96 hr - 1.7×10^3 PFU*	
FRhL/1/Day 7**	2.2×10^1
FRhL/1/Day 14**-7	1.3×10^2
FRhL/1/Day 1°-14-7	2.4×10^2

INOCULUM: FRhL/1/Day 7		INOCULUM: FRhL/1/Day 14-7		INOCULUM: FRhL/1/Day 19-14-7	
FRhL/2/Day 7**	0	FRhL/2/Day 7**	1.3×10^1	FRhL/2/Day 4**	1.1×10^1
FRhL/2/Day 14**-7	1.5×10^1	FRhL/2/Day 14**-7	2.6×10^2	FRhL/2/Day 7**-4	1.6×10^2
FRhL/2/Day 18-14-7	5.4×10^2	FRhL/2/Day 18-14-7	2.4×10^3	FRhL/2/Day 14**-7-4	2.7×10^2
				FRhL/2/Day 18-14-7-4	4.4×10^3

INOCULUM: FRhL/2/Day 18-14-7-4					
FRhL/3/Day 4**	5.1×10^2	FRhL/3/Day 7**	2.7×10^3	FRhL/3/Day 10**	2.2×10^3
FRhL/3/Day 12-4	3.6×10^4	FRhL/3/Day 12**-7	2.4×10^5	FRhL/3/Day 17-10	1.7×10^5
		FRhL/3/Day 14-12-7	1.5×10^5		

* Volume of inoculum was 0.5 ml; harvest titers in terms of PFU/0.2 ml.
** Culture fluids harvested and cell sheets refed with MEM+2% FBS.

The FRhL/3/day 14-12-7 material was found to consist entirely of small plaques in LLC-MK_2 cells, was temperature sensitive, had a lower mouse neurovirulence and produced no viremia but good antibody responses in primates. This virus is being passaged further to determine its stability in FRhL cells.

This small plaque variant appears to be an ideal candidate for an attenuated vaccine. Therefore, the passage procedure will be repeated in FRhL cells certified to be suitable for the preparation of vaccines for human use.

c. Studies were also initiated to determine whether dengue 2 virus (PR159) can be induced to replicate in other types of cells by applying the same passage procedures. WI-38, primary dog kidney and chick fibroblast cells were inoculated with dengue 2 virus that had been previously passaged in FRhL cells. Tables XI, XII, and XIII show the results of these passages to date.

TABLE XI

Passages of Dengue 2 Virus (PR159) in WI-38 Cells

Inoculum:	FRhL/1/Day 14-8	1.1×10^3 PFU*
	WI-38/1/Day 7**	1.0×10^1
	WI-38/1/Day 12-7	8.5×10^2
Inoculum:	WI-38/1/Day 12-7	
	WI-38/2/Day 8**	8.6×10^3
	WI-38/2/Day 11-8	3.7×10^5
Inoculum:	WI-38/2/Day 11-8	
	WI-38/3/Day 3**	5.1×10^4
	WI-38/3/Day 5	1.6×10^5
Inoculum:	WI-38/3/Day 5	
	WI-38/4/Day 4	1.4×10^5

* Volume of inoculum was 0.5 ml; harvest titers in terms of PFU/0.2 ml.

** Culture fluids harvested and cell sheets refed with MEM+2% FBS.

TABLE XII

Passage of Dengue 2 Virus (PR159) in Primary Dog Kidney Cells

Inoculum: FRhL/7/Day 6-4	1.4×10^7 PFU*
DK/1/Day 7**	4.3×10^2
DK/1/Day 13**-7	1.3×10^2
DK/1/Day 20**-13-7	2.1×10^2
DK/1/Day 27-20-7	3.2×10^2

* Volume of inoculum was 0.5 ml; harvest titers in terms of PFU/0.2 ml.

** Culture fluids harvested and cell sheets refed with MEM+2% FBS.

TABLE XIII

Passage of Dengue 2 Virus (PR159) in Chick Fibroblast Cells

Inoculum: FRhL/7/Day 4	3.7×10^6 PFU*
Chick/1/Day 5**	0
Chick/1/Day 12**-5	0
Chick/1/Day 19**-12-5	0
Chick/1/Day 26-19-12-5	0

* Volume of inoculum was 0.5 ml; harvest titers in terms of PFU/0.2 ml.

** Culture fluids harvested and cell sheets refed with MEM+2% FBS.

As can be seen from the above tables, the dengue 2 virus showed evidence of replication in the WI-38 cells, and the virus persisted during the first passage in primary dog kidney cells. The chick fibroblast cells appeared to be refractory to the dengue 2 virus. Further studies will be done with WI-38 cells and dog kidney cells in order to determine if these cells are suitable hosts for dengue vaccine production.

d. It was also of interest to determine if dengue 3 virus (strain CH3489) would replicate in FRhL cells. Using the procedure described above, dengue 3 virus which had been passaged once in

certified primary African green monkey cells was used for further passages in FRhL cells. Table XIV represents a summary of the results of these passages.

The results indicate that dengue 3 virus does replicate in FRhL cells. Further work with this virus will be carried out in certified cells.

TABLE XIV

Passages of Dengue 3 Virus (CH53489) in FRhL Cells

Inoculum: Dengue-3/1/Day 10, Undiluted: $5x10^4$PFU*			
FRhL/1/Day 6**	$6.7x10^2$	FRhL/1/Day 10**	$1.0x10^1$
FRhL/1/Day 13**-6	$1.0x10^1$	FRhL/1/Day 16**-10	$4.0x10^3$
FRhL/1/Day 20**-13-6	$3.1x10^3$	FRhL/1/Day 23**-16-10	$1.9x10^3$
FRhL/1/Day 27**-20-13-6	$5.4x10^3$	FRhL/1/Day 31**-23-16-10	$7.5x10^3$
FRhL/1/Day 35-27-20-13-6	$4.1x10^3$	FRhL/1/Day 38-31-23-16-10	$1.5x10^4$
Inoculum: FRhL/1/Day 20-13-6: $7.7x10^3$PFU*			
FRhL/2/Day 6**	$3.8x10^3$	FRhL/2/Day 10**	$1.4x10^3$
FRhL/2/Day 14**-6	$4.3x10^3$	FRhL/2/Day 17**-10	$3.4x10^3$
FRhL/2/Day 20**-14-6	$6.7x10^3$	FRhL/2/Day 23**-17-10	$8.7x10^3$
FRhL/2/Day 27-20-14-6	$8.1x10^3$	FRhL/2/Day 27-23-17-10	$4.4x10^3$

* Volume of inoculum was 0.5 ml; harvest titers in terms of PFU/0.2 ml.
** Culture fluids harvested and cell sheets refed with MEM+2% FBS.

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 171 Development of biological products

Literature Cited.

References.

1. Wallace, R. E., Vasington, P. J., Petricciani, J. C., Hopps, H. E., Lorenz, D. E. and Kadanka, Z.: Development of a diploid cell line from fetal rhesus monkey lung for virus vaccine production. In Vitro 8:323-332, 1973.

Publications.

1. Altieri, P. L., Berman, S. and Lowenthal, J. P.: Neutralization of Formalin in Biological Products. Developments in Biological Standardization, vol. 24 - Preservatives in Biological Products, 71-76, 1973.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6449	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8A. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D.CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71Q	00	172
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114(F)			

11. TITLE (Precede with Security Classification Code)

(U) Immunological Mechanisms in Microbial Infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 003400 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	3	120
FISCAL YEAR CURRENT 75	5.1	120

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Infectious Diseases; (U) Immunity; (U) Antibodies; (U) Complement Fixation; (U) Radioimmunoassay; (U) Serodiagnosis

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) The objective of this work unit is to elucidate the mechanisms operative in the natural and artificial induction of immunity to a variety of microbial infections of military importance. This includes the study of infections in model systems and the development of methodologies for the study of the immune reaction in humans for research as well as diagnostic evaluations.

24 (U) The approaches used for these studies involve the measurement of various parameters of disease and of the immune response to disease both in in vivo and in vitro experiments. A variety of diseases are attacked with current emphasis on schistosomiasis and hepatitis. Immunological phenomena common to a variety of different diseases are also studied.

25 (U) 73 07 - 74 06 New methodology for the isolation, purification, and analysis of hepatitis associated antigen have been developed. A radioimmunoassay system has been devised for the measurement of antibodies to African trypanosomes and is being adapted to the study of other infectious diseases. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1973 to 30 June 1974.

* Available to contractors upon originator's approval

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 172 Immunological mechanisms in microbial infections

Investigators.
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Effort in this work unit during the preceding year was divided between service and research. The service function consists of the performance of diagnostic tests in support of patient care or research projects reported in other work units. These services are summarized in Table 1. Research activity was directed towards an increased understanding of the immunology of infectious disease through the development and exploitation of improved assay procedures and diagnostic tests. These research projects are described below.

1. Radioimmunoassay for detection of class-specific antibody.

Objective. These studies were designed to develop and evaluate a radioimmunoassay (RIA) for the quantitation of IgG or IgM antibodies against particulate or soluble antigens. The advantage is that quantitation in milligram amounts of antibody directed against a particular antigen is possible. Using the proper reagents, the amount of antibody belonging to a particular immunological class can be determined.

Description. Radioimmunoassay techniques began receiving widespread attention after the observations of Berson and Yalow (1) that radiolabeled insulin could be bound by low concentrations of antibodies to that hormone. Unknown concentrations of insulin could be determined because the unlabeled hormone would compete with the labeled hormone for sites on the antibody.

Radioimmunoassay procedures have been developed most extensively for the detection of hormones, but have also been applied to measurement of other substances including drugs, pathological agents, viral proteins and enzymes. RIA has also been used to quantitate total immunoglobulin levels (2,3) In addition to total immunoglobulin quantitation, RIA procedures have been used to detect antibodies to rabies (4), schistosomiasis (5), ragweed antigen E (6), KLH (7) and other antigens. These assays, although using radiolabeled antigens or antibodies, do not use the competition type assay leading to class specific antibody quantitation and many do not lend themselves to ease of routine performance necessary for diagnostic purposes.

Table 1

NUMBER OF SPECIMENS TESTED BY DIAGNOSTIC SERVICE, DEPARTMENT OF IMMUNOLOGY, 1 JUNE 73 - 31 MAY 74

	COMPLEMENT FIXATION TESTS[5]						FLOCCULATION TESTS		
	Viruses	Fungi[1]	Protozoa	Helminths	DNA[2] Nucleo-protein	SYPHILIS[3] Serology	Amebiasis[4] IHA	Helminths	Rheumatoid Factor
WRGH	---		101	18	1192	1574	99	18	259
WRAIR	2004		1	205	----	----	--	2	---
Military Installation DC Area	----		1	14	383	219	118	14	---
Military Installations Outside DC Area	----		3	20	2267	116	87	20	---
Other	----		---	60	870	---	11	60	---
Total	2004	2506	106	317	4712	1909	325	114	259

[1]Tests performed with four fungal antigens; gel diffusion tests also performed on all sera.

[2]Sera tested with both antigens.

[3]Treponema pallidum immobilization test and fluorescent treponemal antibody tests; includes serum and spinal fluid.

[4]Indirect hemagglutination tests for amebiasis.

[5]A total of 1393 serum complement titrations were also performed.

a) RIA for class-specific detection of antibody directed against whole Trypanosoma rhodesiense organisms.

Progress. The ability to quantitate IgG or IgM is based on the principles used in many of the hormone assays. Radiolabeled IgG can be inhibited from binding to a limited amount of anti human IgG by addition of standard or unknown amounts of unlabeled IgG. Thus, a standard curve can be established for quantitation of unknown immunoglobulin concentration.

The method used for separation of free labeled IgG from antibody-bound labeled IgG is the membrane filtration technique of Borella (8). Membrane filters with a pore size of 0.22 μ, are used to separate ^{125}I-IgG from ^{125}I-IgG -antibody complexes. The radioactivity of the membrane after filtration is an indication of the amount of radiolabeled antigen, either IgG or IgM, that is bound to a standard amount of antibody in each reaction mixture.

Replacement of labeled IgG or IgM by unlabeled immunoglobulin lowers the radioactivity trapped on the membrane and allows a standard curve to be constructed. The amount of IgG or IgM in an unknown solution can be determined by reading the percent radioactivity bound on the standard curve versus the amount of standard IgG or IgM which gives that percentage of binding of the radiolabeled IgG or IgM. A standard IgG curve is shown in Figure 1.

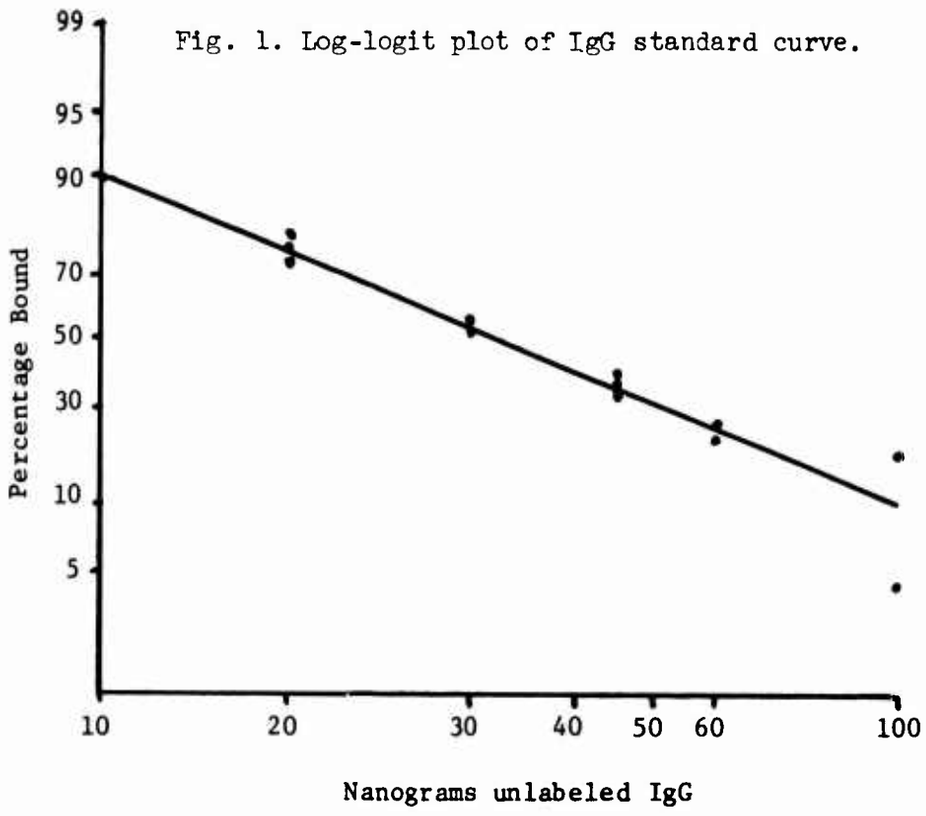

Fig. 1. Log-logit plot of IgG standard curve.

To measure specific antibody, such as antibody to a trypanosome organism the antibody must be isolated from other immunoglobulins found in the serum. After isolation, it can be quantitated through the immunoglobulin class-specific assay just described. The procedure used for class-specific quantitation of an antibody to T. rhodesiense is shown in Table 2.

In order to quantitate immunoglobulin according to class, several criteria for the reagents must be met. No cross reactions between the IgM and IgG antigen and antibodies used were detectable by immunodiffusion. Table 3 shows the inhibition of each reaction with non-specific immunoglobulin reagents. IgM reagents are approximately 100 times more sensitive for detection of IgM than for IgG. IgG reagents on the other hand are less than 50 times as sensitive for IgG as for IgM. Another criteria is sensitivity of the reaction. The sensitivity of the reagents was demonstrated by the ability of the IgM reagents to detect 4 nanograms of IgM, the standard curve having a range of 4-40 nanograms. IgG could be detected at a concentration as low as 10 nanograms with a range of the standard curve between 10 and 200 nanograms. Table 4 shows the precision of the determination for standards run in different assays. These amounts were determined in separate experiments with a standard curve run at the same time. The standard curves are parallel from one experiment to the next; however, they are not always superimposable, indicating that a standard curve should be set up each time the assay is run.

Table 5 shows that the total IgG antibody appears lower if too much (1/200) antibody is used in the assay. Excess antibody is lost during washing of the antibody-trypanosomes complex and causes an erroneously low final measurement once the dilution of serum is considered.

Non-antibody immunoglobulin must not be bound to the trypanosome antigen or it would be detected as specific antibody. When normal serum at a 1/200 dilution was used no IgG could be detected on the washed trypanosome antigen.

The assay was used to measure the IgG and IgM antibody response in Rhesus monkeys infected with 10,000 T. rhodesiense organisms. The antibody amounts are shown on Table 6. The values are the average of antibody determination at two dilutions of antibody. Each dilution was run in triplicate and compared to a standard curve performed at the same time as the antibody assay. No antibody was detected in uninfected rhesus sera.

Table 2

Quantitative Determination of Rhesus Immunoglobulin Class-Specific Antibodies to T. rhodesiense

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Part A. Binding of antibody (anti-trypanosome) to antigen (glutaraldehyde fixed trypanosomes).

1. 0.1 ml of varying dilutions of rhesus sera (infected with T. rhodesiense were added to a pellet of 3.5×10^8 trypanosome organisms.
2. The mixture was shaken at 37°C for 1 hour.
3. The mixture was centrifuged at 770G for 10 minutes. The supernatant was removed and the pellet washed 5 times with 3 ml of PBS containing 1% rabbit serum (NRS).

Part B. Binding of goat anti-human immunoglobulin (e.g. anti-human IgG or IgM) to the antigen-antibody complex (trypanosome-anti-trypanosome).

4. 0.4 ml of a standard dilution[a] of goat anti-human IgG or IgM was added to the washed pellet of antigen-antibody complex (trypanosome-anti-trypanosome) from step 3.
5. The mixtures were shaken at 37°C for one hour and then placed at 4°C for an additional 23 hours.
6. The mixtures were centrifuged at 770G for 10 minutes and decanted into a new set of tubes.

Part C. Indirect measurement of immunoglobulin class-specific antibodies (e.g. IgG or IgM antibodies) bound to trypanosome antigen.

7. 0.2 ml of a standard amount[b] of ^{125}I-IgG or ^{125}I-IgM was added to the mixtures in each of the tubes. Controls included:
   a) 0.2 ml of standard amount of labeled IgG or IgM and 0.4 ml 1% NRS PBS.
   b) 0.2 ml of each standard labeled antigen and 0.4 ml standard dilution of each antibody (goat anti-human IgG or IgM).
   c) The standard curve was set up in step 4 by addition of 1-10 lambda of appropriately diluted standard unlabeled antigens (IgG or IgM) to tubes containing trypanosome antigen and 0.4 ml standard dilution of antisera (anti-IgG or anti-IgM).
8. The mixtures were shaken at 37°C for 1 hour and then incubated at 4°C for 18 hours.
9. The mixtures were filtered through 0.22 μ Millipore filters previously soaked in 10% NRS-PBS. The filters were held on a 30 place filter manifold and counted in polyethylene tubes on a Beckman Biogamma system.

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[a]That dilution of antiserum at which 0.4 ml will bind 50% of the standard homologous labeled antigen.

[b]The standard amounts of radiolabeled immunoglobulins of each class were 3.6 μg of ^{125}I-IgG/0.2 ml and 0.1 μg of ^{125}I-Igm/0.2 ml.

Table 3

Specificity of IgG and IgM Reagents

μg Std. Added	% Binding of ^{125}I-IgM	Apparent μg Value on:
		IgM Std. Curve
0.01 IgM	65	.0096
0.01 IgG	94	----
0.1 IgG	90	----
1.0 IgG	77	.0055
	% of ^{125}I-IgG	IgG Std. Curve
0.02 IgG	68	.021
0.02 IgM	100	---
0.1 IgM	85	.012
1.0 IgM	4	>.2 μg

Table 4

Reproducibility of IgG and IgM Determinations

μg Added	Apparent μg Determined from Standard Curve	Mean in μg	Standard Deviation
IgG			
.06	.066, .06, .05	.059	.007
.02	.021, .019, .024, .03	.024	.004
IgM			
.01	.0076, .013, .01	.010	.002
.02	.011, .016, .02	.016	.004

Table 5

Milligrams Anti-trypanosome IgG Calculated

Serum Dilution	μg IgG/0.1 ml	IgG (mg/dl) Whole Serum
1/200	.105	21
1/400	.079	32
1/800	.035	28

Table 6

IgG and IgM Antibody Response of Rhesus Monkeys Infected with T. rhodesiense

Monkey Number		Days after Inoculation 10	20	35
1	IgG	5.2[a]	9.6	7.0
	IgM	8.6	4.7	6.2
2	IgG	2.9	12.4	9.3
	IgM	7.8	<1	<1
3	IgG	7.4	15.5	16.4
	IgM	14.2	9.8	9.2

[a] Mg/dl IgM or IgG antibody

Discussion. In evaluation of the class-specific antibody assay, several criteria must be considered. Three critical points exist for the theoretical function of the assay. These are: (a) all the antibody (anti-trypanosome) must be adsorbed; (b) non-antibody immunoglobulins must be removed; and (c) the antigen (trypanosome) antibody complex must be sedimentable. Using the glutaraldehyde-fixed whole trypanosome antigen, these points seem to be met. The serum dilution which must be used in order to fall on the standard curve is greater than 1/100. This is advantageous since dilution of the non-antibody immunoglobulin which at higher concentration may be detected on the trypanosome antigen is also accomplished. If sera with lower concentrations of antibody were tested, non-antibody immunoglobulin might be detected on the trypanosome antigen. A standard dilution of sera would have to be chosen to prevent detection of non-antibody immunoglobulins as antibody. The fixed trypanosome antigen is easily sedimentable and washable even though extensive washing is time consuming.

The indicator system (quantitation of IgG or IgM) meets technical standards required of a diagnostic assay. Using the Millipore filtration manifold, several samples can be rapidly processed. The other alternative to this separation procedure would be the double antibody method (9). The two separate overnight incubation steps are perhaps the least desirable points in the assay. Perhaps these times may be shortened by varying incubation conditions.

The antibody values determined on infected monkey sera show only slight variations between sera, though general trends are apparent. IgM antibody declines throughout the course of infection, while IgG antibody is on the increase. The ability to measure class specific antibodies is present. Through more purified reagents and further descriptions of the precision and accuracy of the technique by statistical means, this procedure should provide a valuable tool to diagnosis and quantitative study of trypanosomiasis.

Several avenues of approach are available for future studies. The available technology of the quantitative immunoglobulin indicator system can easily be applied to other antigens of interest. Further characterization of the system can be accomplished through the use of an antigen such as sheep erythrocytes which is more readily available than trypanosome organisms. Furthermore, the technique lends itself to automated techniques at many steps. Automated steps in the procedure will be advantageous for use in epidemiological or diagnostic studies.

b) Quantitative immunoglobulin class-specific antibody assay for use with soluble antigens.

The immunoglobulin indicator system described above can be used to quantitate antibodies against a soluble antigen as well as against whole trypanosome organisms. The only additional requirement is that of a solid-matrix for attachment of the soluble antigen so that antigen specific antibody can be separated from non-antibody immunoglobulin.

The antigen chosen for study was bovine serum albumin (BSA). Porous glass beads from Corning Glass Works were chosen as the solid matrix for retention of the BSA because of their large surface area available for interaction between antibody and antigen. This would fulfill the criteria of antigen (BSA) excess as discussed above. BSA was attached to the glass beads by adsorption or glutaraldehyde fixation. After incubation of the antigen coated beads with varying dilutions of normal rhesus sera or rhesus anti-BSA sera, the beads were washed and used in place of antibody coated trypanosomes in the IgG RIA assay as described above.

In several experiments, IgG was detected on the beads, however little difference was noted between normal sera and sera containing antibody against BSA. This could mean that little BSA was actually attached to the beads although experiments using ^{125}I-BSA showed retention of BSA after several washings. Because of the binding of normal IgG to the beads, low dilutions of sera could not be used. Experiments using various concentrations of rabbit serum, or Tween 80 in the buffer were only partially successful in blocking the adherence of non-antibody IgG to the BSA coated beads, even at 1/100 dilution of whole serum. Thus, the glass beads do not meet this requirement for an antibody determination by RIA.

A second type of solid support for soluble antigen was studied. Cellulose acetate discs were used as a matrix for attachment of BSA according to procedures used for the soluble antigen fluorescent antibody test (10).

Using air drying followed by 95% ethanol and 1% acetic acid treatment, BSA can be attached to the discs with little removal after washing. Differences in amount of IgG bound to the discs were noted between normal sera and anti-BSA sera. Two percent Tween 80 in phosphate buffered saline prevented non-antibody IgG from attaching to the discs with serum dilutions as low as 1/20. The cellulose acetate solid support has many advantages for their use in the RIA system. They are: 1) ease of handling; 2) retention of soluble antigen and 3) ease of removal of non-antibody immunoglobulin. Further studies will determine if the lack of surface area as compared to porous glass beads causes any difficulties in obtaining antigen excess for any soluble antigens tested.

2. Removal of Hepatitis B antigen from protein solutions in a recognizable form by an insoluble lipophilic matrix of silicic acid.

Hepatitis B antigen (HB-Ag) was originally discovered as the result of a search for unique lipid containing proteins and the detection of antibodies directed against them (11). Since that time, several investigators have demonstrated the lipid staining characteristics of HB-Ag (12, 13). Gerin (14) using the detergents sodium deoxycholate and Tween 80, was able to alter the bouyant density of HB-Ag without destroying its complement fixing antigenic properties. This study

utilizes the lipid like properties of the antigen to remove it from protein containing solutions in a recognizable form, by binding it to an insoluble lipophilic matrix of silicic acid.

Materials and Methods

Chemicals. Silicic acid in an insolubilized form was purchased as "Liposorb" from Custom Reagents Laboratory, Inc., San Diego, California 92111. Plasdone-C, (P.V.P.) was purchased from General Aniline and Film Corporation, Calvert City, Kentucky.

HB-Ag Antigen and HB-Ag Antibodies. Plasma obtained from patients with serum hepatitis were used in all experiments. The immunoelectrophoresis titers ranged from 1:4 to 1:16. The tagged antiserum used in this study was the commercially available Abbott Ausria I^{125} anti HB-Ag. The "cold" anti HB-Ag (R933) had an antibody titer of 1:4 in IEOP.

HB-Ag Absorption. The absorption of HB-Ag from 0.1 ml aliquots of IEOP positive plasma was done at room temperature (25°C) using 10 mgs. of silicic acid in 5 ml of phosphate buffered saline (pH 7.4). Variations of absorption times and the amounts of silicic acid were used to define certain parameters and are explained in the text. After binding of HB-Ag to silicic acid any potentially unoccupied silicic acid sites were blocked by the further addition of 1 ml of pooled normal serum. One hour later the tubes were centrifuged at a relative centrifugal force of 1873 and washed once with phosphate buffered saline. Following a five minute recentrifugation, the pellets were resuspended in 0.5 ml of plasdone-C and 0.1 ml of tagged anti HB-Ag was added. The final incubation lasts one hour. Three washings with phosphate buffered saline were done prior to counting.

Results

The absorption phenomenon by which HB-Ag was removed from solution has been demonstrated in two ways. The first was by an indirect assay in which identical aliquots of a representative sample of HB-Ag plasma, positive by counter immunoelectrophoresis were absorbed with varying concentrations of silicic acid. The supernatants following centrifugation were tested for the presence or absence of HB-Ag (Fig. 2). At a concentration of 5 to 10 milligrams of silicic acid per 0.1 milliliter of plasma recognizable HB-Ag was no longer present in the supernatant.

Since HB-Ag was no longer present in the previously positive supernatant, it seemed reasonable that it would be found in the insoluble lipophilic pellet of silicic acid. In addition, if indeed the lipid component of HB-Ag was an antigenically silent moiety as suggested by Gerin's work (14), the HB-Ag bound to the lipophilic matrix would be serologically recognizable.

Direct evidence for this was obtained (Fig. 3). The binding of HB-Ag to the insoluble matrix of silicic acid takes place very rapidly

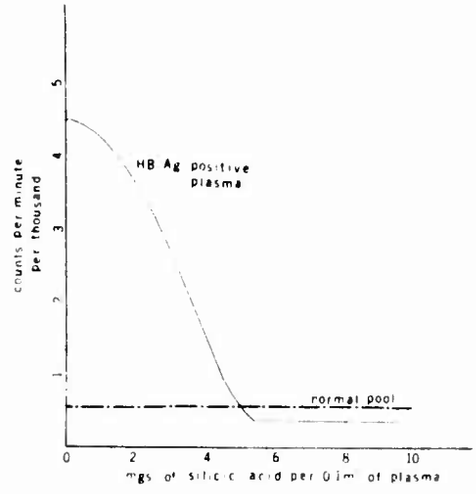

Fig. 2

Absorption of HB-Ag from Plasma. HB-Ag plasma, positive by counter-immunoelectrophoresis, was absorbed at 25°C on varying amounts of silicic acid. After four hours, the aliquots were pelletted at a relative centrifugal force of 1677 and the supernatants examined for HB-Ag by the standard Abbott-Ausria-125 radioimmune assay. Each point was done in triplicate and the normal control pool was made up from 9 normal individuals.

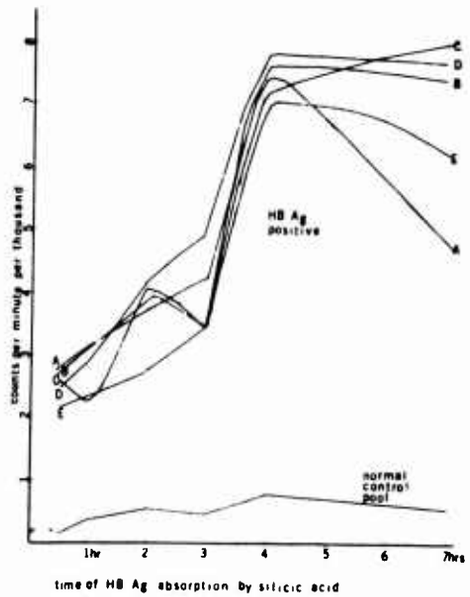

Fig. 3

Direct assay of HB-Ag absorption on silicic acid as influenced by time. One-tenth milliliter aliquots of five HB-Ag positive plasma (A-E, previously subtyped as adw and ayw were absorbed at 25°C on 10 milligrams of silicic acid for varying periods of time. At the end of each period, the absorption mixture was spun at a relative centrifugal force of 1873, resuspended in phosphate buffered saline and washed three time. After the final wash, the pellet was resuspended in 1% Plasdone-C (Polyvinylpyrrolidine) in phosphate buffered saline. This was found to limit non-specific binding of protein to the matrix. Then a fixed amount of radio-actively tagged anti HB-Ag was added to each tube. Following a one hour antibody incubation period, the suspension was centrifuged and the pellet wasned three times with phosphate buffered saline prior to counting.

at 25°C. Within thirty minutes the average of the counts for the HB-Ag positive plasma were twenty-one times the normal control values, or a difference of two hundred and twenty standard deviations above the normal mean. The average differences between HB-Ag positive plasma and the normal controls progressively increase up to four hours at which time absorption appears complete.

Once the silicic acid-HB-Ag bond has been established, the recognition of the bound antigen by specific antibody was very rapid and essentially complete within thirty minutes. No significant increase was observed with further incubation (Fig. 4).

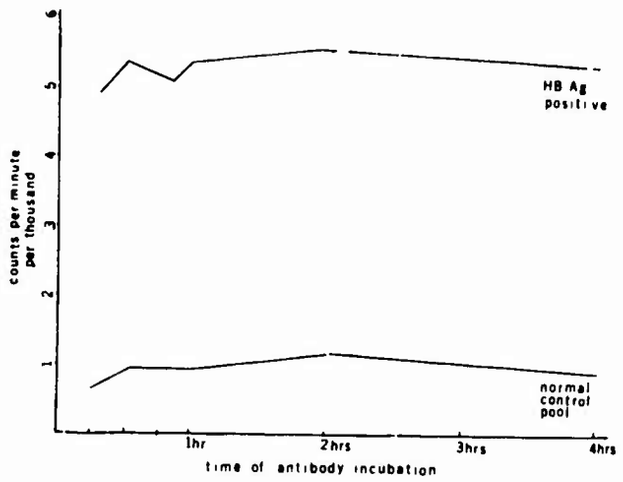

Fig. 4

Effect of time upon antibody recognition. One-tenth milliliter aliquots of different plasma from three different HB-Ag positive patients were absorbed on ten milligrams of silicic acid for four hours at 25°C. The subsequent antibody incubation and washings were identical to those outlined in Fig. 2. Each point represents the average of the individual determinations done on three separate HB-Ag positive patients for each specific time interval. The variable in this experiment was the time of antibody incubation.

The stability of the bond between HB-Ag and the lipophilic matrix was demonstrated by the final set of experiments. One-tenth milliliter aliquots of an HB-Ag (subtype ayw) positive plasma were absorbed on 10 milligrams of silicic acid for four hours at 25°C. Three replicate groups were set up and each group subjected to a different treatment. Following HB-Ag absorption, the first group was simply washed three times with phosphate buffered saline. The second group was incubated for thirty minutes with 3 molar potassium iodide and then washed three times in the same 3 molar potassium iodide solution. The third group

was incubated with 0.2 ml of "cold" anti HB-Ag for 2 hours and then washed with phosphate buffered saline prior to the addition of "hot" tagged anti-HB-Ag. The fourth group is really the third group which has had the "cold" blocking anti HB-Ag stripped from it after it was counted by pH 1.8 glycine HCl buffer. After these basic manipulations all the tubes were re-washed with phosphate buffered saline and restored to a pH of 7.2 - 7.4 and isotonicity. At this point, the final pellets were resuspended in 1% Plasdone-C (polyvinylpyrrolidine) and tagged anti-HB-Ag was added to each tube. Aliquots taken from a plasma pool of nine normal individuals were subjected to the same set of conditions and used as the normal controls (see Table 7).

Table 7

Stability of HB-Ag Binding to Silicic Acid

		(Standard Deviations above the control mean)
GROUP 1	Untreated	103
GROUP 2	Pretreated with 3M KI	99.4
GROUP 3	Blocked with cold anti-HB-Ag	0.7
GROUP 4	Group 3 from which cold blocking anti HB-Ag has been stripped* and reincubated with hot tagged HB-Ag	106

*After counting.

It is apparent from the data in Table 7 that neither pre-treatment with 3 molar KI or post absorption treatment with pH 1.8 glycine-HCl disrupts the bond between the silicic acid matrix and the HB-Ag. The antigen-antibody bond, however, can be disrupted and indeed in going from group 3 to group 4 cold antibody is stripped away and tagged antibody reapplied to the stable antigen which remains solidly bound to the silicic acid matrix.

Discussion

Silicic acid is a selective but not exclusive absorber of lipids and lipoproteins. Following the absorption of HB-Ag from plasma, the potentially unoccupied silicic acid sites must be saturated by the addition of an excess of lipoproteins supplied in the form of normal serum. After that, "tagged" specific anti-HB-Ag can be used to identify the bound HB-Ag. The ability of the absorption bond between HB-Ag and

silicic acid to withstand acid elution treatment allows an in built specificity control check to be done on the same specimen as illustrated in Table 7.

The present state of the art does not allow any correlations to be drawn between infectivity and the reduction of HB-Ag content of solutions as reflected by radioimmune assay or by any other assay for that matter. Nonetheless, this paper provides direct and indirect evidence of the absorption of HB-Ag from protein containing solutions in a recognizable and manipulable form. At present, HB-Ag degradation studies are in progress and the system is being adapted to other lipid containing antigen systems.

3. Isolation and assay of Hepatitis B antigen from urine.

Hepatitis B antigen (HB-Ag) was discovered by Blumberg (11) in 1965. Six years later, using affinity chromatographic techniques developed by Axen et al. (15), Tripatzis (16) demonstrated that HB-Ag was also present in the urine of patients with serum hepatitis. In addition, he established the fact that the HB-Ag present in both the serum and urine of the infected patient were antigenically identical by gel diffusion. The subject of this report is a method for the efficient removal of HB-Ag from urine and its rapid identification by radioimmunoassay. Although the procedures specifically concern HB Ag found in urine, it has applicability to other dilute biologic materials. The technique exploits the fact that HB-Ag contains a lipid moiety (12, 13) which can be firmly bound by adsorption onto an insoluble lipophilic silicic acid matrix. We have recently described a method (17) for the isolation of HB-Ag from serum which is based on the well-established affinity of silicic acid for lipid containing materials (18). This present report describes additional aspects of the techniques involved and demonstrates that the bond between HB-Ag and silicic acid allows each specimen to be used as its own specificity control.

Materials and Methods

HB-Ag positive serum: The sera of ten serum hepatitis patients, positive by counter immunoelectrophoresis (CIEP) at titers of 1:4 to 1:8 were used. Ten normal sera, negative by CIEP and the Abbott Ausria-125 radioimmunoassay were pooled and used as the controls.

Normal urine pool: The urine of ten individuals whose serum and urine were negative by CIEP and the Abbott Ausria-125 assay were pooled and used as the normal urine pool. Aliquots of this pool were used for all dilutions of normal and HB-Ag positive sera.

Chemicals: Insolubilized silicic acid, commercially available as "Liposorb" was obtained from Custom Reagents Lab, San Diego, CA. Polyvinylpyrrolidine, commercially available as "Plasdone C" was obtained from General Analine and Film Corporation, Calvert City, Kentucky.

Antiserum to HB-Ag: Commercially available anti-HB-Ag antibody labeled with I^{125} was obtained from Abbott Labs, North Chicago, IL.

Adsorption of HB-Ag onto silicic acid: Three milliliter aliquots of urine containing HB-Ag were incubated at room temperature (25°C) for two hours with ten milligrams of silicic acid. This amount of silicic acid has been found by previous experiments to be sufficient to adsorb all the recognizable HB-Ag from 0.1 ml of serum having a CIEP titer of 1:8 (17). The adsorbing pellet of silicic acid was then sedimented at a relative centrifugal force of 1800 xg for five minutes and the supernatant fluid removed. In order to saturate any unoccupied silicic acid binding sites 0.5 ml of normal guinea pig serum was then added to each tube and incubated for one hour. The final steps were to wash the tubes with 0.15 molar phosphate buffered saline, pH 7.4 (P.B.S.) and, following centrifugation, to resuspend the insolubilized pellet of silicic acid to which HB-Ag was now bound in 0.5 ml of polyvinylpyrrolidine. 0.1 ml of anti-HB-Ag antibody labeled with I^{125} was added to each tube and incubated with the specimen for one hour at room temperature (25°C). All the tubes were then washed three times with P.B.S. and counted in a dry well scintillation counter. The controls for each experiment consisted of normal urine containing an equivalent amount of normal serum. Variations in the procedure employed to evaluate the effect of different parameters on the system are explained in the appropriate section.

Results

Effect of volume on adsorbing efficiency: To compare the effect of total urine volume on the efficiency of adsorption, 0.050 ml aliquots of HB-Ag positive serum (CIEP titer 1:4) were added to 5, 50, 100, 500, and 1000 mls of pooled normal urine. The controls were 0.050 ml aliquots of pooled normal serum added to equivalent volumes of the same normal urine pool. In all cases, 10 milligrams of silicic acid was used as the adsorbing matrix. Following a two-hour adsorption period at room temperature, the silicic acid to which HB-Ag was now bound, was processed as previously outlined. The only variation was that one set of replicates was partially blocked after the HB-Ag adsorption phase by the addition of unlabeled anti-HB-Ag two hours prior to the addition of radiolabeled anti-HB-Ag.

The results of the experiments are shown in Table 8. There is a rather high and uniform efficiency of HB-Ag binding by insolubilized silicic acid even when the antigen is dispersed in relatively large volumes. The specificity of the reaction is demonstrated by the partially inhibited uptake following pre-exposure to unlabeled anti-HB-Ag. The addition of unlabeled antibody did not alter the normal control values.

Effect of time upon the completeness of HB-Ag adsorption: To determine the optimum adsorption times for silicic acid and HB-Ag mixtures, the two components were allowed to react for varying intervals. In this experiment CIEP positive serum was diluted one hundred fold in

normal pooled urine. Multiple three ml replicates were set up and processed exactly as described in the Methods Section, except that varying adsorption times were used. The controls were prepared by substituting pooled normal serum for CIEP positive serum before dilution with the pooled normal urine. Table 9 demonstrates that the reaction is essentially complete at the end of one to two hours.

Table 8

Effect of Volume on Adsorbing Efficiency

Volume of Urine	Counts per Minute*	
	Uninhibited	Partial Inhibition
Undiluted	2337	731
5 ml	1864	982
50 ml	2047	708
500 ml	1724	612
1000 ml	1932	1099

*Control values (233-253 CPM) were subtracted in each case.

Table 9

Effect of Time Upon Efficiency of Adsorption

Treatment	Time of Adsorption (hours)					
	1/2	1	2	3	4	5
	Counts per minute					
Detection of HB-Ag (1)	737	1427	1350	1631	1391	1627
Inhibition of Detection (2)	0	13	0	0	162	0
Re-establishment of Detection (3)	1536	2613	2840	2764	2673	2085

*Control values were subtracted in each case.

(1) HB-Ag was adsorbed onto silicic acid and detected with labeled anti HB-Ag.

(2) Labeled anti-HB-Ag was eluted off with acid washes and then HB-Ag recognition blocked by unlabeled antibody prior to the re-addition of radioiodinated anti-HB-Ag.

(3) The unlabeled anti-HB-Ag was again eluted by acid washed and the presence of HB-Ag re-established by the uptake of labeled anti-HB-Ag.

Specificity of the reaction and stability of the lipophilic bond: After the replicates used in determining optimum adsorption times were counted, all tubes were washed twice with glycine-HCl buffer pH 1.8 to elute off the labeled anti-HB-Ag. The pH was restored to 7.4 by two more washes with phosphate buffered saline. Unlabeled anti-HB-Ag was then added and the tubes incubated for two hours at room temperature. Labeled anti-HB-Ag was added after a preliminary phosphate buffered saline wash and the specimens were reprocessed as outlined previously.

The results of this inhibition procedure are also demonstrated in Table 9. A marked reduction HB-Ag recognition by the radiolabeled anti-HB-Ag is clearly shown. To prove that HB-Ag was not lost from the silicic acid matrix by acid elution the inhibiting unlabeled anti-HB-Ag was once again eluted from the bound HB-Ag and labeled anti-HB-Ag was reapplied. The third set of counts in Table 2 shows that HB-Ag is still bound in a recognizable form. Similar results were found when 0.02 per cent pepsin digestion is used in place of the acid washes.

Discussion

The first isolation of HB-Ag from urine by Tripatzis (16) required large volumes of urine and prolonged affinity chromatography procedures. This paper outlines a quick and efficient method of removing HB-Ag from urine in a recognizable form by means of an insoluble lipophilic matrix. Silicic acid provides an inexpensive adsorption matrix for HB-Ag, its small volume and relative inertness appears to offer a potentially feasible way to screen large suspect populations in the field without having to transport large volumes of urine. It is evident that the technique can be applied to other experimental situations including the isolation and concentration of HB-Ag from a variety of biologic materials.

The stability of the bond between HB-Ag and silicic acid allows experiments in which antibody can be added to or removed from the same specimen under investigation. The removal of antibody to HB-Ag by either acid washes or pepsin digestion does not result in a loss of antigenicity, but in fact appears to result in more efficient binding of antibody. This "increase in recognition" has been consistently found using a variety of individual specimens. Similar treatment of HB-Ag by acid washes or pepsin digestion has been shown not to degrade HB-Ag (13,19). Whether or not they improve recognition as suggested in this study has not been previously explored. One of the possible explanations for an increase in recognition following these procedures is that protein or proteins are being removed from the surfaces of the HB-Ag. When purified HB-Ag was treated with the detergent Tween 80, Millman, et al (20), noted that it partially dissociated into soluble components. These released products appeared to include IgG (both heavy and light chains), complement, beta-lipoprotein, transferrin and albumin. In addition, Prince (21) has found macroglobulin (IgM) associated with a significant fraction of HB-Ag in whole serum. It would seem reasonable, then that the removal of proteins, whether they are specific antibodies or not, might reveal previously hidden antigenic sites. Experiments are

presently underway to detect and characterize these possible masking proteins. Particulary scrutiny is being directed to the possible role of proteins found on the surfaces of HB-Ag in chronic antigen carriers.

4. Hepatitis B immune complexes in antibody negative patient sera.

Hepatitis B antigen (HBA) was first detected by Blumberg et al (11) in 1965. Since that time several studies have characterized the biochemical and biophysical nature of the antigen, its relationship to serum hepatitis (13) and its ability to produce precipitating (14) as well as complement fixing antibodies (12). An increasing interest in the capacity of immune complexes of HB-Ag to affect the clinical courses of the disease has provided the impetus for the development of a variety of techniques to demonstrate these complexes. Almeida (22) employed electron microscopy to demonstrate immune complexes in the sera of several patients in various stages of serum hepatitis B antigenemia. Shulman and Barker (23) reported that 95% of the sera in a series of 130 patients with serum hepatitis were anti-complementary. After protein stripping with dichloro-difluormethane they were led to the conclusion that the anti-complementary activity was attributable to the presence of antigen-antibody complexes. Alpert et al (24) using immunofluorescent techniques has shown immune complexes of HB Ag, IgG, and C3 fixed in the vascular epithelium of patients with serum hepatitis. Gocke et al (25) has identified immune complexes containing IgM, HB Ag, and C3 in serum hepatitis patients with polyarteritis using the same technique. Recently, Madalinski et al. (26) have employed DEAE cellulose chromatography to dissociate immunoglobulins of IgG, IgM, and IgA classes from the soluble immune HB Ag complexes of four patients.

Taking advantage of the fact that HB Ag contains a lipid moiety, Hedlund (17) introduced a radioimmunoassay in which HB Ag was adsorbed onto an insoluble lipophilic matrix of silicic acid. It has subsequently been possible to demonstrate that a significant increase in antigenic recognition occurs in a majority of asymptomatic carriers and acute serum hepatitis patients if one performs this assay before and after acid pepsin digestion. Other protein dissociation procedures such as treatment with 8 molar urea, 3 molar KCL, etc., are equally feasible. These proteins which limit recognition can be shown to specifically bind to HB Ag by direct as well as indirect assays. This method for uncovering immune complexes requires only 0.1 ml of patient sera and can be done in a relatively short time period. Because of the high percentage of sera which appear to contain complexes the data are reported in this communication.

Materials and Methods

HB Ag sources and controls: Three groups of sera were studied. The first consisted of 31 asymptomatic blood donors who were not clinically ill, but who did have circulating HB Ag by either counter immunoelectrophoresis or the Abbott Ausria I-125 radioimmune assay. The second group of sera were obtained from 15 patients who had clinical

signs and symptoms of acute hepatitis and compatible laboratory data. HB Ag was detected by either counter immunoelectrophoresis or Abbott Ausria I-125 assay. The third group comprised 10 normal individuals who had no prior history of hepatitis and who were negative for HB Ag by counter immunoelectrophoresis and Ausria I-125 assay. Standard passive hemagglutination examination of the donor, patient and normal control groups were negative for circulating anti-HB-Ag antibodies.

Chemicals: Insolubilized silicic acid, commercially available as "Liposorb" was obtained from Hagadorn Labs, San Diego, CA. Polyvinylpyrrolidine (PVP), commercially available as "Plasdone C" was obtained from General Analine and Film Corporation, Calvert City, KY. Antiserum to HB Ag: Commercially available guinea pig anti-HB-Ag antibody labeled with I-125 was obtained from Abbott Labs, North Chicago, IL.

Radioimmune assay of HB Ag bound to silicic acid: As previously (17), 0.1 ml aliquots of sera were bound to 10 mg of silicic acid at 25°C for 3 hours. The adsorbing pellet of silicic acid was then sedimented at a relative centrifugal force of 1800 xg for five minutes and the supernatant fluid removed. In order to saturate any unoccupied silicic acid binding sites 0.3 ml of pooled normal human and guinea pig serum was then added to each tube and incubated for one hour. The next step was to wash the tubes with 0.15 molar phosphate buffered saline, pH 7.4 (P.B.S.) and, following centrifugation, to resuspend the insolubilized pellet of silicic acid to which HB Ag was now bound in 0.5 ml of PVP. 0.1 ml of anti-HB-Ag antibody labeled with I-125 was added to each tube and incubated with the specimen for one hour at room temperature (25°C). All the tubes were then washed three times with P.B.S. and counted in a dry well scintillation counter. The normal controls consist of 0.1 ml aliquots of pooled human serum treated in an identical fashion. This constituted the first recognition step. To strip away any blocking proteins the pellets were resuspended in 5 ml of .02% pepsin in 0.02 HCl (pH 2.3) or 8 molar urea and incubated for 1 hour at 37°C. Following three P.B.S. washings, the tubes were reprocessed as previously described, starting with the readdition of 0.3 ml of pooled normal human serum.

Dissociation of the immune complexes: In those experiments where recovery blocking proteins was desired, dissociation was carried out by the addition of 3 ml of either glycine HCl (pH 2.3) or 3 molar KCl to the well washed pellet. These maneuvers do not disrupt the HB Ag silicic acid bond. After a two hour period the tubes were recentrifuged and the supernatant containing the eluate was removed and dialysed against phosphate buffered saline.

Results

The effect of protein removal by acid pepsin digestion upon the recognition of bound HB Ag by radioimmune assay: The work of Kim and Tilles (13) and Millman et al. (27) have clearly demonstrated that HB Ag is resistant to acid pepsin digestion. Radioimmune assays done

on the same sample before and after enzymatic protein stripping method results in increased recognition in terms of counts per minute in 11 of 15 acute serum hepatitis patients and 30 of 31 asymptomatic carriers.

Statistical comparison by paired t test of all the members of each group before and after acid pepsin digestion indicated a significant increase at the 99% confidence level for both the 15 acute hepatitis sera and for the 31 asymptomatic carriers. In contrast there is no significant difference between the pre- and post-treatment members of the normal control group. Table 10 illustrates a portion of the data.

Table 10

Group*		Pre-Acid Pepsin Digestion	Post-Acid Pepsin Digestion
A	1	818	1029
	2	605	1132
	3	747	1095
	4	894	816
	5	777	1017
B	6	1081	1438
	7	1243	1498
	8	1313	1311
	9	1206	1577
	10	1549	1905
C	11	222	228
	12	204	198
	13	246	283
	14	229	248
	15	220	204

*Group A represents sera from patients with acute serum hepatitis, group B were from asymptomatic carriers, and group C were normal controls.

Inhibition of HB Ag recognition by proteins dissociated from the surfaces of HB Ag: The experiments above demonstrate that in a majority of cases, proteolytic enzyme treatment appears to uncover previously masked antigenic sites and improves the recognition of HB Ag bound to silicic acid.

To investigate the functional ability of these blocking proteins to inhibit the reaction of HB Ag with radiolabelled homologous antibody, experiments were performed as follows: HB Ag sera which were negative by passive hemagglutination for anti-HB Ag antibodies were adsorbed onto silicic acid. After three hours the tubes were centrifuged and the pellets resuspended, w shed and extracted with 3M KCl. Following

recentrifugation, the supernatant fluids containing the dissociated proteins were equilibrated with P.B.S. by dialysis and then allowed to interact for four hours with purified HB Ag which was adsorbed onto silicic acid. The specimens were then recentrifuged, washed with P.B.S., incubated with anti-HB Ag antibody labeled with I^{125}, washed three times with P.B.S., and counted. Pooled normal human serum adsorbed onto silicic acid and treated in an identical fashion was used as a control in place of the purified HB Ag. The purified HB Ag used in these experiments, which was obtained by the method of Gerin and Purcell (14), was negative by complement fixation and did not induce production of anti-human antibodies after repeated injections in animals. A representative inhibition experiment is shown in Table 11. Complete inhibition of recognition occurs when the protein material eluted from the surface of #BK HB Ag interacts with HB Ag (#392) from a different source. The #435 HB Ag eluted material yields only partial inhibition. To further test the hypothesis that inhibition of recognition occurred when experimentally dissociated proteins were taken from one crude HB Ag preparation and reacted with purified HB Ag from another source, the following additional manipulations were performed: all the samples were acid pepsin digested; this procedure removes any blocking proteins but does not effect the HB Ag silicic acid bond; as is seen in Table 11, recognition is restored as evaluated by the reactivity of the preparations with radiolabeled antibody.

Table 11

Inhibition of HB-Anti-HB-Ag Binding by KCl Extracts of HB Ag-Serum-Silicic Acid Complexes

	Material Eluted From Silicic Acid to Which was Bound:		
	Normal Serum	#435 (HB Ag serum)	#BK (HB Ag serum)
#392 Purified HB Ag	568 cpm	362 cpm	164 cpm
	*617	*562	*596
Control (Normal human serum)	197 cpm	145 cpm	148 cpm
	*158	*165	*160

*CPM observed following acid pepsin digestion and the addition of I^{125} anti-HB Ag.

Discussion and future plans

In total, 41 of 46 acute and carrier sera containing HB Ag showed statistically significant improvement in antigen recognition following maneuvers which dissociate or destroy proteins but do not effect HB Ag;

no changes are seen in the normal population. The biological activity of these dissociated proteins has been clearly established by their ability to specifically inhibit the recognition of HB Ag.

The final phases of these studies will involve the characterization of these blocking proteins (which are most probably immunoglobulins) and to determine if there is a correlation of surface immunoglobulin and antibodies to the clinical course of the antigenemia; i.e. do asymptomatic carriers have different antibodies on the surface of their HB Ag than those patients who quickly recover from the acute illness?

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 172 Immunological mechanisms in microbial infections

Literature Cited.

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Publications:

Hedlund, K. W.: Hepatitis B antigen: Removal from protein solutions in a recognizable form by an insoluble lipophilic matrix of silicic acid. Life Sciences 13:1491, 1974.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6530	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. CHG	U	U	N/A	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71Q	00	173
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Vaccine Development in Trypanosomiasis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 09	CONT	DA	C In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: N/A EXPIRATION

b. NUMBER:

c. TYPE: d. AMOUNT

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	1.0	102
	CURRENT 75	1.0	102

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Kenya; (U) Trypanosomiasis; (U) Vaccine; (U) Africa; (U) Irradiation; (U) Immunity

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The objective of the research program is to develop an effective and practical vaccine against African Trypanosomiasis, useful to military and civilian agencies. Related benefits include acquisition of knowledge pertaining to trypanosome immunology, biology, host response and the pathology of the disease in natural and experimental hosts

24. (U) Experiments conducted at WRAIR and Kabete,Kenya during the past 3 years have demonstrated that experimental animals can be successfully immunized with irradiated trypanosomes. Rodents, cattle and monkeys could be rendered completely resistant to a challenging infection with T. rhodesiense by this method. Partial immunity was obtained in rodents and cattle to T. congolense. Immunization using this method, as well as passive protection of antibodies, will be studied with relation to field use.

25. (U) Initial results from a study in an area endemic for T. rhodesiense, Lambwe, Kenya, indicate that a single strain of trypanosomes is causing the disease in man and many domestic animals are infected with this same strain. Passively transferred convalescent serum has been found to confer resistance to T. rhodesiense in calves.

THIS RESEARCH IS COMPLEMENTARY TO DAOB 7521, WORK UNIT 096, ENTITLED "STUDY OF AFRICAN TRYPANOSOMIASIS"

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

PROJECT 3A161102B71Q Communicable Diseases and Immunology

Task 00 Communicable Diseases and Immunology

Work Unit 173 Vaccine Development in Trypanosomiasis

Investigators.
Principal: COL Dale E. Wykoff, MSC
Associate: Bruce T. Wellde, GS 12

Description. The US Army Medical Research Unit (WRAIR) Kenya became operational in the Fall of 1973. It now consists of two US personnel. The major effort has been to establish the USAMRU-K within the Veterinary Research Laboratory, Ministry of Agriculture, Nairobi, Kenya. Adequate electrical, plumbing and security facilities were achieved in Spring 1974 whereupon the Government of Kenya donated the laboratory to the Rockefeller-sponsored International Laboratory for Research on Animal Diseases (ILRAD). The new space provided for USAMRU-K will require extensive efforts to reduce the rat population and to provide adequate electrical and plumbing facilities. The move to the new area will be complete by 31 Jul 74. Several months of research time will be lost as a result of this move. In spite of delays a research program has been initiated which addresses four major problems.

1. Immunization of Calves by Passive Transfer of Immune Serum.

Problem. To determine whether otherwise susceptible calves can be protected against infection with Trypanosoma rhodesiense by passively immunizing them with antisera from cattle which have undergone infection and self cure with the same organism.

Background. Although it has been shown that laboratory animals can be protected against trypanosomiasis by receiving antisera in a heterologous system (i.e. bovine to mouse) controlled experiments using homologous transfers between large animals have not been reported. The protection afforded in heterologous transfers persists for relatively long periods (Soltys 1964). It is reasonable to assume that the degree of protection would be higher and of longer duration in a homologous system. Cattle are ideal experimental hosts because, in

contrast to most other animals they are readily susceptible, they undergo a long duration of infection and eventually self cure. The self cured animals are resistant to re-infection by the same strain of trypanosome for at least 14 months (Wellde et al. 1973).

Results. Adult cattle which had self cured T. rhodesiense infections were rechallenged and subsequent subinoculations of their blood into mice indicated complete resistance. These cattle were bled weekly and serum was collected, pooled and stored at -20°C. When sufficient serum had been collected it was filtered through bacteriologic filters and stored in sterile containers. The protection afforded by this serum against the homologous strain of T. rhodesiense can be seen in Table I.

TABLE I

NEUTRALIZATION OF T. RHODESIENSE BY ANTISERUM FROM IMMUNE CATTLE*

	No. trypanosomes per mouse			
Serum:	10^6	10^4	10^2	10^0
Immune	0/5**	0/5	0/5	0/5
Normal	5/5 (3)	5/5 (4)	5/5 (5)	5/5 (6)
Fetal calf	5/5 (3)	5/5 (4)	5/5 (5)	4/5 (6)

* Electrophoresis showed 2.58 gr gamma globulin per 100ml
** No. mice dying over number inoculated
() Mean survival time

Approximately 3,500 ml of immune serum was collected from four self cured donor cattle during the three week period. Four calves weighing between 300 and 375 lbs were selected and two received 1,500ml each via the jugular vein, with no untoward reaction. These two experimental and two normal calves were challenged later the same day with 1×10^4 T. rhodesiense from infected rats. Neither experimental calf developed a detectable infection after challenge whereas both control calves developed patent infections which were characterized by fever and positive subinoculations to mice (Table II)

TABLE II

RESULTS OF SUBINOCULATIONS OF IMMUNIZED AND CONTROL CALVES AFTER CHALLENGE

	Day of subinoculation						
Group:	-1	9	20	115	139	173	179
Immune 1	-	-	-	-	-	-	-
Immune 2	-	-	-	-	-	-	-
Control 1	-	⊕	⊕	-	-	-	-
Control 2	-	⊕	⊕	+	+	-	-

⊕= Patent infection

Control calf No. 2 developed pathological CNS symptoms and was subsequently sacrificed. Histopathological examination of the brain showed heavy perivascular infiltration and meningitis. An assay of serological antibodies in the four calves was conducted using a previously described complement fixation test (Lötzsch and Deindl 1974) (Table III).

TABLE III

RECIPROCALS OF COMPLEMENT FIXATION TITERS OF IMMUNE SERUM RECIPIENTS AND CONTROLS

	Days After Challenge					
Group:	-1	2	11	16	22	179
Immune 1	5	5	5	5	5	10
Immune 2	<5	5	10	5	5	<5
Control 1	<5	<5	160	640	1280	160
Control 2	40	40	160	160	320	1280

Serological antibodies were not detectable in the immune serum recipients although they were completely resistant to the challenging infection. Both control calves had developed elevated titers by the eleventh day of infection. Increased antibody levels persisted for relatively long periods. Six months after the primary challenge the two experimental animals and the remaining control were rechallenged along with new control animals. The two previously immune calves (Immune 1 & 2) and the new control animal all developed typical infections but Control 1 was resistant. A second pool of immune serum has been collected and the duration of immunity will be determined by challenging immune serum recipients at periods of 1 day, one week and one month after transfer of serum.

2. Antigenic Relationships Between Organisms of the T. brucei Group

Problem. To define antigenic differences and similarities between trypanosomes collected from persons infected with T. rhodesiense in the Lambwe Valley of Western Kenya. T. brucei was collected fromcattle in the same area and similarly studied. During the coming two years it is expected that the number of strains of T. rhodesiense occurring in man; the number of strains of T. brucei occurring in cattle and the antigenic relationships between these morphologically identical parasites will be disclosed. The study is designed to determine whether there are so many strains that immunization may be unfeasable; whether immunity produced against strains in Kenya will protect against infection acquired in neighboring areas, and whether cattle in Lambwe Valley harbor parasites antigenitically similar to those infecting man.

Background. Practical immunization against African Trypanosomiasis is largely dependent on the number of strains of a given species found in an endemic area. Gray (1970) worked five years with the same herd of cattle in Nigeria and reported the existence of many strains of T. brucei, thus making immunization impractical. It is possible, however, that the techniques employed were not specific enough to select all variants belonging to a common strain. In Lambwe Valley human trypanosomiasis of the rhodesian type is endemic and cattle and wild game harbor T. brucei. These two species are morphologically identical and their relationship to the disease in man is unclear. In nearby Alego Station, Kenya, T. brucei-like organisms were isolated from cattle and injected into human volunteers. The volunteers developed typical T. rhodesiense-like infections (Onyango 1966).

Results. Isolates of T. rhodesiense were collected from patients in the Homa Bay Hospital by Kenya Medical Department personnel. Rats injected with the blood from patients were shipped to USAMRU-K. The strain of T.rhodesiense from Gambella, Ethiopia, was collected by NAMRU-3, Addis Ababa. Animal trypanosomes were collected by USAMRU-K. Isolates of trypanosomes were tested by neutralization (Soltys 1957) with antisera collected from bovines which had undergone long-term infection with various isolates. The first series of experiments was done with antiserum against a strain of T. rhodesiense (LVH-1) collected in Lambwe Valley from a patient in 1972. Table IV shows the neutralizing effect of

antiserum on the infecting strain of T. rhodesiense.

TABLE IV

EFFECT OF ANTISERUM ON HOMOLOGOUS T. RHODESIENSE (LVH-1)

	No. of trypanosomes per mouse				
Serum:	10^4	10^3	10^2	10^1	10^0
Immune	0*	0	0	0	0
Normal	5	5	1	1	0

*No. of mice developing patent infection, of 5 inoculated

The results derived from testing the same antisera on T. rhodesiense isolated from three different patients from Lambwe Valley are presented in Table V.

TABLE V

EFFECT OF ANTISERUM TO LVH-1 ON PARASITES OBTAINED FROM OTHER PATIENTS (LVH-2) (LVH-3) (LVH-4)

		No. of trypanosomes per mouse				
Isolate:	Serum:	10^4	10^3	10^2	10^1	10^0
LVH-2	Immune	0*	0	0	0	0
	Normal	5	5	2	0	0
LVH-3	Immune	0	0	0	0	0
	Normal	5	5	5	3	0
LVH-4	Immune	0	0	0	0	0
	Normal	5	5	5	5	5

* No. of mice developing patent infection, of 5 inoculated

Two other strains of known T. rhodesiense were also tested, one from southern Ethiopia and a laboratory strain (Wellcome) which had been isolated in Tanzania and maintained in rodents for many years. The results are presented in Table VI.

TABLE VI

EFFECTS OF ANTISERUM ON PARASITES ISOLATED FROM DIFFERENT GEOGRAPHICAL REGIONS

Isolate:	Serum:	No. trypanosomes per mouse 10^4	10^3	10^2	10^1	10^0
Ethiopia	Immune	5	5	5	1	0
	Normal	4	5	5	5	3
Wellcome	Immune	5	4	4	2	0
	Normal	5	5	4	5	1

Nine different isolates of T. brucei were made from 83 cattle by subinoculation of their blood into rats. Each of these isolates was then tested against the antiserum prepared against the known T. rhodesiense (LVH-1). Results are in Table VII.

TABLE VII

EFFECTS OF ANTISERUM TO T. RHODESIENSE (LVH-1) ON SUPPOSED T. BRUCEI ISOLATED FROM CATTLE IN LAMBWE VALLEY

Isolate:	Serum:	No. trypanosomes per mouse 10^4	10^3	10^2	10^1	10^0
LVB-1	Immune	0	0	0	0	0
	Normal	5	5	5	2	0
LVB-2	Immune	0	0	0	0	0
	Normal	5	5	5	4	0
LVB-3	Immune	4	1	2	0	0
	Normal	5	4	2	0	0
LVB-4	Immune	4	0	0	0	0
	Normal	5	5	4	5	3
LVB-6	Immune	5	5	5	3	3
	Normal	5	5	5	5	5
LVB-7	Immune	5	5	4	1	0
	Normal	5	5	5	5	3
LVB-8	Immune	5	5	5	2	0
	Normal	5	5	5	5	5
LVB-9	Immune	5	5	5	0	3
	Normal	5	5	5	4	5

It is interesting to note that all four isolates of T. rhodesiense from Lambwe Valley appear to be antigenically similar since antiserum to one neutralized them all within the limits of our test. Since antiserum to trypanosomiasis is strain specific, this indicates the persistence of this strain since at least 1972. Many more isolates need to be tested but these preliminary findings indicate that trypanosomiasis in the Valley is caused by a single strain of T. rhodesiense which is of a persistent nature. The antiserum had only a slight effect on trypanosomes of different geographical areas. When isolates of T. brucei from cattle were tested the antiserum showed a strong effect against 3 of the 9 isolates indicating that these cattle were infected with the same strain as the four humans. Whether these isolates are indeed infective for man could only be determined by subinoculation into human volunteers, but given the specificity of the antibody it is reasonable to assume that they are in fact T. rhodesiense. Further isolates of trypanosomes from humans and bovines will be taken and studied throughout the coming year in an effort to clarify and extend our findings.

3. The Feasibility of Practical Artificial Immunization by the Use of Irradiated Trypanosomes

Problem. Although immunization with irradiated parasites has proved successful under laboratory conditions studies using the naturally transmitted disease have not been undertaken.

Background. Research conducted in Kenya by WRAIR personnel has indicated that cattle immunized with a sufficient number of partially purified irradiated trypanosomes were refractive to a challenge infection with non-irradiated trypanosomes of the same strain of T. rhodesiense. Immunized animals were completely resistant to challenges given one week and eight months after immunization. A partial immunity was observed after challenge at 14 months. No resistance was found to challenge with a heterologous strain of T. rhodesiense (Wellde et al. 1973). Similar trials with T. congolense failed to produce a strong resistance in cattle (Wellde et al. 1974), however, mice were highly resistant (Duxbury et al. 1973). Definition of antigenic relationships existing between stock strains of trypanosomes maintained in rodents and metacyclic trypanosomes from the tsetse will be attempted. Tsetse will be reared from pupae collected with the assistance of the Tsetse Department staff. Mice will be immunized with the irradiated stock strain of T. congolense and challenged by the bite of flies fed on animals fed with the stock strain. If the mice are resistant the challenge will be made by tsetse fed on chronic (varient strain) infections.

It has been reported by Gray (1970) that varient trypanosomes return to a basic antigenic composition in tsetse. If these methods are successful in mice attempts will be made by column purification and adjuvants to increase the immune response in bovines. The above studies will then be repeated in calves.

Progress. One field trip to Aitong Station (Masai Mara) has been undertaken for the purpose of learning techniques and methods involved in the collection of tsetse pupae. With cooperation and help from the Tsetse Department, 120 pupae of Glossina swynnertoni were collected in about eight hours. The arrival of a WRAIR entomologist on TDY in mid January 1975 will give impetus to these experiments. Prior to his arrival incubators, cages and other necessary equipment will be obtained.

4. Pathophysiology of Trypanosomiasis

Problem. To attempt to clarify and document the pathological process occurring in trypanosomiasis by a systematic study of appropriate animal models.

Background. Many important pathological aspects of trypanosomiasis in animals and man have not been properly studied. Although many pathological changes occurring in host animals have been attributed to the trypanosomes, in some instances the findings are fragmentary and inconclusive.This may be due in part to differences in virulence of separate species and strains of trypanosomes as well as by host factors such as age, condition, sex, breed, presence of concomittant infections, etc. We are now establishing the capability to support hematological and clinical chemistry studies by a veterinary pathologist to be assigned by WRAIR in August 1974. Preliminary studies conducted over the past three years (Wellde et al. 1974, Kaliner 1974) indicate several areas which deserve concentrated study.

Project 3A161102B71Q Communicable Diseases and Immunology

Task 00 Communicable Diseases and Immunology

Work Unit 173 Vaccine Development in Trypanosomiasis

LITERATURE CITED

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23. (U) Determine the factors influencing outbreaks of plague infection and the most appropriate methods to prevent the infection of troops engaged in field operation.

24. (U) Using standard methods, sera from humans and animals are tested for the presence of F-1 antibody to Y. pestis.

25. (U) 73 07 - 74 06 - Rats with titers in excess of 1:32 following vaccination with F-1 antigen, plague vaccine USP and attenuated Y. pestis usually survived subcutaneous and intranasal challenge with virulent Y. pestis. Rats challenged by the intranasal route were infected with Y. pestis cultivated in vivo in the mouse peritoneum to obtain bacilli of antigenic composition similar to those found in the lung in terminal pneumonic plague, thus closely simulating the type of respiratory infection likely to be encountered in nature. Clonal analysis of the Y. pestis isolated from several vaccinated rats dying of plague infection many months after experimental challenge shows that the bacilli are virulent non-encapsulated variants lacking the F-1 antigen. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

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Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 174 Ecology of plague

Investigators.
Principal: COL Dan C. Cavanaugh, MSC
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Progress.

I. Vaccines and immunization against plague in Rattus norvegicus.

A program of experimental studies was undertaken during the period covered by this report to determine the efficacy of various vaccines against plague in the laboratory rat (Rattus norvegicus Berkenhout, Wistar strain).

In addition to new information on vaccine efficacy, a significant body of background data has resulted that is relevant to understanding the role of rats in the epidemiology and ecology of plague, in which Rattus norvegicus assumes an especially important function as the urban link between wild-rodent plague and human infection. Although rats have been used in the past for laboratory investigations of plague, the majority of information on vaccines and immunization against plague has come from work conducted with mice, guinea pigs and various species of monkeys. Mice have been used because small size made them less expensive to obtain and maintain, and mice were found by some investigations to be more susceptible to plague than were rats. The guinea pig has been of interest because its' immunology is different from that of mice. Monkeys have been studied as hosts most nearly representative of man, although it is now recognized that this assumption is not justified for some species. In effect, past work on plague immunization has demonstrated that species respond differently to vaccination or infection, and even when differences have been subtle, new insights were obtained.

The albino rats used in our work came from the inbred colony maintained by this Institute. We have found these rats to be exquisitely susceptible to fatal infection with fully virulent strains of Y. pestis (LD_{50} in the range of 1-10 plague organisms). Whereas difficult, if not impossible with mice, complete serological histories can be and were obtained in experiments with rats. Emphasis was placed on the investigation of bubonic plague. In most experiments, challenge of animals was carried out subcutaneously to mimic infection by fleabite. Strains of Y. pestis for subcutaneous inoculation were grown at 25°C to obtain phagocytosis-sensitive organisms similar to

those acquired through fleabite. In addition, intranasal challenges were done with fully virulent bacilli grown in vivo (i.e., in rat peritoneum). Infection induced with in vivo grown organisms by this route of challenge is nasopharyngeal and pulmonary in character and is representative of infection acquired by rats through feeding upon infected carcasses or of man via plague pneumonia.

In the discussion of work that follows, serum titers for antibody are given as the reciprocals of the highest serum dilutions that gave a positive reaction in the test used (IHA or CF). Zero titer indicates that no positive reaction occurred either because no antibody was produced or because a previous titer was lost with time.

A. Rats vaccinated with a living attenuated plague vaccine. An experiment was conducted to test the relationship between dose of living vaccine administered and the serologic response in rats in terms of the frequency with which antibody is produced and of the titers achieved. Two groups of rats were vaccinated. In group A, the living attenuated strain EV76(51f) of Y. pestis was injected directly into the hearts of anesthetized rats. All animals in group A produced antibody after inoculation, but the geometric-mean titer of this group was only 32 (Table 1). Rats of group B were inoculated subcutaneously with serial dilutions of EV76(51f). Those inoculated with smaller numbers did not produce antibody as frequently as rats given larger doses. Furthermore, a relationship between dose administered and geometric-mean titer was obtained. Rats that received a dose of 25.4 million viable EV76(51f) developed titers with a geometric mean of 91, whereas rats given lesser amounts of vaccine produced lower geometric mean titers. Rats given equivalent doses by subcutaneous inoculation and by inoculation into the heart responded with geometric-mean titers of the same magnitude, although only 30% of the animals inoculated subcutaneously produced antibody.

Rats vaccinated with EV76(51f) were challenge with the virulent strain 195/P of Y. pestis to determine if serum titer was any indication of resistance to infection or ability to survive infection (Table 2). Rats that did not have detectable IHA titer or that had titers of 8 or less all died. However, on the average, they did not die as rapidly as rats that had never been vaccinated. Rats with titers of 16-32 showed a 46% survival rate following challenge, and deaths were delayed longer compared to rats with lower IHA titers. Most rats (94%) with titers of 64 or higher survived challenge. All survivors, irregardless of prechallenge titer, developed four-fold or greater increases in IHA titer afterwards.

Male rats surviving the challenge experiments were subsequently bled at 1-2 month intervals to determine how long antibodies to the Fraction 1 antigen of Y. pestis persist in Rattus norvegicus (Table 3). Female rats immunized with EV76(51f) and challenged with 195/P were studied similarly (Table 4). In these animals, IHA titers continued to increase up to 115 days after virulent challenge. Thereafter, a decline in titer occurred, but the decline leveled off between 160-240 days following challenge. Subsequently, very little, if any, change occurred in IHA titer, as measured over two years from challenge in some rats. Only one rat, a male with a titer of 512 at 22 days post challenge, lost all detectable titer, and that occurred approximately 480 days after challenge.

Serum samples were not tested routinely for CF activity. However, determinations were made for samples taken from female rats 241 days and from male rats 424 days after challenge. Of four females tested, three had CF titers of 32-64. Eight of nine male rats also had CF antibody; five with titers of 32-64.

The serological data indicated that rats which survive an infection generally retain antibodies for the remainder of their lives. Following these observations, experiments were performed to determine if the antibody retained long after an infection would prevent reinfection with Y. pestis. Reinfection was attempted 5-10 months after the initial subcutaneous challenge. Subcutaneous and intranasal routes were investigated, and the rats challenged survived (Table 5). Animals with titers of 512 or more at the time of rechallenge usually did not display a significant increase in IHA titer subsequently (i.e., four-fold or greater increase), whereas rats with titers of 32-256 generally did. Animals with CF titers of 128 or more at the time of rechallenge did not demonstrate significant change in CF titer afterwards, while rats with CF titers of 64 or less usually had a drop in titer after rechallenge, frequently to zero (i.e., negative test reaction at a serum dilution of 1:2).

B. Rats infected with virulent Y. pestis and treated with antiserum. Rats immunized with the attenuated strain EV76(51f) and challenged with the virulent strain 195/P subsequently demonstrated a high degree of resistance to reinfection. Following this result, additional work was carried out to determine if rats which survive a virulent infection without benefit of prior immunization subsequently display a similar degree of immunity. For this experiment, 90 male rats were infected with the virulent strain 195/P by subcutaneous inoculation of approximately 48 organisms. Two days later, antiserum to Y. pestis was administered to the rats intraperitoneally. Twelve of the 90 rats later died of plague. The survivors were studied to determine their IHA response after the infection and for resistance to reinfection.

Most of the rats that survived the infection developed IHA titers of 128-256. Surprisingly, some survivors did not produce a detectable titer, while others developed only low titers (Table 6). IHA titers changed little from 57-92 days after infection, except that decreases were observed for animals with high titers. At 180 days post infection, increased IHA titers were found for a significant proportion of the test animals. An examination of the data revealed that most of the rats displaying an increased titer between days 92 and 180 were rats with buboes. Buboes were present in some animals on days 92 and/or 180, but some rats which did not have buboes on day 92 did have buboes on day 180. IHA titers also increased in a few rats that did not have externally discernible buboes, but the majority of rats without buboes displayed titers on day 180 that were less than the titers seen on day 92, again especially in rats with high titers.

Rats without discernible buboes on day 180 were used in experiments to assess resistance to reinfection. Subcutaneous and intranasal challenges were given (Table 7). Most rats with titers less than 64 died. However, death was delayed compared to previously uninfected controls. All rats with titers of 64 or more survived (only intranasal challenge was done). Some of the survivors of the subcutaneous challenge developed four-fold or greater increases in IHA titer, whereas the rats surviving intranasal challenge did not.

C. Rats vaccinated with Plague Vaccine USP. A study with Plague Vaccine USP was directed first to determining the serologic response of the Walter Reed strain of Rattus norvegicus to vaccination. Groups of male and female rats were vaccinated intraperitoneally, subcutaneously, and intramuscularly. The effect of antigenic mass of vaccine administered was investigated by comparison of high and low intramuscular doses. The vaccination schedules used for both sexes is given in Table 8.

In male rats, intraperitoneal vaccination was far superior to the other routes of inoculation investigated. The geometric-mean titer for rats vaccinated intraperitoneally was high and a greater percentage of rats responded to intraperitoneal administration of vaccine by producing IHA antibody (Table 9). Some males achieved titers as high as 1024 after only one booster inoculation. The subcutaneous route of vaccination was second in effectiveness. There was no apparent difference in the IHA response of male rats inoculated intramuscularly with high and low vaccine doses.

Results similar to those found in males were obtained for female rats (Table 9). The intraperitoneal route of vaccination was significantly more effective for inducing high titered IHA antibody. Female rats that responded to intraperitoneal vaccination produced significantly higher titers than those found in males, but fewer females than

males produced antibody. Unlike results for males, the subcutaneous route was only slightly better than the intramuscular route for vaccinating females. Little difference was found between females given high and low doses of vaccine.

IHA titers in rats vaccinated with the Plague Vaccine USP declined considerably over 3 ½ months, at which time many rats did not have a detectable titer that previously possessed one.

Three groups of rats vaccinated with Plague Vaccine USP were challenged subcutaneously with ten-fold quantities of virulent Y. pestis (Table 10). Results of this challenge demonstrated for rats vaccinated with Plague Vaccine USP the same relationship between titer and resistance to disease seen in rats immunized by other means. Whereas most rats with titers of 8 or less did not survive, animals with prechallenge titers of 64 or more usually survived, although survival was not quite as good as in rats vaccinated with living attenuated plague vaccine or in rats that had survived an earlier infection with virulent Y. pestis. The lower rate of survival could have been due to sex, since the rats with high titers challenged after Plague Vaccine USP were females, and in other experiments male rats were employed. All the rats that survived challenge following vaccination with Plague Vaccine USP demonstrated four-fold or greater increases in IHA titer.

D. Rats vaccinated with the Fraction 1 antigen of Y. pestis. This experiment was designed to determine if vaccination with the purified Fraction 1 antigen of Y. pestis could achieve the efficacy obtained with the whole-organism Plague Vaccine USP. As inoculum, freeze-dried Fraction 1 antigen was reconstituted with saline and mixed 1:1 with Freund's complete adjuvant. Rats were inoculated subcutaneously with doses equivalent to 1 ½ mg Fraction 1/K body weight. Subcutaneous booster inoculations were given at 7 and 14 days equal to ½ mg Fraction 1/K body weight. The rats were bled 28 days after the second booster inoculation. Of 78 animals vaccinated, 77 produced IHA titers of 16-2048, with a geometric-mean titer equal to 272. One rat did not develop a titer.

Rats vaccinated with the Fraction 1 antigen were challenged with virulent plague bacilli by subcutaneous and intranasal routes (Table 11). Rats with titers of 64 or higher survived. Survival was somewhat better than for rats with similar titers after vaccination with Plague Vaccine USP.

E. Discussion: Ecology and epidemiology. It has been shown that Rattus norvegicus vaccinated with living attenuated plague vaccine, whole-organism killed plague vaccine and the Fraction 1 antigen, or rats which survive infection without prior immunization because of serum therapy, respond with the production of IHA antibody. In all cases the same relationship between antibody titer and survival pertains. Rats with very low titers usually do not survive, although death may be delayed, suggesting partial resistance to infection. Survival rates are higher for rats with titers of 16-32, whereas most rats with titers of 64 or more survive. Without indications to the contrary, it can be assumed that adult Rattus norvegicus caught in the wild displaying IHA titers of 64 or more have experienced and survived infection with Y. pestis and are refractory to reinfection by either fleabite or cannabalism.

The laboratory work has also indicated that, after infection, IHA and CF antibodies may be retained for the life of a rat, irregardless of sex. Therefore, titers of these antibodies are not indications of when infection occurred. In experiments where rats were given Plague Vaccine USP or infected rats were administered antiserum treatment, IHA titers subsequently showed rapidly decline. These circumstances are not met with by rats in nature, of course. The laboratory data on rats immunized first with EV76(51f) and then challenged with virulent 195/P probably reflect more closely what might occur in the wild, such as immunization of wild rats by sublethal infection or with avirulent or attenuated strains of Y. pestis followed by exposure to virulent plague bacilli.

Clear evidence of cryptic infection with Y. pestis was obtained when rats without buboes three months after infection were observed with buboes at six months, with concomitant increases in IHA titer. Additional instances of the establishment of cryptic plague in another group of laboratory Rattus norvegicus will be discussed in the next section (II). As long as plague bacilli persist in rodent tissues, a mechanism for rodent-to-rodent transfer within a species or among different species exists. That mechanism is cannibalism or feeding upon infected carcasses. This mechanism may be very significant for the maintenance of Y. pestis through inter-epizootic and enzootic periods. The present studies are important because they demonstrate that IHA titer and the absence of detectable buboes does not necessarily indicate freedom from infection with Y. pestis. Sites of infection could be small and/or hidden internally where their presence would not be suspected.

F. Discussion: Vaccines and immunity to plague. Efficacy of plague vaccines may be a measure of different things. It may be the ability to induce IHA or mouse-protective antibodies in blood constituents. These type of data have been applied to estimate the

value of vaccines in man. Efficacy can also be the measure of resistance to disease; a value that can be estimated directly through challenge experiments with laboratory animals. Indirect evidence of resistance to disease has been obtained for man from vaccine trials and from other observations, such as the lack of clinical disease from Y. pestis in vaccinated American troops in Vietnam. The experiments reported here indicate that, at least in the laboratory rat, vaccines with an ability to induce IHA antibody will also protect a larger number of animals against fatal disease, since IHA titer was correlated with survival. An important point is that IHA titer is an indication of immune status, in R. norvegicus, irregardless of how the titer was induced. The Fraction 1 vaccine was an effective as the whole-organism Plague Vaccine USP or even the immunity derived from previous infection with virulent organisms.

The results of our experiments can be related to pneumonic plague, although only approximately since it is impossible to know exactly how many virulent Y. pestis reached the lungs by inhalation. Intranasal challenge produces infection of the tonsils, larynx and other structures of the upper respiratory tract, as well as of the lungs if sufficient numbers of virulent organisms are given at challenge. Since large numbers were used in the present studies, probably some Y. pestis did reach the lungs. Rats vaccinated with living attenuated EV-vaccine or with Fraction 1 antigen, and rats actively immunized from subcutaneous infection with virulent Y. pestis, showed no signs of disease after intranasal challenge in those rats with IHA titers of 64 or higher. Thus, these studies suggest that vaccination effective against bubonic plague will also protect against plague pneumonia, and that IHA titer may be an important indication of immune status to pneumonic plague.

The vaccines tested were composed of encapsulated organisms, alive or dead, or immunogenic subunit Fraction 1 of Y. pestis. The challenge experiments described in this report were all done with the classical virulent encapsulated strain 195/P of Y. pestis. Strain 195/P, of Indian origin, is representative of most of the strains of Y. pestis that have been found in nature. Thus, the results of our investigations apply to circumstances most frequently encountered with plague in rats. However, unusual strains of Y. pestis have also been found, such as non-encapsulated organisms, which retain virulence. It is uncertain how well IHA titer correlates with resistance to infection with or disease from these unusual types of plague bacilli.

II. Variants of Y. pestis derived in vivo from Rattus norvegicus.

It has been routine procedure in the plague laboratory to investigate the deaths of laboratory animals for infection with Y. pestis. In the course of such work, isolations of plague bacilli were obtained from two rats 14-months and 19-months after challenge. Both animals

had been vaccinated with the attenuated strain EV76(51f) of Y. pestis prior to challenge with the virulent strain 195/P. Plague, which had persisted in these two rats in some cryptic form, was considered to be the direct cause of death. Bacteriological studies of the Y. pestis isolated from the rats indicated that they were non-encapsulated strains, whereas EV76(51f) and 195/P used for immunization and challenge are encapsulated. Non-encapsulated strains of Y. pestis were not being studied in the laboratory at any time during the histories of these rats. Apparently, the non-encapsulated variants had arisen spontaneously during latent infection. In addition, both rats had high titers of IHA antibody at the time of death.

The variants caused deaths in mice, almost invariably with terminal septicemia, from one week to several months after inoculation. Mice often appeared sick several days after inoculation, but subsequently showed no signs of illness until time of death. Mice rarely developed buboes after subcutaneous inoculation of the non-encapsulated strains.

Work on the variants continues.

III. The significance of maternal antibody in Rattus norvegicus for the epidemiology and ecology of plague.

This investigation was completed early in the period covered by this report. Results have been described in manuscripts published or submitted to journals. In summary, it was demonstrated that maternal antibody increases the probability of surviving infection with Y. pestis, and some rodents may survive devastating epizootics because of maternal immunization. Furthermore, maternal antibodies should be considered in epidemiological surveys for antibodies to Y. pestis to avoid misinterpreting serologic data obtained from young animals.

Table 1 . Vaccination of male rats with the attenuated strain EV 76(51f) of *Y. pestis*

	Dose of EV 76(51f) administered	Number developing antibody/Number vaccinated	GMT of rats with titer (range in titer)
Group A	Inoculated into the heart:		
	17,800	24/24	31 (4-512)
Group B	Inoculated subcutaneously:		
	25,400,000	6/6	91 (4-512)
	2,540,000	3/6	40 (16-256)
	254,000	2/6	45 (32-64)
	25,400	2/6	32 (8-128)
	2,540	1/6	32 (32)

Table 2. Challenges of male rats vaccinated with the attenuated strain EV 76(51f) of *Y. pestis*

IHA titer at time of challenge	No. of *Y. pestis* strain 195/P inoculated subcutaneously		Average day of death		Percent survival (Groups A + B)
	497,000 (Group A)	217,000 (Group B)	Group A	Group B	
Unvaccinated (controls)					
0	0/4*	0/19	3.0	2.8	0
Vaccinated					
0-8	0/6	0/18	3.7	4.4	0
16-32	3/8	3/5	5.2	6.0	46
64-512	9/10	7/7	6	-	94

* Number survived/number challenged. Group A was challenged 26 days after vaccination; Group B was challenged 72 days after vaccination. All survivors of both groups developed 4-fold or greater increases in IHA titer.

Table 3. Persistence of antibody to the Fraction 1 antigen of Y. pestis in male rats immunized with the attenuated strain EV 76(51f) and challenged with the virulent strain 195/P

Rat (Group A)	IHA titer at time of challenge*	IHA titer at intervals after 195/P challenge								
		Day 22	Day 88	Day 115	Day 175	Day 240	Day 301	Day 361	Day 424	Day 484
22	16	8,192	8,192	16,384	2,048	1,024	512	dead		
19	32	512	128	32	4	16	8	16	8	0
4	32	2,048	16,384	8,192	4,096	2,048	1,024	4,096	256	128
24	64	1,024	128	128	64	64	128	64	16	16
5	64	2,048	16,384	16,384	4,096	1,024	1,024	2,048	256	256
17	64	4,096	8,192	16,384	2,048	4,096	4,096	4,096	1,024	4,096
10	128	1,024	2,048	2,048	1,024	512	1,024	dead		
6	128	2,048	16,384	16,384	2,048	1,024	512	2,048	512	512
1	128	2,048	16,384	16,384	4,096	2,048	512	512	128	64
21	128	8,192	8,192	4,096	1,024	1,024	512	1,024	256	dead
8	128	32,768	32,768	32,768	4,096	2,048	2,048	1,024	1,024	1,024
23	512	1,024	2,048	512	256	128	128	256	128	128

* 497,000 Y. pestis 195/P inoculated subcutaneously (day 1). All males were vaccinated with 17,800 Y. pestis EV 76(51f) in 0.1 cc of inoculum injected into the heart 26 days before the challenge with 195/P.

Table 3. Persistence of antibody to the Fraction 1 antigen of Y. pestis in male rats immunized with the attenuated strain EV 76(51f) and challenged with the virulent strain 195/P (Continued)

Rat (Group A)	IHA titer at time of challenge*	IHA titer at intervals after 195/P challenge				
		Day 546	Day 602	Day 662	Day 728	Day 787
22	16					
19	32	0	0	0	0	0
4	32	128	dead			
24	64	16	8	0	8	4
5	64	128	128	64	dead	
17	64	dead				
10	128					
6	128	dead				
1	128	64	128	128	64	64
21	128					
8	128	512	1,024	512	dead	
23	512	128	dead			

* 497,000 Y. pestis 195/P inoculated subcutaneously (day 1). All males were vaccinated with 17,800 Y. pestis EV 76(51f) in 0.1 cc of inoculum injected into the heart 26 days before the challenge with 195/P.

Table 4. Persistence of antibody to the Fraction 1 antigen of Y. pestis in female rats immunized with the attenuated strain EV 76(51f) and challenged with the virulent strain 195/P

Rat No.	IHA titer at time of challenge*	IHA titer at intervals after 195/P challenge								
		Day 63	Day 160	Day 241	Day 426	Day 482	Day 541	Day 607	Day 644	Day 729
39	16	2048	4096	2048	512	2048	1024	1024	512	256
35	32	1024	512	512	dead					
15	64	4096	1024	2048	dead					
19	1024	4096	8192	8192	dead					
48	1024	128,000	8192	8192	4096	8192	8192	8192	8192	dead

* 217,000 Y. pestis 195/P inoculated subcutaneously (day 1). Female rats were vaccinated with different doses of EV 76(51f) between two and six months before challenge with 195/P.

Table 5 . Intranasal challenges of male rats previously infected subcutaneously with virulent *Y. pestis*

	IHA titer		CF titer	
	pre-challenge	48 days post	pre-challenge	48 days post
Challenge No. 1 (4.1×10^4 *Y. pestis* 195/P)				
- subcutaneous inoculation:*				
rat N-25 (EV)	512	512	64	0
N-28 (EV)	512	512	16	0
N-30 (EV)	1024	4096	256	128
- intranasal instillation:*				
rat N-5 (MA)	512	1024	32	0
N-36 (EV)	512	1024	64	32
N-39 (EV)	1024	2048	128	128
	pre-challenge	31 days post	pre-challenge	31 days post
Challenge No. 2 (3.7×10^6 *Y. pestis* 195/P)				
- intranasal instillation:*				
rat N-19 (MA)	32	64	8	4
N-7 (MA)	32	4096	0	0
N-3 (MA)	32	8192	2	0
N-1 (MA)	32	8192	4	0
N-18 (EV)	128	512	64	32
N-31 (EV)	256	1024	64	64
N-38 (EV)	512	2048	128	256
N-26 (EV)	1024	512	0	0
N-37 (EV)	4096	8192	256	256
N-40 (EV)	8192	4096	4	0
N-27 (EV)	8192	8192	512	256
N-9 (MA)	8192	8192	256	256

* Non-immune rats (2) also included as controls (IHA and CF = 0), which died within 3 days after challenge. Symbols in parentheses indicate factor which probably permitted survival after subcutaneous challenge done 5-10 months prior to this experiment. EV = vaccination with attenuated *Y. pestis* strain EV 76(51f); MA = maternal antibody to *Y. pestis* obtained from dam.

Table 6. Correlation between IHA titer and buboes in rats infected with virulent *Y. pestis* and treated with anti-plague serum

IHA titer 57 days after infection (number rats)	GMT on day 92	GMT on day 180	
		Rats without detectable buboes	Rats with buboes*
0 (12)	0	1**	256
4-8 (2)	8	8	
16-32 (3)	64	81	
64 (6)	23	20	40
128 (20)	39	12	114
256 (27)	71	10	128
512-1024 (6)	72	8	

* Rats seen with buboes on day 92 and/or day 180 included.

** One rat without titer on days 57 and 92 developed an IHA titer of 8 by day 180.

Table 7. Challenges of male rats previously infected with Y. pestis and treated with anti-plague serum

IHA titer at time of challenge	No. of Y. pestis strain 195/P* (Route of challenge) 3,500 (Subcutaneous)	88,700 (Intranasal)	Average day of death: Subcutaneous challenge	Average day of death: Intranasal challenge	Percent survival (Combined data)
Nonimmune (control)					
0	0/5**	0/4	3.6	3.0	0
Previously infected					
0-8	4/35	0/3	6.3	3.7	11
16-32	3/15	0/4	7.2	6.2	16
64-512	-	9/9	-	-	100

* Subcutaneous challenge was 181 days after previous infection; intranasal challenge was 187 days after previous infection.

** Number survived/number challenged. Some survivors of the subcutaneous challenge developed 4-fold or greater increases in IHA titer, whereas survivors of intranasal challenge did not.

Table 8. Vaccination schedule for study of Plague Vaccine USP in rats

Groups for each sex	Route of inoculation	No. rats of each sex	Ml. Plague Vaccine USP inoculated (Lot K9703, exp. 3 May 1974)			
			Day 1*	Day 32	Day 49	Day 83
ip	Intraperitoneal	16	1	.5	.5	1
sc	Subcutaneous	16	1	.5	.5	1
im (high)	Intramuscular	16	1	.5	.5	1
im (low)	Intramuscular	12	.2	.1	.1	.2

* 26 July 1973

Table 9. Antibody to the Fraction 1 antigen of *Y. pestis* in rats vaccinated with Plague Vaccine USP

Serological result	Day of Expmt.	Male rats				Female rats			
		ip	sc	im (high)	im (low)	ip	sc	im (high)	im (lo
Percent of group with no detectable antibody*									
18 days after initial inoculation of vaccine	19	6	63	81	92	50	31	88	10
15 days after first booster inoculation	47	0	31	44	83	38	25	63	6
32 days after second booster inoculation	81	0	20	75	83	38	31	69	4
13 days after third booster inoculation	96	0	7	44	17	25	25	33	8
Geometric-mean IHA titer of rats with antibody**	19	22	6	4	4	35	11	11	0
	47	31	6	5	6	60	14	6	5
	81	61	11	7	4	208	21	9	13
	96	128	30	5	10	542	51	38	21
Range in IHA titer among rats with antibody	19	4-128	4-8	4	4	16-64	4-128	8-16	0
	47	4-1024	4-16	4-16	4-8	8-512	4-512	4-32	4-8
	81	4-1024	4-128	4-16	4	64-512	4-512	4-256	4-64
	96	8-1024	4-256	4-64	4-32	4-8192	4-4096	4-512	4-256

* No hemagglutination at a serum dilution of 1:4.

** Reciprocal of highest serum dilution giving 100% hemagglutination of antigen.

Table 10. Rats vaccinated with Plague Vaccine USP and challenged with virulent Y. pestis

IHA titer at time of challenge	No. of Y. pestis strain 195/P inoculated subcutaneously			Average day of death	Percent survival
	1,090	10,900	109,000		
Unvaccinated ♂ (controls)					
0	0/2*	0/2	0/2	3.2	0
Vaccinated ♂					
0-8	0/12	2/12	0/9	4.0	6
Vaccinated ♀					
0-8	1/13	1/13	2/13	4.6	11
16-32	0/2	2/2	1/2	13.3	50
64-512	4/6	6/6	2/6	11.5	86

* Number survived/number challenged. Challenge done 100 days after third booster inoculation of Plague Vaccine USP.

Table 11. Challenges of male rats vaccinated with the Fraction 1 antigen of Y. pestis in adjuvant

IHA titer at time of challenge	No. of Y. pestis strain 195/P* (Route of challenge)		Average day of death		Percent survival (Combined data)
	3,500 (Subcutaneous)	88,700 (Intranasal)	Subcutaneous challenge	Intranasal challenge	
Nonvaccinated (control)					
0	0/5**	0/3	4.2	3.0	0
Vaccinated					
16-32	2/5	0/4	9.0	9.2	22
64-256	14/15	8/9	11	13	92
512-2048	20/20	7/7	-	-	100

* Subcutaneous challenge was 37 days after the second booster inoculation of Fraction 1 antigen; intranasal challenge was 43 days afterwards.

** Number survived/number challenged. A 4-fold or greater increase in IHA titer was observed in 11% of the survivors from the subcutaneous challenge and in 20% from the intranasal challenge.

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 174 Ecology of plague

Literature Cited.

Publications:

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Presentations:

1. Cavanaugh, D.C. 1973. Plague survey and surveillance: A critical evaluation of current procedures recommended to detect plague in rodent populations. Presented at the Ninth International Congress on Tropical Medicine and Malaria, Athens, Greece, 14-21 Oct.

2. Cavanaugh, D.C. Plague. 1974. Presented at the Haffkine Institute Platinum Jubilee, Bombay, India, 7-16 Jan.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6537	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
NIS	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71Q	00	175
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Histopathologic Manifestations of Zoonotic Diseases of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 02	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	2	116
FISCAL YEAR CURRENT 75	2	116

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Enterocolitica; (U) Yersinia; (U) Peritonitis; (U) Bronchopneumonia;

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) This study is being conducted to determine the pathogenesis of Yersinia enterocolitica infection in laboratory animals and determine the zoonotic implications of this bacterium.

24(U) Pilot studies in rats will be performed to determine the pathogenicity of Yersinia enterocolitica via different parenteral and oral routes of inoculation. The study will then be initiated in dogs and the following information obtained: LD fifty, clinical disease, gross lesions, histopathologic lesions, serologic conversions, and determination of shedding capacity of infected host.

25(U) 74 02-74 06 Pilot studies in rats have been completed. Animals infected by intratracheal, intraperitoneal, subcutaneous and intravenous routes had systemic involvement characterized by embolic or bronchopneumonia, peritonitis, and abscesses or pyogranulomas in liver, spleen, kidneys, adrenals, mesenteric lymph nodes and subcutis. Orally, exposed rats and controls were free of lesions. Dog inoculation is pending until the availability of animal holding space.
For technical reports, see Walter Reed Army Institute of Research Annual Report 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 175 Histopathologic Manifestations of Zoonotic Diseases of Military Importance

Investigators.
Principal: MAJ Charles A. Montgomery, VC
Associates: CPT Walter P. Trevethan, VC; MAJ Brice C. Redington, MSC; Helen R. Jervis, Dr.Nat.Sc; MAJ James H. Thrall, MC; LTC Paul K. Hildebrandt, VC.

Background

To define, study, diagnose and control known and potential bacterial and parasitic diseases common to both man and animals of potential military significance. The major effort is directed toward defining the pathogenesis of these diseases utilizing gross pathology, microscopic pathology, histochemical and ultrastructural methods.

During the reporting period, research activities have been concerned with: (1) histochemical differentiation of Brugia malayi and Brugia pahangi; (2) the pathology of Brugia malayi infection in the Patas monkey; (3) the pathology of experimental Yersinia enterocolitica infection in rats.

Results and Discussion

Histochemical Differentiation of Brugia malayi and Brugia pahangi. Brugia malayi and Brugia pahangi coexist in western Malaysia where the former is considered a zoonosis and produces in man the classic lesions of filariasis. It is very difficult to differentiate the microfilariae of these two species because of similar and overlapping morphologic characteristics. Previous workers have attempted to distinguish them on the basis of frequency of sheath casting, morphology of the rectal protusion, measurement of the "Innenkorper," and ultrastructural characteristics. These methods are tedious and/or unreliable for differentiating the two species. In our study, smears of peripheral blood from B. malayi-infected Patas monkeys and gerbils were compared with smears from B. pahangi-infected cats and dogs after processing for the demonstration of acid phosphatase (AP) activity. B. pahangi showed strong diffuse AP activity throughout the with stronger activity at the excretory and anal pores. In B. malayi, AP activity was concentrated at the two pores and less strongly in the cephalic

space and in a restricted area equidistant from anal pore and tail tip. The rest of the body showed no or minimal activity. This method provides a practical, rapid, and reliable laboratory technique for obtaining a definitive etiologic diagnosis in the host.

Pathology of Brugia malayi Infection in the Patas Monkey. Four adult female Patas monkeys were each inoculated subcutaneously on the dorsum of both feet with 175 third stage larvae of B. malayi. The source of the parasites was Aedes aegypti mosquitoes (Black eye strain) which had been membrane-fed 11 days previously on infective Patas monkey blood. All exposed monkeys developed patent infections 10-12 weeks post-exposure and remained patent throughout the experiment. The clinical course of the lymphatic disease was followed by the use of radiopharmaceutical technetium-sulfur colloid scans of the lower extremities during the 36th to 40th week of infection. This work was done in collaboration with the Department of Medical Zoology, WRAIR.

Infected animals were euthanized and necropsied 14-18 months post-exposure. Gross lesions consisted of enlarged, tortorous, distal lymphatic vessels. Lymphatic varices were quite prominent. Individual vessels showed intermittent zones of dilatation and constriction. Histologic examination of affected lymphatic vessels revealed viable adult nematodes in the dilated areas with mild to moderate proliferative endolymphangitis. Constricted areas observed grossly were characterized microscopically by obliterative, granulomatous lymphangitis with dead nematodes within their lumina. Collateral branches containing viable nematodes were observed adjacent to these granulomatous lesions. An occasional focus of endophlebitis was present. The lymphatic lesions were limited to that area distal to the popliteal lymph node. A multifocal granulomatous splenitis was observed in two of the four infected monkeys. Granulomata contained dying microfilaria.

Twelve additional Patas monkeys have since been infected and pathology data is pending termination of the experiment.

Pathology of Experimental Yersinia enterocolitica Infection in Rats. Yersinia enterocolitica has been recognized as a cause of disease in man and animals for the past ten years. Reported occurrences of disease attributable to Y. enterocolitica have recently been on the increase in man and animals. To date, the organism has been isolated from chinchillas, hares, gerbils, guinea pigs, rats, galagos, vervet monkeys, dogs, cats, sheep, pigs, horses, cattle, frogs, birds, snails and man.

To date, more than 1000 cases of diseases caused by Y. enterocolitica have been diagnosed. Manifestations include appendicitis, ileitis, intestinal ulceration and acute lymphadenitis of the mesenteric lymph nodes. Yersinia enterocolitica has also been implicated in human cases of erythema nodosum, arthritis, myocarditis, pharyngitis, and septicemia.

In the spring of 1972, an outbreak of Y. enterocolitica caused two deaths, an infant and a young adult, among eighteen individuals reporting clinical signs. Although the source of the outbreak was not proven, a bitch with a litter of sick pups was implicated.

Because of the proven association of Y. enterocolitica with disease outbreaks in man and animals, the similarity of lesions and organs infected in these species, and the uncertainties of the source of infection, it was considered necessary to acquire more information about this bacterial disease. Laboratory rats were to be used initially, and, if the results warranted, further work would be undertaken using dogs.

Twelve male rats were divided into six groups of two rats each. The two rats in each group were infected with Yersinia enterocolitica organisms (from the North Carolina outbreak) by either the intraperitoneal, intratracheal, subcutaneous, intravenous or oral route of administration. One non-infected control group was maintained. Using a plate count method, it was determined that 8.8×10^8 organisms were given to each test animal.

One of the rats in the intratracheal group was found dead the next morning (day 1) while the two rats in the intraperitoneal group were found dead on day 2. All the other animals survived for 18 days at which time they were killed and necropsied. No significant lesions were observed in either the control group or in the animals infected orally. The lesions observed in the other animals consisted of pyogranulomatous foci of inflammation. Although the organs affected varied between individual rats, there was no consistent

variation between groups. The organs which most consistently contained pyogranulomatous lesions were lung, liver, spleen, lymph node, kidney and pancreas. The adrenal glands and bone marrow were affected less frequently. Subcutaneous abscesses were present in the two rats injected subcutaneously and in one rat injected intravenously.

The results of the rat inoculation indicate that Y. enterocolitica is indeed pathogenic for rats by all of the tested parenteral routes of inoculation. In light of the pathogenicity of Y. enterocolitica for rats and the implication of sick puppies as the source of infection in the North Carolina outbreak, it became important to continue the experimental work utilizing dogs. The protocol utilizing dogs has been approved and the studies are pending, awaiting the availability of suitable animal holding facilities.

PROJECT 3A161102B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 01
Surgery

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6527	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A162110A821	00	125
b. CONTRIBUTING	61102A	3A161102B71R	01	
c. [redacted]	CARDS 114(f)			[redacted]

11. TITLE (Precede with Security Classification Code)

(U) Control Mechanisms of Regional Circulation

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	2.5	240
FISCAL YEAR CURRENT 75	2.5	151

19. RESPONSIBLE DOD ORGANIZATION

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NAME: DA

21. GENERAL USE

Foreign Intelligence Not Considered.

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Peripheral Vascular Injury; (U) Venous Reconstruction; (U) Venous Occlusion; (U) Combat Injury

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To define mechanism regulating regional circulation, effects thereon of injury, and methods of restoring function. These studies apply directly to the technology of reconstructive vascular surgery necessary to repair combat wounds.

24 (U) Physiological techniques have been employed to document the hemodynamic response of peripheral vascular beds when subjected to venous occlusion and also when subjected to intraarterial injection of abuse drugs. Effectiveness of various materials as vein grafts has been evaluated by venography.

25 (U) 73 07 - 74 06. Previous title: Pathogenesis and treatment of shock. Completed studies in subhuman primates demonstrate that femoral venous ligation is associated with a significant reduction of arterial inflow and increases in venous pressure and vascular resistance; responses detrimental to an injured extremity. Lumbar sympathectomy causes reduced resistance and increased arterial flow; an effect that might be beneficial in cases of venous obstruction. Intraarterial injection of amphetamines, methadone, Darvon, barbiturates and morphine, all dilate the peripheral vasculature. This observation demonstrates that distal gangrene secondary to drug injection is not a result of vasoconstriction. A suitable prosthetic material has yet to be developed that can serve as grafts in peripheral veins. Collagen tubes, bovine heterografts, Dacron arterial prostheses and Dacron coated with a hydrophilic polymer all have been evaluated in femoral veins of dogs and have demonstrated poor results in terms of maintained patency.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*Available to contractors upon originator's approval

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PII Redacted

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01 Surgery

Work Unit 092 Pathogenesis and treatment of shock

Investigators.
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PFC John C. Kerr

1. Gastrointestinal Responses to Shock

a. Statement of the Problem and Background. Previous reports from this laboratory have demonstrated species differences in the hemodynamic response of the splanchnic vasculature to shock.[1,2,3] In canine models of endotoxin shock, mesenteric blood flow falls markedly while in subhuman primate models mesenteric flow remains unchanged for a prolonged period. Studies have been conducted to evaluate a mechanism that possibly maintains mesenteric flow in primates during endotoxemia.

Gastric mucosal ulceration has been observed in canine endotoxin shock. Although the etiology remains unclear, local mucosal ischemia has been implicated. The reported effects of endotoxin shock on canine gastric arterial blood flow are controversial and parallel studies have not been conducted in subhuman primates. Studies have been initiated to describe the effect of this form of shock on gastric hemodynamics in both dogs and baboons.

b. Experimental Approach. Mesenteric arterial blood flow was measured electromagnetically in baboons and the responses to intraarterial injection and infusions of catecholamine recorded. Arterial and portal venous pressures were monitored for calculation of vascular resistance. The effect of endotoxin shock was studied in splenectomized dogs with flow transducers placed on the left gastric and splenic arteries. The right gastric artery was ligated and catheters were placed appropriately to collect blood samples to determine gastric oxygen consumption during the course of the experiment.

c. Results and Discussion. Epinephrine and norepinephrine were injected into the superior mesenteric arteries of baboons in a log dose range from 10^{-3} to 10^{0} μg (base) kg^{-1} and infused at a rate of 0.05 μg kg^{-1} min^{-1}. The drug injections and infusions had no effect on control pressures except at the dose of 10^{0} μg kg^{-1} at which aortic pressure increased by 10-15 mm Hg. Norepinephrine injections caused dose dependent constrictor responses while infusion of the drug caused significant constriction that did not exhibit autoregulatory escape over a 10 minute period. When delivered as bolus injections, the two lower doses of epinephrine caused vasodilation while the higher doses caused

constriction. Epinephrine was infused at a rate calculated to mimic the amount perfusing the gut during experimental shock. At that concentration, epinephrine caused a progressive increase in mesenteric blood flow. Alpha adrenergic blockade with phenoxybenzamine potentiated dilator responses and attenuated constrictor responses while beta adrenergic blockade with propranolol exerted the opposite effect. The data thus suggest the possibility that the elevation of plasma epinephrine concentration during endotoxemia might account for the sustained mesenteric blood flow previously reported for baboons. These results have been published in preliminary form.

The studies on gastric blood flow are not complete and only canine data have been collected. Subsequent to the intravenous injection of a lethal dose of endotoxin, arterial pressure and blood flow to the stomach fell precipitously and remain at low values. The decrease in pressure and flow are in proportion and thus vascular resistance is not significantly increased; a response that is unusual to other canine splanchnic regional circulations. Oxygen consumption of the stomach increased during the first hour of shock and remained elevated during the remaining three hours of observation. This later observation is a paradox that has not been explained but is being subjected to further investigation.

2. Effect of Endotoxin on Tissue Oxygen Consumption

a. Background and Statement of the Problem. Many groups have recognized and documented differences in the shock produced by bacterial sepsis and shock due to other causes. The effect of endotoxin at the organ or tissue level of investigation remains controversial. Various suggestions of the effect of endotoxin have included a depressant effect on the heart (4), the systemic circulation (5), neither (6), and both (7). At the tissue level, endotoxin has been reported to detrimentally affect oxidative metabolism by liver mitochondria. A series of studies have been conducted to describe the direct effect of endotoxin on the oxidative metabolism of an isolated vascular bed and slices of rabbit liver.

b. Experimental Approach. Following surgical isolation of a rear leg of the dog, the femoral circulation was perfused by pump to maintain constant blood flow. A membrane lung and heat exchanger were used to maintain the arterial blood at normal levels of oxygen content, pH, PCO_2, and temperature. Oxygen and glucose consumption, lactate production, and arterial and venous pressures were measured over a one hour control period and for an hour subsequent to the intraarterial delivery of graded doses of endotoxin.

Rabbits were sacrificed with intravenous air, and liver slices (0.3 gm) were placed in cuvettes containing a balanced electrolyte solution, maintained at 37° C, and agitated. As the liver slice consumed oxygen, the oxygen tension was continuously monitored with an oxygen electrode. Oxygen content was calculated from PO_2, pH, and hemoglobin

concentration. Oxygen consumption (change in content) was calculated at various oxygen tensions. When the oxygen tension had fallen to 10 torr the cuvette was reoxygenated, endotoxin introduced, and the liver permitted to consume oxygen in the same manner.

c. Results and Discussion. These studies are nearing completion. The results demonstrate the following: (1) In the constantly perfused vascular bed, the intraarterial injection of endotoxin has no effect on arterial (perfusion) or venous pressures. (2) Oxygen consumption during the control period was constant and fell significantly following the injection of endotoxin. (3) Liver slices consumed oxygen at identical rates during duplicate runs but consumption was significantly reduced when endotoxin was present during the second run. The results suggest that endotoxin influences tissue metabolism by reducing oxygen consumption.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 01 Surgery

Work Unit Pathogenesis and treatment of shock

Literature Cited.

References:

1. Swan, K. G., Barton, R. W., and Reynolds, D. G.: Mesenteric hemodynamics during endotoxemia in the baboon. Gastroenterology 61: 872-876, 1971.

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3. Selmyer, J. P., Reynolds, D. G., and Swan, K. G.: Renal blood flow during endotoxin shock in the subhuman primate. Surg. Gynec. Obst. 137: 2-6, 1973.

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PROJECT 3A161102B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 02
Internal Medicine

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6451	74 07 01	DD-R&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71R	02	085
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with security Classification Code)

(U) Circulatory Responses to Disease and Injury

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 Physiology 002400 Bioengineering 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	7	251
FISCAL YEAR CURRENT 75	7	251

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with security Classification Code)

(U) Blood; (U) Coronary vessels; (U) Myocardium; (U) Oxygen

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Research is devoted to studies of the hemodynamic and biochemical controls of the heart and circulation particularly during stresses of military importance such as exercise and shock.

24. (U) This program integrates studies of subcellular metabolism with physiological studies in anesthetized and also conscious trained animals. While standard preparations are used, new ones are developed to meet specific program requirements.

25. (U) 73 07 - 74 06. An assay technique for 5'-nucleotidase has been improved and used in studies of the carbohydrate structure of this enzyme. Cyclic AMP appears to stimulate calcium uptake of sarcoplasmic reticulum via a low molecular weight protein. The stereochemical determinants of the coronary vasoactivity of adenosine have been established. Potassium deficiency does not appear to impair exercise performance. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 085 Circulatory responses to disease and injury

Investigators.

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Description.

Development and application of standardized biological preparations to long-term biophysical and biochemical studies of the controls of the circulation in the normal state and under the influences of physiological and pathological stresses.

Progress and Results.

During this reporting period major efforts have been directed toward further defining the relationship between coronary blood flow rate and myocardial metabolism, the factor(s) initiating collateral development in responses to coronary insufficiency, and the effects of hypokalemia on physical performance.

1. Metabolic Regulation of Coronary Blood Flow Rate.

Ventricular pacing at various rates in open-chest anesthetized dogs with surgical heart block is being used to examine the relationship between coronary flow rate, myocardial oxygen consumption rate and cardiac muscle adenosine levels. These experiments are designed to test the adenosine hypotheses of the regulation of coronary blood flow under normoxic conditions.

Efforts continue to develop a sensitive and reliable assay of adenosine which is applicable to biological specimens. Methods which work well when applied to pure aqueous solutions of this nucleoside (1-3) are unsatisfactory when applied to tissue extracts. An enzymatic isotope-dilution method based on the conversion of adenosine to 5'-AMP by adenosine kinase (1) gives very high and variable results when applied to tissue extracts, probably because of inhibitory substances in the extracts. A double isotope dilution enzymatic assay was developed based on the adenosine kinase catalyzed formation of ^{14}C-^{32}P-5'AMP from ^{14}C-adenosine (diluted by ^{12}C-adenosine in the sample) and γ-^{32}-P-ATP. While this method is insensitive to the presence of inhibitors of adenosine kinase in tissue extracts, it

requires stringent purification of the reaction product. The 5'-AMP analyzed must be contaminated by less than 0.0001 percent of the ATP originally present, a degree of separation which is unattainable by modern chromatographic methods. Recently described microbiological (2) and fluorometric (3) assay methods were found to be too insensitive to measure the (picomolar) amounts of adenosine present in tissue extracts, and both required tedious purification of adenosine tissue extracts, and both required tedious purification of adenosine prior to assay. At the present time the insensitive and relatively imprecise enzymatic spectrophotometric method (4) remains the best available method for the assay of adenosine in tissue extracts.

The assay method for 5'-nucleotidase developed last year has been further modified and improved, and a detailed report is being prepared for publication in the scientific literature. This method is convenient; over 100 assays can be performed in a day. It is specific for 5'-nucleotidase, which makes it especially useful for assaying 5'-nucleotidase in the presence of other phosphatases which can utilize 5'-AMP as a substrate. The method is precise, having an intra-sample correlation coefficient $\geq$ 0.97 over a substrate concentration range of 1-100 μM. This method compares favorably with standard optical assays for 5'-nucleotidase (Fig. 1).

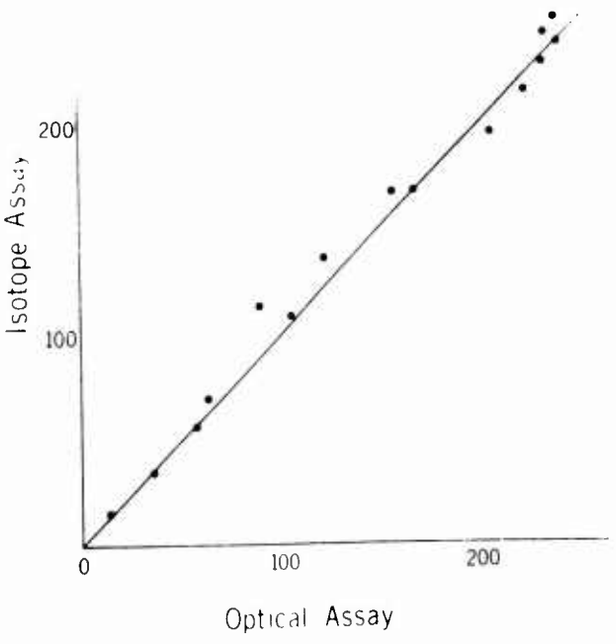

Figure 1. Comparison of optical and radioisotopic assays of 5'-nucleotidase $r^2 = 0.998$.

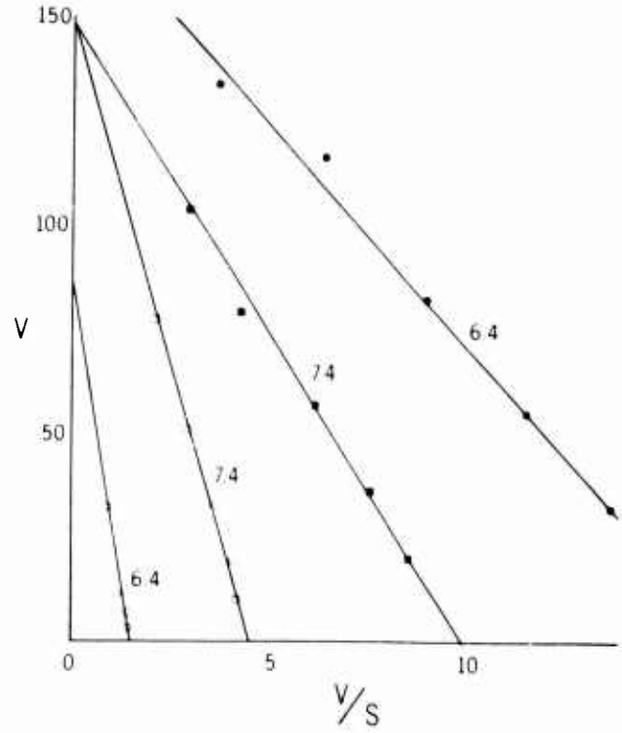

Figure 2. Effect of 61 nM AOPCP on 5'-nucleotidase activity at pH 6.4 and pH 7.4. Open symbols indicate velocity in the presence of AOPCP.

The kinetics of 5'-nucleotidase inhibition by adenosine-5'-α,β-methylene diphosphonate (AOPCP) were studied in detail during the development of the enzyme assay method. Figure 2 shows the results of a typical experiment. AOPCP is a competitive inhibitor in 60 mM TRIS·HCl pH 7.4 having a Ki of 0.05 μM. The enzyme is slightly (20%) activated in 60 mM TRIS·MES pH 6.4, which may be an expression of the remarkable sensitivity of this enzyme to buffer anion species (5). At pH 6.4 AOPCP is a mixed inhibitor, decreasing Vmax and increasing Km significantly. The type of inhibition found in our studies confirms the results of Burger and Lowenstein (5) which have recently been challenged by Evans and Gurd, who found AOPCP to be a noncompetitive inhibitor (6). Because AOPCP is a competitive inhibitor at pH 7.4 we have covalently linked it to Sepharose 4B (Affigel 10 ® purchased from Bio-Rad Laboratories) through the purinyl 6-amino group, and will use this in attempts to purify the enzyme by affinity chromatography.

Table 1: Effect of Lectins on Cardiac Microsomal 5'-Nucleotidase

Lectin	Sugars Bound	Concentration µg/ml	Cardiac Microsomal 5'-Nucleotidase 100% = 190 nmole/mg protein·min		
			Washed	Neuraminidase	Lysozyme
			100	96	55
Con A	α-D-Man α-D-Glc α-D-GlcNAc	10	53	45	22
WGA	$(\alpha\text{-D-GlcNAc})_{\geq 2}$	50	37	91	18
SBA	α-D-GalNAc	50	100	75	58
FBP	α-D-Fuc	50	109	105	64

Abbreviations: Con A, concanavalin A; WGA, wheat germ agglutinin; SBA, soybean agglutinin, and FBP, fucose binding protein. Carbohydrate nomenclature conforms with IUPAC Rules for Nomenclature of Carbohydrates (7).

Lectins and glycosidases were employed to examine the structure and functional importance of the carbohydrate moiety of cardiac microsomal 5'-nucleotidase. The results of one of two studies giving identical results is summarized in Table 1. The inhibition produced by lectins and lysozyme digestion was noncompetitive (Fig. 3), indicating two things: the carbohydrate moiety is not at the active site of the enzyme, and it is essential for enzyme activity. The loss of activity caused by lysozyme digestion is evidence for a carbohydrate chain containing 2 or more GlcNAc residues, and although both WGA and particularly Con A bind to other carbohydrates besides GlcNAc, their inhibitory effect, which is additive with that of lysozyme digestion, could also be explained by binding to $(GlcNAc)_{\geq 1}$. Although treatment of the microsomes with neuraminidase had an insignificant effect on 5'-nucleotidase activity, this treatment rendered the enzyme susceptible to inhibition by soybean agglutinin, which is evidence for a carbohydrate chain containing a penultimate GalNAc and terminating in sialic acid. Paradoxically, neuraminidase digestion of the microsomes rendered 5'-nucleotidase insensitive to inhibition by WGA; an amount of WGA which reduced 5'-nucleotidase activity of native microsomes by two thirds produced only 10 percent inhibition in neuraminidase-treated microsomes. Neuraminidase had no effect on Con A inhibition. These results are consistent with the complexity of the WGA binding site, which may consist of up to 4 subsites with differing binding affinities (8). In the study cited, the affinity of WGA for polymers of GlcNAc decreased as a function of chain length. The concentrations of GlcNAc, GlcNAc·NANA and $(GlcNAc\text{-}NANA\text{-})_2$ required for 50% inhibition of hemagglutination

by WGA were in the ratio 3000:50:1, indicating that shortening the length of the GlcNAc chain by cleavge of NANA or a NANA-containing residue will reduce the binding affinity of the lectin, and could explain the loss of lectin inhibition observed. The insensitivity of Con A inhibition to neuraminidase treatment suggests that this lectin may be binding to a different portion of the carbohydrate chain and perhaps to a different sugar, e.g., α-D-Man.

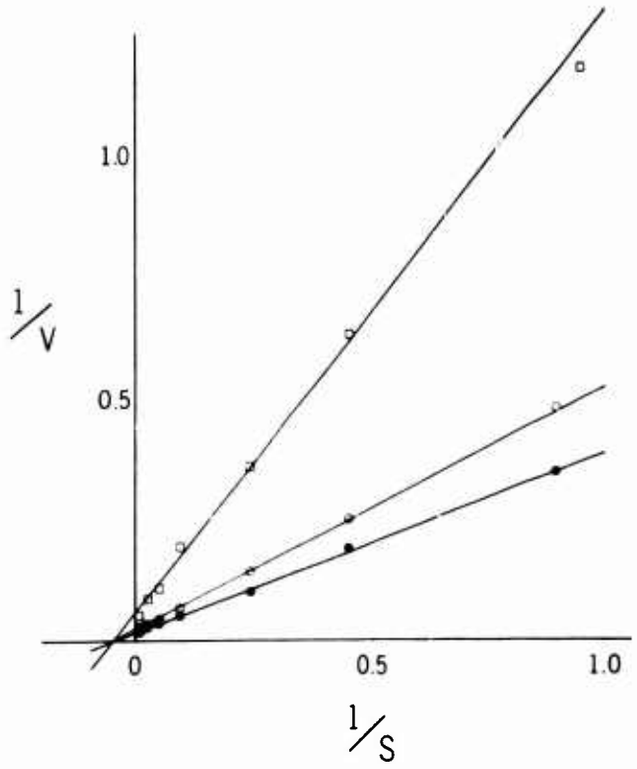

Figure 3. Effect of concanavalin A (2.5 μg/ml ○; 25 μg/ml □) on 5'-nucleotidase activity.

The lectin from Lens culinaris has been purified and preliminary studies have shown that this lectin binds to 5'-nucleotidase. The resulting inhibition of enzyme activity can be reversed by methyl-α-D-glucopyranoside. We plan to use this lectin in the preparation of affinity chromatography columns for purifying large amounts of the enzyme.

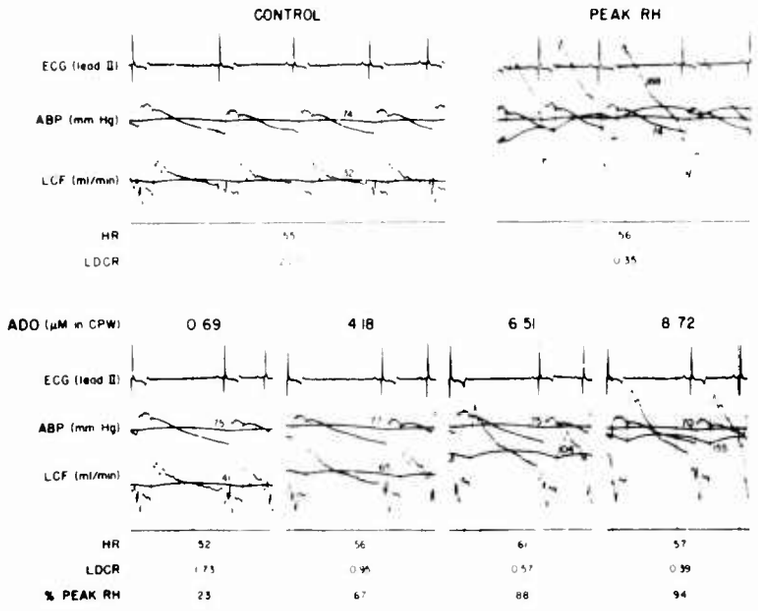

Figure 4. Effect of adenosine on coronary vascular resistance, showing that 8.72 μM adenosine (lower right) causes maximum coronary vasodilation, i.e., equal to peak reactive hyperemia (upper right).

The stereochemical determinants of the coronary vasoactivity of adenosine were investigated by intracoronary infusions of various purine ribosides and adenine nucleosides in conscious resting dogs (Fig. 4). Comparisons of dose-response curves for these compounds yielded the following structure-activity rules:

1. Purine substitutions at N^1 reduce vasoactivity.
2. Purine substitutions at C^2 enhance activity.
3. Replacement of the purine 6-amino-group generally decreases vasoactivity. The order of potency of the nucleosides tested was NH_2 > MeNH > Me_2N > H > Cl > $SCH_2\phi NO_2$ > Mes > S=O. Inosine was approximately 10^3 less potent than adenosine.
4. Alteration of the sugar moiety decreased vasoactivity. The vasoactivity of the deoxy ribosides decreased in the order 5'd > 3'd > 2'd. While epimerization at C'2, C'3 or C'4 completely abolished activity, substitution of a hydroxymethyl group for the anomeric hydrogen or 2'-O or 3'-O-methylation reduced but did not abolish vasoactivity.
5. Introduction of a bulky bromine atom at C-8 of the purine, which restricts the glycosidic torsion angle to the <u>syn</u> range, abolishes activity.

This study shows that the coronary vasoactivity of adenosine is determined by the erythro configuration of the 2'- and 3'-hydroxyls of D-ribose, and the relative potency is influenced by the chemical nature of the substituent in the purinyl 6-position. Electron-withdrawing substituents at purinyl C-2 enhance activity, whereas substitutions at N-1 decrease activity. The anti conformation at the glycosidic bond appears to be essential for activity. The determinants for coronary vasoactivity are not the same as those for binding to the putative membrane carrier system in cardiac sarcolemma (9).

Studies of the chemistry of energy-dependent processes in cardiac cell membranes have shown that the stimulation of Ca^{++}, Mg^{++}-ATPase of sarcoplasmic reticulum by cyclic AMP is Ca^{++}-dependent, being maximal at [Ca^{++}] medium of ca.10 μM and insignificant at > 100 μM. Under identical assay conditions the cyclic AMP-stimulated phosphorylation of membrane proteins was Ca^{++}-dependent and paralleled the cyclic AMP stimulation of Ca^{++}, Mg^{++}-ATPase. Polyacrylamide gel electrophoretic fractionation of the phosphoesterified proteins formed by incubation of sarcoplasmic reticulum with γ^{-32}P-ATP yields labeled proteins having molecular weights of 18-20,000, 45-50,000 and > 125,000 daltons, respectively. Phosphorylation of the 18-20,000 dalton protein is stimulated by cyclic AMP, suggesting that the enhancement of Ca^{++} uptake by sarcoplasmic reticulum may be mediated by a relatively low molecular weight protein.

2. Factor(s) Initiating Coronary Collateral Development.

Experiments have been started to determine whether the development of a collateral circulation by coronary constriction (10) is initiated by decreased pO_2 in the ischemic bed or by the pressure gradient established between the constricted vessel and neighboring coronary branches. Left-anterior descending coronary artery constriction is applied in conscious resting dogs by means of a hydraulic occlusive device. A fine silastic catheter implanted distal to this device is used to monitor the pressure gradient, and silastic catheters implanted in the great cardiac vein and in the coronary sinus are used to monitor the pO_2 of the venous effluent of the constricted descendens and normally perfused circumflex beds. Occlusion of the venous system during blood sampling by inflation of a pneumatic cuff at the terminus of the great cardiac vein minimizes contamination of coronary sinus blood by great cardiac vein blood. Initial studies show that this preparation is technically feasible and suitable, that the pO_2 of coronary sinus blood is 1-2 mm Hg higher than that of the great cardiac vein, that coronary artery constriction appears to decrease the pO_2 of the venous effluent simultaneously with the appearance of an intercoronary pressure gradient. Subsequent complete coronary artery occlusion caused an elevation of pO_2 in the venous effluent from the ischemic bed which disappeared after 2 days.

3. Effect of Potassium Depletion on Exercise.

Heavy physical training in a hot climate causes a loss of about 20 percent of exchangeable potassium in man (11). Dogs depleted of potassium by feeding a potassium-free diet together with the administration of deoxycorticosterone have markedly reduced skeletal muscle exercise hyperemia (12) and evidence for impaired cardiac performance (Knochel, J.P.: personal communication). In order to more precisely define the effect of potassium depletion on physical performance, the oxygen cost of performing a fixed program of treadmill exercise is being assessed in dogs undergoing dietary potassium depletion. Results to date indicate that heart rate, blood pressure, cardiac output and total body oxygen consumption during exercise (9 mph at an 11 percent grade for 20 minutes) are not affected by 15-25 percent depletion of body potassium stores.

Parallel studies of the effect of potassium depletion on skeletal muscle glycogen metabolism are too preliminary to give definitive results.

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10. Elliot, E.D., Jones, E.L., Bloor, C.M., Leon, A.S., and Gregg, D.E.: Day-to-day changes in coronary hemodynamics secondary to constriction of circumflex branch of left coronary artery in conscious dogs. Circ. Res. 22:237-250, 1968.

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Publications:

1. Wray, H.L., and Harris, A.W.: Adenosine 3',5'-monophosphate-dependent protein kinase in adipose tissue: Inhibition by tolbutamide. Biochem. Biophys. Res. Com. 53:291-294, 1973.

2. Elliot, E.C., Khouri, E.M., Snow, J.A., and Gregg, D.E.: Direct measurement of coronary collateral blood flow in conscious dogs by an electromagnetic flowmeter. Circ. Res. 34:374-383, 1974.

3. McLean, J.D., Khouri, E.M., and Olsson, R.A.: Stereochemical determinants of coronary vasoactivity of adenosine. Fed. Proc. 33:395A, 1974. (abstract)

4. Olsson, R.A., and Gentry, M.K.: Inhibition of cardiac 5'-nucleotidase by concanavalin A. Fed. Proc. 33:344A, 1974. (abstract)

5. Wray, H.L., and Gray, R.R.: Effect of triton on cyclic AMP stimulation of Ca^{2+}, Mg^{2+}ATPase in cardiac microsomes. Fed. Proc. 33:479, 1974. (abstract)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA 0A 6450	74 07 01	DD-DR&E(AR)636

3. DATE PRV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71R	02	086
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 11 F			

11. TITLE (Precede with Security Classification Code)

(U) Military Hematology

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

0800 Life Support 002600 Biology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
58 05	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		c. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	10	310
	CURRENT 75	10	171

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign intelligence not considered

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Coagulation; (U) Malaria; (U) Blood; (U) Blood Transfusion; (U) Anemia

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Studies of the pathophysiology of diseases with hematologic manifestations in soldiers and the investigation of those that occur as a direct military problem.

24. (U) Studies of hematologic abnormalities produced by chemicals, drugs, and infectious agents encountered primarily in military populations and in natives of geographic areas of potential military operations. Studies of blood, blood products, and blood substitutes used for the treatment of casualties and the prevention and diagnosis of diseases in soldiers.

25. (U) 73 07 - 74 06 Biochemical and biophysical studies of erythrocytes in normal blood, in certain disease states, and following biochemical and immunologic treatment of the cells were undertaken to identify factors affecting red blood cell survival in vivo and in banked blood. New methods for typing platelets using serotonin release were developed and studies of better methods for storage of platelets were initiated. The production of procoagulants by lymphocytes following exposure to endotoxin was identified and quantified. The toxicity of agents proposed for the treatment of sickle cell disease was investigated. Cyanate was shown to produce liver damage with glycogen accumulation, and the affected enzyme was identified. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 Jun 74.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 67 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 086 Military hematology

Investigators:

Principal: Marcel E. Conrad

Associate: Daniel B. Kimball, David J. Ahr, Jeffrey L. Berenberg, Patrick R. Bergevin, Johannes Blom, David H. Boldt, Robert P. Brouillard, John R. Durocher, Jon P. Gockerman, Michael J. Haut, John A. Kark, Robert G. Knodell, Frederick R. Rickles, Harold L. Williams.

Objectives: Basic and clinical investigation in the field of hematology.

Technical Approach: Basic and clinical studies were performed to investigate the functions of blood and blood-forming organs. These include investigations in the fields of immunohematology, nutritional hematology, coagulation, and red blood cell biochemistry and biophysics. The diversity of investigative undertakings and publications reflects the fact that this laboratory serves as the primary site for hematologic studies, referral of difficult diagnostic problems, training of hematologists, and standardization and quality control of laboratory reagents for hematological parameters for the U.S. Army.

Progress and Results: Cyanate therapy has been proposed as a treatment for patients with sickle cell anemia both to prevent and treat crisis, and is presently being used in human subjects to determine its safety and efficacy at other institutions. It has been postulated that cyanate prevents sickling by carbamylating the terminal valine residue of sickle hemoglobin. This reaction is nonspecific because cyanate reacts with the amino and thiol groups of many proteins and produces irreversible inactivation of G6PD activity in erythrocytes in vitro. We undertook studies in rodents in order to delineate the toxicity of cyanate. During the previous year we demonstrated that rats fed 50 mg/kg for 8 weeks developed mild lethargy and had excessive amounts of glycogen in the liver. Larger doses of sodium cyanate (100-200 mg/kg) caused severe lethargy and hind limb paralysis with pronounced glycogen deposition in the liver. Therefore the activities of various hepatic enzymes involved in glycogen synthesis and degradation were measured in rats receiving either oral or parenteral doses of sodium cyanate. In orally dosed animals, significant decreases in the activities of glucose-6-phosphatase and glucose-6-phosphate dehydrogenase were observed in the liver of treated animals. There was no significant effect upon phosphorylase, UDPG-pyrophosphorylase, glycogen synthetase,

phosphoglucomutase or debranching enzyme. In intraperitoneally dosed rats, significant decreases were observed in the activities of glucose-6-phosphatase, phosphorylase, and UDPG-pyrophosphorylase but not in glycogen synthetase, phosphoglucomutase, or debranching enzyme. In vitro studies on purified enzymes showed strong inhibition of glucose-6-phosphatase and glucose-6-phosphate dehydrogenase, and in both cases this was competitive with glucose-6-phosphatase. Cyanate inhibition of phosphorylase and UDPG-pyrophosphorylase was less potent and noncompetitive. At high concentrations, cyanate inhibited phosphoglucomutase. These data suggest that (a) in vivo administration of cyanate affects glycogen metabolism by a direct effect on the enzymes, (b) in vivo inhibition of enzymes by cyanate corresponds to in vitro sensitivities of the enzymes to cyanate, and (c) cyanate interacts with the glucose-6-phosphate binding site at the active center of the glucose-6-phosphatase and glucose-6-phosphate dehydrogenase enzymes. The effect of cyanate on muscle glycogen metabolism is currently under investigation. Preliminary studies reveal that cyanate affects the in vivo stimulation of muscle phosphorylase by AMP. A preliminary study of the pharmacologic effects of water-soluble vitamins on the neuromuscular toxicity of cyanate was conducted with no apparent beneficial effects. Further biochemical studies showed that cyanate caused no change in thiamine-dependent glycolysis. Preliminary studies of rats dosed with radiolabeled sodium cyanate demonstrated differences in the subcellular localization in the livers of animals that had previously received cyanate, with the greatest concentrations of radiolabeled cyanate in mitochondrial and microsomal fractions of hepatic cells.

In vitro studies of the effect of sodium cyanate upon red blood cells were undertaken. Cyanate was found to increase the net negative surface charge of human erythrocytes. In addition cyanate increased the deformability of erythrocytes from patients with sickle cell disease irrespective of their oxygen tension. Similar effects of cyanate upon deformability were observed in stored red blood cells. These changes in deformability may be related to a decreased calcium content of carbamylated membranes. Similar biophysical studies were performed in normal, pregnant women and in women using oral contraceptives. Although women taking oral contraceptives allegedly have decreased erythrocyte deformability, we were unable to confirm these results and attributed this to improvements in the composition of newer chemical formulation of contraceptives. On the contrary, erythrocyte deformability was decreased in pregnant women and the erythrocytes became less deformable as pregnancy progressed; these changes could be correlated to increasing levels of estrogen in the later stages of pregnancy. Studies were undertaken of the membrane of irreversibly sickled cells because sickle cell ghosts maintain their abnormal shape. Polyacrylamide gel electrophoresis of membrane proteins from sickle cells failed to show any significant abnormalities.

Studies of the mechanism by which the reticuloendothelial system recognizes aged and injured red blood cells were continued. Studies were undertaken to ascertain the mechanism by which complement fixation predisposes erythrocytes to hemolysis. It has been previously reported that red blood cells coated with a complement are temporarily sequestered in the reticuloendothelial organs of man. However, the mechanism of sequestration of the complement-coated red cells has not been defined. We studied two membrane properties possibly associated with the sequestration and extravascular destruction of human erythrocytes: surface charge and deformability. Complement was fixed to red cells by low ionic strength or cold agglutination antibody. Surface charge was measured as electrophoretic mobility in a Zeiss cytopherometer. Deformability was measured as filtration time of 2% red cell suspensions through 3 micron polycarbonate filters. No difference was found in the electrophoretic mobility of either normal or complement-coated red cells. However, red cells that were coated with complement by either method were found to be significantly less deformable than normal erythrocytes. Increasing amounts of complement as measured by the degree of agglutination with a specific anti-C3 antibody increased filtration time. Contrariwise, incubating the complement-coated red cells with serum returned the filtration time towards control values. It was postulated that decreased deformability of complement-coated red cells could retard their passage through the microvasculature, resulting in their rapid clearance from the circulation and prolonging their exposure to macrophages with C3 receptors. Alteration of C3 by incubation of complement-coated red cells with serum and associated return of deformability towards normal could explain the delayed return of nonphagocytized complement-coated erythrocytes to the general circulation.

Biophysical and biochemical studies of erythrocytes obtained from patients with various hemolytic states were undertaken to elucidate the characteristics that rendered these cells effete and made them recognizable as abnormal cells doomed to destruction either in the microvasculature or by the reticuloendothelial system. Red blood cells from patients with hereditary erythrocytic multinuclearity were selected as an example of a hemolytic disorder with grossly abnormal physiological functions. Abnormal amounts of phosphatidyl choline and glycosphingolipids have been identified in the membranes of these cells with a significant decrease in neuraminic acid concentration. These chemical changes appeared directly related to altered physiological characteristics of surface charge and membrane deformability which appeared to correlate with the altered immunological characteristics these membranes display. Studies of erythrocytes in spur cell anemia showed abnormalities in lipid content, osmotic fragility, and deformability. Correlation of changes pre- and post-splenectomy provided support for the concept of membrane conditioning by the spleen as a

factor in the etiology of hemolysis. Studies of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria showed defects in the binding of complement components to the membrane. A normal lipid composition was found and the lipids were functionally normal regarding complement binding. Further data is being obtained because this is the first strong evidence suggesting abnormalities in the protein components affecting complement binding in this disease.

Investigations are currently in progress to assess the physiologic importance of exterior membrane galactose moieties. Galactose will be removed or modified enzymatically on rat erythrocytes and these changes correlated with in vivo ^{51}Cr erythrocyte survivals. Microwave warming of banked human blood appears to decrease erythrocyte deformability without altering membrane proteins or surface charge. These changes may be related to the production of Heinz bodies. Efforts are being made to determine if the passage of banked canine blood through Swank or Pall filters prior to infusion interferes with erythrocyte viability. A study on the stability of the human erythrocyte membrane during ACD storage for 6 weeks is being completed. Special attention was given to deformability, surface charge, sialic acid content; cholesterol and phospholipid content; and membrane protein composition on polyacrylamide gel electrophoresis.

The participation of cell membrane in infections and inflammatory states was studied. In an attempt to identify a membrane receptor for the malaria parasite, erythrocytes were treated with various proteolytic enzymes and the effect on surface charge and merozoite invasion monitored. Chymotrypsin and primase eliminated invasion, but trypsin and neuraminidase did not. This suggested that the sialoglycoprotein is not the membrane receptor for the parasite. The negative surface charge of penicillin-sensitive, penicillin-resistant, and methicillin-resistant staph aureus was found to increase with increasing antibiotic resistance. Methicillin-resistant staph isolated from plates of penicillin-sensitive staph had a greater negative surface charge than its progenitor. The significance of these studies is not clear but might lend itself to rapid in vitro identification of methicillin-resistant staph.

A study is being completed that correlated the in vitro invasion of malarial parasite into enzymatically treated human and monkey erythrocytes and the changes in red blood cell membrane proteins on polyacrylamide gel electrophoresis. An attempt is being made to correlate pathogenicity of meningococcus with its net negative surface charge. Preliminary studies have suggested that increased electronegativity correlates with pathogenicity. Finally, a study is being completed correlating decreases in surface charge of human leukocytes following exposure to chemotactic substances--c.f. CSA, kallikrein, and transfer factor.

The effect of hypokalemia on glycogen metabolism of skeletal and myocardial muscle in dogs fed a potassium-free diet is being studied in collaboration with Dr. Randolph Patterson of the cardiorespiratory section at Forest Glen. Control values for enzyme activities have been established on tissue from normal dogs, and one dog has been maintained on the K^+-deficient diet for more than 60 days. This animal has had twice-weekly specimens obtained for sequential metabolic studies and potassium levels. Its total body potassium is approximately 50% depleted.

Application of our glycogen assays to the study of a patient with McArdle's syndrome has had two significant offshoots. (1) We have prepared pure rabbit muscle phosphorylase and will be generating antiserum in roosters. This may enable us to determine whether a given patient with absent muscle phosphorylase has inactive but immunologically reactive phosphorylase present; (2) We have normal values in our laboratory for glycogen-related enzymes in human muscle, and will be studying glycogen metabolism in muscles of patients with malignancy-associated weakness.

Over the past year, we have performed approximately 60 studies of red cell metabolism in patients with unexplained hemolytic anemia. Of the patients with detectable abnormalities, two families are particularly noteworthy. One family was found to have an unstable hemoglobin; this family is being studied in conjunction with Dr. Titus Huisman. Another family has a unique hereditary pattern of pyruvate kinase deficiency. In this family it appears that the father is a double heterozygote (the classic gene for PK deficiency and a silent mutation) and the mother is heterozygous for a silent mutation at the PK locus.

During the Fall, a TB-med on hemoglobin variants and hereditary enzyme defects was prepared.

Current work is focused on the interaction between human peripheral blood lymphocytes and sheep erythrocytes. In recent years the ability of a subpopulation of human lymphocytes to form rosettes spontaneously with sheep red cells has become recognized and has been widely applied as a surface characteristic of thymus-dependent (T) lymphocytes. Despite the increasingly widespread clinical application of this phenomenon, very little is known about the nature of the T-lymphocyte-sheep cell interaction.

Our results indicate that a mean of 60-65% of peripheral blood lymphocytes from normals are rosette-forming cells (RFC), this data deriving from nearly 100 separate determinations on many different subjects. We have chosen to study the nature of this reaction by introducing various perturbations into the system. Our data demonstrate that trypsin treatment of sheep erythrocytes markedly diminishes their ability to participate in

rosette formation. This effect of trypsin is both time- and concentration-dependent. The glycoprotein material released by trypsin is capable of inhibiting rosette formation between lymphocytes and intact sheep cells. This suggests that trypsinization of sheep erythrocytes solubilizes a glycopeptide fragment that serves as a receptor site for the rosette interaction. The tryptic glycopeptides can be fractionated by gel filtration on Sephadex G-75 into two major peaks: a high molecular weight, sialic-rich peak eluting at the front of the column and a hexose peak devoid of sialic acid which elutes after approximately 2-1/2 void volumes. Significant receptor site activity appears to be associated with the high MW fraction (> 50,000 daltons). This fraction can be further subdivided by anion exchange chromatography on DEAE cellulose into several (3 to 5) different species varying in their content of sialic acid. Several of these species appear to contain receptor activity, and present work involves identification of the most active fraction. Currently we are attempting to label the sialic-rich glycopeptide fragments using mild periodate oxidation of the terminal sialic residues followed by reduction with tritiated sodium borohydride. We hope to use this radiolabeled product to demonstrate specific binding to lymphocytes, thus establishing its role as receptor in the rosette interaction.

We have been investigating other means of perturbing the rosette system. Chloroform-methanol extraction of sheep erythrocyte membranes also yields a high molecular weight water-soluble glycoprotein rich in sialic acid. To date we have been unable to convincingly demonstrate receptor activity in this fraction. Experiments are planned to see if tryptic digestion can release this activity. Other experiments are planned to investigate alternative means of receptor release from the erythrocyte membrane including LIS extraction, anionic detergent extraction, and treatment with various protein perturbants to investigate in situ membrane localization of the receptor structure.

We have investigated the ability of a number of carbohydrate compounds to function as haptene inhibitors of rosette formation. We have found that simple sugars such as glucose and mannose are ineffective inhibitors, as is sialic acid. However, fetuin glycopeptide, a complex oligosaccharide, is several orders of magnitude more effective, indicating that rosette formation does involve an interaction between oligosaccharide surface components.

We have also investigated the effect of neuraminidase treatment of sheep erythrocytes on the rosette interaction. In contrast to observations reported in the literature, we observe no increase in percentage of rosettes following this treatment despite release of up to 80% of membrane sialic acid. Further confirming our impression that sialic acid by itself is not of major

importance in the rosette reaction is the fact that the desialyzed tryptic glycopeptides are as effective as native tryptic glycopeptides in inhibiting rosette formation.

We have been interested in attempting to demonstrate separate subpopulations of lymphocytes responsive to different lectins. Cuatrecasas (BBRC 52: 305, 1973) has reported that Con-A stimulation resulted in increased Con-A surface receptors, but no increase in wheat germ agglutinin receptors. We have carried this work further by investigating other lectins in this system. Preliminary data indicates that Con-A stimulation increases not only Con-A receptors, but PHA receptors as well. These data suggest an overlap between lymphocyte populations responsive to these two lectins. Further experiments are planned to investigate receptors for other lectins, especially nonmitogens. These cell lines are NC-37 and PA-3, B cell lines, and Molt-Y T cell line. These cells are available in gram quantities and will be used to evaluate differences in lectin receptor sites. Also surface glycopeptide structures will be radiolabeled (using periodate oxidation of sialic residues or galactose oxidase, both followed by reduction with tritiated sodium borohydride to label terminal sialic and/or galactose residues). The radiolabeled membrane glycopeptides will be extracted using LIS and examined by ion exchange chromatography and/or polyacrylamide gel electrophoresis for significant differences.

Platelets prepared by standard techniques of washing in protein buffers with calcium and fibrinogen frequently lose their ability to react to aggregating agents in standard low concentrations unless resuspended in plasma. Utilizing a ficoll-hypaque density gradient and a resuspending medium containing Tyrode's solution, heparin, fibrinogen with and without albumin and apyrase, we obtained platelets retaining their ability to aggregate in presence of $4 \times 10^{-6}M$ of ADP and epinephrine, collagen suspensions, and thrombin (0.25u/ml). Electron micrographs showed preservation of normal discoid shape with intact α-granules, dense bodies and canalicular systems. No significant difference was seen when these platelets were compared to platelets similarly fixed in platelet rich plasma (PRP). ^{3}H-5HT release after aggregation with bovine thrombin (2.5u/ml) was comparable to that in PRP (62% vs 69%), as were platelet ATP, ADP, and serotonin content and ATP/ADP ratios (1.2 vs 1.3). After two washes using this method, response to low concentrations of aggregating agents remained unchanged for at least 4 hours with incubation at 37°C. In contrast, platelets prepared either without a gradient or with a bovine serum albumin gradient frequently showed poor reactivity to aggregating agents and marked disruption and aggregation when examined by electron microscopy. Ficoll-hypaque density gradient separation is a simple and reproducible method for collection of washed platelets free of plasma that retain functional and morphologic characteristics of platelets in autologous plasma.

Studies were undertaken to find better methods for platelet typing for use in platelet transfusions. Patients receiving random platelet transfusions could become alloimmunized and show no rise in platelet counts for the poor hemostatic effect from transfused platelets. A method proposed to avoid the effect of alloimmunization disease is the use of HLA lymphocyte typing to find an HLA-compatible donor. This method requires specialized reagents and techniques. To bypass this problem we have developed a modification of the radioactive serotonin platelet release technique originally developed for ITP factor. Modification of this technique allows use of small quantities of serum from the alloimmunized patient as antibody and tritium-labeled serotonin-labeled platelets as the antigen. Presence of an alloantibody in the sera specific for platelets results in the release of labeled serotonin and indicates an incompatible match. This technique was evaluated clinically, and when no serotonin release occurred between the patient's sera and platelet samples, there was a normal platelet rise after platelet transfusion. However, when serotonin release occurred, no or little platelet rise was seen. Patients who showed no rise in platelet counts when given random platelets usually caused release of serotonin from random donor platelets when their sera were added. In these same patients, platelet typing of the families resulted in the finding of compatible donors whose platelet transfusions caused a rise in the platelet counts of the recipient. Chromium 51 tagging of platelets was performed; platelet survival studies revealed (1) an extremely short persistence of randomly obtained platelets showing abnormal serotonin release, and (2) a normal survival of platelets in which no serotonin release was observed. This new technique allows rapid matching of platelets and requires no specialized reagents. We found that platelets may be stored for as long as 48 hours at room temperature before the test is performed, thus allowing blood samples to be mailed for testing. It is believed that the technique may be of aid in the problem of tissue compatibility and evaluating HLA antigen patterns on platelets vs lymphocytes.

Studies of the isoantibody specificity in post-transfusion purpura were undertaken using serum from a fatal case of post-transfusion purpura. Antiplatelet antibody in this serum specimen showed specificity for the PL^{A1} platelet isoantigen in that the antibody was adsorbed by all PL^{A1} positive platelets tested but not in PL^{A1} negative platelets. Some PL^{A1} positive platelets that fix complement with the reference anti-PL^{A1} did not fix complement with this antibody. When used with these platelets, the patient's antibody competitively blocked the complement-fixing activity of reference anti-PL^{A1}. There was no evidence for lack of antigenic sites on the noncomplement-fixing platelets or for antibodies with more than

one specificity in the patient's serum. These studies, therefore, indicated that complement-fixing antibody of different samples of anti-PL^{A1} are variable and that this variation is probably due to distribution of PL^{A1} antigenic sites on cell surfaces rather than differences in antigenic structure that affect antibody affinity.

A quantitative assay for the plasma factor deficient in von Willebrand's disease (VWD) was developed in collaboration with Columbia University and the University of Connecticut. We assessed this new procedure (quantitative correction of ristocetin-induced platelet aggregation) in 15 patients with von Willebrand's disease and 20 normal subjects as well as 14 patients with Hemophilia A. Comparisons were made between the bleeding time, factor VIII procoagulant activity ($VIII_{ACT}$), factor VIII antigen content ($VIII_{AGN}$), and the von Willebrand factor ($VIII_{VWF}$) as measured by our assay. The correlation coefficient between $VIII_{VWF}$ and $VIII_{AGN}$ was 0.94 but bleeding time prolongation in von Willebrand's disease correlated best with $VIII_{VWF}$.

In an attempt to further elucidate the molecular abnormalities in von Willebrand's disease, $VIII_{AGN}$, $VIII_{ACT}$, and $VIII_{VWF}$ survivals have been studied in six patients with von Willebrand's disease following cryoprecipitate therapy or epinephrine infusion. Variable response to factor VIII transfusion has produced conflicting results, but it is clear that some patients process infused material quite differently than was postulated in the literature and that they maintain levels of $VIII_{AGN}$ for longer periods of time. Epinephrine appears to induce release (synthesis?) of both $VIII_{ACT}$ and $VIII_{AGN}$ in patients with both VWD and Hemophilia A. $VIII_{VWF}$ studies are yet to be completed and depend upon FDA release of Ristocetin. The final results of this study should provide some insight as to the number of molecules involved in the defects of VWD.

Cryoprecipitate from normal volunteers has been prepared and re-infused to determine the "normal" T 1/2 of both $VIII_{ACT}$ and $VIII_{AGN}$. Most of the studies of VIII survival in the literature fail to measure the normal values for these parameters or lack the $VIII_{AGN}$ data. This project is continuing and will hopefully provide baseline information regarding the in vivo survival of this (these) molecule(s).

In addition to VWD we have had the opportunity to study in detail three families with defects of both hemostasis (platelets-vascular factors) and coagulation (clotting factors). A large family with both Hageman factor deficiency and a thrombocytopathy is being reported as well as two families with thrombocytopathies in association with hemophilia A. These cases are particularly instructive in that all patients had prolonged bleeding

times, abnormal PTT's, and defective platelet retention by glass bead filters; they were referred, therefore, with the diagnosis of von Willebrand's disease. A review of such combined defects is contemplated, but such cases are exceedingly rare.

Although organ infarction due to vascular occlusion is a hallmark of sickle cell disease (SCD), the role of the coagulation system in the pathophysiology of this disease remains uncertain. We have recently reviewed the current status of investigation in this area of sickle cell research, constructed a hypothetical model for hypercoagulability, and recommended new avenues for therapeutic trials and clinical research.

Lymphocytes have recently been found to be a source of the extrinsic coagulation protein tissue factor. The role of this clotting protein in biology remains unknown, but recent work in our laboratory has suggested its importance in the mediation of cellular immune function.

Lymphocytes recovered from a rejected human renal allograft were cultured in vitro and assessed for their ability to generate both soluble mediators and tissue factor. The lymphocytes were shown to be of recipient origin by sex keryotyping. Unlike control cells (peripheral blood lymphocytes) which required antigenic or mitogenic stimulation in vitro to produce mediators, the kidney lymphocytes or so-called "rejection cells" were capable of generating macrophage migratory inhibition factor (MIF), mitogenic factor (MF), chemotactic factors (CF), and tissue factor (TF) without exogenous stimulation. It was of interest that shortly before clinical rejection of the allograft was noted, the patient excreted large quantities of fibrin degradation products in her urine. It is our postulate that blood coagulation plays an important role in the chronic rejection reaction and that the tissue factor pathway may be initiated by sensitized lymphocytes.

Studies on the interaction of endotoxin (lipopolysaccharide) and lymphocyte membranes have been completed with the collaborative efforts of Dr. John Hardin of the National Institutes of Health and Dr. Jack Levin of The Johns Hopkins University. Endotoxin has been shown to be mitogenic for human lymphocytes and a potent activator of lymphocyte tissue factor. Dissociation exists, however, between the membrane effects (where TF resides) of endotoxin and subsequent nuclear events. Further ultrastructural studies and binding experiments are now underway to clarify the effects of endotoxin on the cell surface coat and thereby provide information on tissue factor activation.

Trypanosomiasis is a devastating parasitic infection that has been reported to produce disseminated intravascular coagulation (DIC) in a single human case but thrombocytopenia without DIC in an experimental rat model utilizing the same strain. We have investigated the coagulation changes in both monkeys (using T. rhodesiense) and dogs (using T. congolense) and have found marked differences in the responses. Alhough T. rhodesiense produced thrombocytopenia in monkeys, no evidence for DIC was found. T. congolense, however, while producing similar thrombocytopenia, regularly induced profound changes of DIC in beagle and German shepherd dogs. The similarities and differences of these strains are currently under study but may provide important models for the study of the effects of parasitic diseases on platelets and coagulation factors.

During the last 6 years this laboratory has made and distributed serum specimens to nine international laboratories participating in a collaborative endeavor to establish standards and standard referee methods for the measurement of iron and iron-binding protein in serum specimens. A simple method that permits the reproducible measurement of iron in serum specimens in the various collaborating laboratories was developed and reported. Methods for the measurement of iron-binding protein in serum specimens have not been reproducible. Methods for the measurement of iron-binding protein are important because they aid in the differentiation of patients with a low serum iron and iron deficiency from patients with infection or an inflammatory condition that have a low serum iron concentration despite a normal body store of this element. The collaborating group has failed to improve existent techniques sufficiently so that they could be used as a referee method. However, the methods have been improved so that reproducible results with about 5% error can be obtained in different laboratories. The error factor seems to be due to an iron-binding fraction in serum other than transferrin. Solid phase radioimmunoassay methods are being developed for both ferritin and transferrin. These methods may be more practical than biochemical methodology for establishing a standard biochemical technique.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 086 Military hematology

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6453	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	N/A	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71R	02	088
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Military Nursing Research

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Internal Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
58 03	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

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c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 74	4.92	74
	CURRENT 75	5.0	74

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military Nursing; (U) Clinical Assessment; (U) Patient Teaching; (U) Survey; (U) COMPSY.

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Develop rational underlying military nursing and provide a basis for clinical nursing practitioners to improve the quality of health care services; determine the effectiveness of patient teaching; and use of computers in military psychiatry.

24. (U) Assessment of methods enhancing sleep, nurse-patient communications, differences in knowledge scores of hypertensive patients treated in different treatment facilities, biorhythmicity of heart rate in MI patient, quality of nursing care, patient satisfaction with hospitalization; structured vs. unstructured preop teaching; and testing the use of computerized nursing applications.

25. (U) 73 07-74 06 Differences in knowledge scores of hypertensive patients treated in different treatment facilities, quality of nursing care, and patient satisfaction with hospitalization are completed; data have been collected for methods enhancing sleep, nurse-patient communications and pilot study, biorhythmicity of heart rate in MI patient; structured vs. unstructured preop teaching is in progress; computerized nursing applications in psychiatry continue to be used and assessed. Portions of this work have been submitted to Walter Reed Army Medical Center under Work Units 9026, 9027, 9031, and 9032. For technical reports, see Walter Reed Army Medical Center and Walter Reed Army Institute of Research Annual Progress Reports, 1 July 1973 - 30 June 1974.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 088, Military Nursing Research

Investigators:
Principal: LTC Margaret L. O'Dell, ANC
Associates: LTC Geraldene Felton, ANC
MAJ Waltraut M. Hurd, ANC
MAJ Eunice J. Kennedy, ANC
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Description.

Research in military nursing is concerned with both direct and indirect patient care, and with identifying and testing principles underlying nursing care. The research endeavors being reported consist of the following: continuous heart rate monitoring of myocardial infarction patients; comfort measures to promote a physiological state; structured versus unstructured pre-operative nursing preparation of patients for surgery; knowledge hypertensive patients have concerning their illness and prescribed treatment program; hospitalized adults' understanding of selected terms and abbreviations used by nursing personnel; selected factors associated with patient satisfaction in Army Medical Facilities; and computer support in military psychiatry.

Progress and Results.

1. Continuous Heart Rate Monitoring of Myocardial Infarction Patients.

Within the last two decades, numerous studies have demonstrated the existence of biological rhythmicities in living organisms at all levels of structural organization. Recognition of the importance of the temporal aspects of biological functioning has lead to the emergence of a discipline known as biorhythmology or chronobiology. The purpose of this study is to investigate the circadian and ultradian rhythms of heart rate in myocardial infarction patients and to determine if there are detectable factors which alter these rhythms. The pilot study consisting of four subjects has been completed. Data is in process of being analyzed. Findings from the pilot data will determine whether or not a definitive study will be conducted. This research is being reported under Work Unit Number 9031.

2. Comfort Measures to Promote a Physiological State.

Nurses use both physical and mental comfort measures in giving patient care. This study is designed to observe the effectiveness of two relaxation techniques in promoting sleep. A skeletal muscle relaxation technique (representing peripheral entry into relaxation) and relaxation via imagery (representing central entry into relaxation) will be compared with two control groups to determine if the methods differ significantly in reducing sleep latency. Adult subjects are randomly assigned to one of the four groups, and written consent of voluntary participation is obtained. The IPAT Anxiety Scale and the MPI Scale are administered for descriptive population indices. A sleep history questionnaire, sleep rating scale, and sleep record are completed. All groups, with the exception of one control group, listen to a 10 to 15 minute audio tape recording twice a day for three days. A sleep record is completed daily on the morning of each study day. On the terminal day, a sleep rating scale, daily sleep record, sleep latency change scale, and exit interview questionnaire are completed. Data has been collected on 120 subjects and is in the process of being analyzed. This research is also being reported under Work Unit Number 9026.

3. Structured Versus Unstructured Pre-Operative Nursing Preparation of Patients for Surgery.

In addition to the stress and depression associated with anesthesia, the trauma of surgery triggers a series of metabolic and endocrine phenomena which are part of the widespread response of the body to injury. Convalescence after injury is completed when the body has returned to as near normal functioning as is possible. There is at present, however, a paucity of knowledge about results of pre-operative structured teaching of patients and its contributions to convalescence. There is a general need for reliable and valid criteria of patient well-being to determine the effectiveness of nursing intervention with the surgical patient. The purpose of this study is to assess the effectiveness of a structured nursing approach for pre-operative teaching of the surgical patient on manifest anxiety level, incidence of post-operative complications, ventilatory function, and patients' perceptions of psychological well-being. Information regarding experimental and control adults requiring surgery will consist of two general types of data: (1) evaluation of circulatory and respiratory status including type and incidence of post-operative circulatory and pulmonary complications and post-operative respiratory functioning using the Wright Respirometer; and (2) comparison of pre-operative and post-operative scores on three Personal

Orientation Inventory Scales that measure psychological well-being, and pre-operative scores on the IPAT Anxiety Scale. Data collection is in progress. Thirty-two subjects have been studied thus far. This research is also being reported under Work Unit Number 9032.

4. Knowledge Hypertensive Patients Have Concerning Their Illness and Prescribed Treatment Program.

Statistics describing the prevalence of chronic disease conditions in the United States are overwhelming. Hypertension is one of the most common chronic diseases, and directly, or indirectly, it constitutes one of the major causes of disability and death in this country. This study was conducted to identify differences in knowledge about the condition and treatment regimen among essential hypertensive patients receiving care in an outpatient medical clinic and a nurse-manned clinic. Data were obtained from the responses to questions on an Interview Guide with three major categories: 1) nature of the disease, 2) medication, and 3) diet. Data were analyzed by descriptive techniques, analysis of variance, and chi-square. One hundred and seventy-five adults, ages 25-83, who attended a medical clinic and a chronic-care clinic in two Army outpatient facilities were subjects for this study. The subjects in both clinics were similar in the following characteristics: the majority were female, dependents, married, between 40 and 59 years of age, had between 12 and 14 years of formal education, a family history of hypertension, had known about their hypertension over five years, kept most of their appointments, and did not smoke. In respect to race disbribution, however, more non-whites were found in the medical clinic than in the chronic-care clinic. There were higher knowledge scores among subjects in the chronic-care clinic than in the medical clinic, significant at $p < .001$ level. More subjects in the medical clinic had diastolic blood pressures which deviated above an acceptable level for their age, than in the chronic-care clinic. This finding was statistically significant. There was no significant difference between smokers and non-smokers in terms of mean knowledge scores. The findings were explained on the basis of difference in compliance in relation to the interaction of the client with the caretaker. This study adds to the increasing evidence that the nurse provides a new dimension in outpatient services in terms of promoting health maintenance and supervision for hypertensive patients. Patient compliance is a major problem in any approach to health maintenance. Admittedly, there are a few studies relating compliance to physician-patient interaction, however, substative studies are needed to determine the influence of the nurse on compliance behavior among chronically-ill outpatients.

5. Hospitalized Adults' Understanding of Selected Terms and Abbreviations Used by Nursing Personnel.

Man, a social animal, communicates with others in order to give information or to receive information with which to guide his actions. To be of value, the information, which may be written, verbal or non-verbal, must be understood by both the sender and receiver. As in other professions, practitioners in medicine and nursing have a language of their own and a shorthand version of it which they use when caring for patients. Communications between patients and nursing personnel is basically verbal, and for it to be effective, it needs to be understood. The purpose of this study is to determine what selected terms and abbreviations require a more comprehensive explanation in order to improve patient-nursing service personnel communications. An interview schedule, using each term and abbreviation in a sentence, was used to obtain verbatim definitions from each subject. Data collection has been completed and is now in process of being analyzed. This research is also being reported under Work Unit Number 9027.

6. Selected Factors Associated with Patient Satisfaction in Army Medical Facilities in the United States.

A vast number of patients are cared for daily in hospitals throughout the United States. Patients are admitted for a variety of reasons ranging from routine physical examinations to serious illnesses and injuries. Regardless of the reason for any hospitalization, each patient is entitled to the best possible care that can be provided, but who defines what that care is to be? Traditionally, it has been defined by hospital personnel. In recent years, a patient revolt has been in progress, and now, patients want to help define their care. The purpose of this study is three-fold: (1) to gain knowledge of the satisfaction and dissatisfaction of patients regarding selected aspects of their hospitalization; (2) to gain knowledge of patients' perceptions of their health; and (3) to obtain information about the perceived importance of selected aspects of patients' hospitalization. Five hundred and sixty-two adults hospitalized on medical and surgical wards in 11 Army medical facilities in the U.S. were the subjects for this study. Forty-four per cent of them preferred to remain in the hospital and 56 per cent preferred to leave. The biographic characteristics of those preferring to leave were: active duty; 18 to 25 years of age; male; white; and no previous hospitalizations.

Eighty-six per cent of all the respondents were satisfied with their hospitalization. The stayers and leavers differed in their overall satisfaction with a larger percentage of the stayers being satisfied. Forty-eight per cent of all patients perceived their health to be poor or fair, while 52 per cent perceived their health as good or excellent. There was little difference, however, between stayers and leavers in perceived health status. Stayers and leavers had similar perceptions of the importance of 87 per cent of the items regarding hospitalization, but the importance they attached to nine items was significantly different. The seven items perceived as more important by stayers than by leavers were: seeing the clergy; having adequate lighting; getting enough sleep; talking with a dietitian; obtaining a bedpan or urinal when needed; having a means to contact ward personnel; and being instructed about care after discharge. Leavers, however, perceived these two items as more important than did stayers; having personal records and recreational activities available. A significant difference existed between stayers and leavers in the satisfaction expressed with 46 of the 68 items (68 per cent) concerning their hospitalization. Stayers were more satisfied. Ten items in the questionnaire, important to many respondents, were dissatisfying to at least 20 per cent of them. One-half of these were items for which patients depend on physicians, nurses, and other hospital personnel for information. Three items related to the environment; having a pleasant environment; getting enough sleep; and having undisturbed rest. Other dissatisfactions were having to wait for diagnostic tests and not having hospital clothing that fits. Fifteen items, important to many respondents, were satisfying to at least 90 per cent of them. Seven of these concerned the adequacy or cleanliness of the physical plant, equipment, and supplies. Six items involved the physical care given by hospital personnel, and two items concerned patients' orientation to the unit and hospital. There was a significant difference in the satisfaction of respondents in relation to six biographic variables. Patients hospitalized two weeks or more were less satisfied than those hospitalized for a shorter time. Respondents on active duty were less satisfied than retired persons or dependents. Patients 18 to 25 years of age were less satisfied than those over 25. The unmarried were not as satisfied as the married, and men were less satisfied than women. Respondents never hospitalized before had less satisfaction than those with previous hospitalizations.

7. Computer Support in Military Psychiatry.

The Computer Support in Military Psychiatry (COMPSY) study is a project in Walter Reed Army Medical Center's Department of Psychiatry and Neurology with full-time nurse support from the Division of Nursing, Walter Reed Army Institute of Research. It is being reported under Project 9076, WRAMC.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02, Internal Medicine

Work Unit 088, Military Nursing Research

Publications

1. Felton, Geraldene and Smith, Roy: "Administrative Guidelines for an Abortion Service." Human Sexuality: Nursing Implications. Mary H. Browning and Edith P. Lewis, Editors. New York: The American Journal of Nursing Company, 1973, pp. 174-175.

2. Felton, Geraldene: "Effect of Time Cycle Change on Blood Pressure and Temperatures in Young Women." A Source Book of Nursing Research. Florence Davis and Margaret A. Newman. Philadelphia: F. A. Davis Company, 1973, pp. 158-173.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71R	02	089
b. CONTRIBUTING				
c. ~~SECONDARY~~	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Pathogenesis of Renal Diseases of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012900 Physiology 003500 Clinical Medicine 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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17. CONTRACT/GRANT

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e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	10	435
FISCAL YEAR CURRENT 75	11	435

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Renal Failure; (U) Renal Hemodynamics; (U) Heat Stress (U) Shock; (U) Fluid and Solute Homeostasis; (U) Dialysis; (U) Kidney Function

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To investigate mechanisms for maintaining fluid, electrolyte and hemodynamic homeostasis in response to disease, injury and environmental stresses of military significance, such as acute renal failure, shock, infectious disease, heat stress, and gastrointestinal disorders in order to provide rational basis for prevention and treatment.

24. (U) Clearance methods, dialysis, externally monitored isotope methods, isotope dilutions, experimental models, in vivo micropuncture, in vitro renal microperfusion, membrane transport, radioimmunoassay, light and electron microscopy and immunopathology.

25. (U) 72 07-74 06 Uranyl nitrate induced acute renal failure results in marked alterations in renal hemodynamics, characterized by a preferential cortical ischemia, possibly mediated by intrarenal renin activity. In addition, there are changes in renal tubular capacity to absorb sodium and water, providing a stimulus for renin release in nephrotoxic acute renal failure. Early elevations of renin activity in juxtaglomerular apparatuses seem to support this hypothesis. Studies have shown potassium has a direct effect on intrarenal handling of sodium and may modulate the renin-angiotensin system. Turtle bladder epithelium studies demonstrated a dual role for acetazolamide in hydrogen ion and sodium transport. Hydrogen ion secretory studies demonstrate short chain fatty acids to be the primary substrate for this transport mechanism. Parathyroid hormone and cyclic adenosine nucleotides may have a primary role in water/sodium absorption in the proximal tubule. Parenteral nutrition studies in trauma patients demonstrated the efficacy of solutions containing essential amino acids in decreasing major catabolic stress. Studies have shown that lymphocytes from uremic patients demonstrate a decreased antigenic responsiveness which can be reversed following correction of the uremia by either transplantation or dialysis. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73 - 30 June 74.

*Available to contractors upon originator's approval

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Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 089 Pathogenesis of renal diseases of military importance

Investigators.

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Description: Studies are directed at investigations of mechanisms for maintaining body fluid, electrolyte and hemodynamic homeostasis or their correction in response to disease, injury and environmental stress of military significance such as acute or chronic renal failure, shock, heat stress, infectious disease and gastrointestinal disorders. The role of adaptive homeostatic mechanisms, including renal and extrarenal mechanisms, whereby body fluid and solute balance is achieved and maintained in the face of stress has been emphasized in order to provide a rational basis for the development of improved methods for prevention and treatment of altered fluid, electrolyte and hemodynamic states and acute and chronic renal failure induced by these stresses.

Progress:

1. Acute Renal Failure:

a. Based on previous work from this laboratory, a postulated sequence of events for the pathophysiology of the initiation of acute renal failure has been developed. In brief, the initiating event whether it be a nephrotoxin, circulatory failure or cryptogenic results in tubular damage and dysfunction. At its earliest stages, prior to the manifestations of total cell necrosis, this tubular epithelial dysfunction results in the altered handling of sodium chloride and fluid along the entire length of the nephron, most importantly in the proximal convoluted tubule and loop of Henle. This abnormality in fluid and electrolyte transport results in an increased load of sodium chloride to the macula densa which, in turn, stimulates the release of renin from its storage sites in the juxtaglomerular apparatus. As a consequence of increased renin release there is a primary effect of the local generation of angiotensin on the local nephron level, as well as an

"overflow" phenomenon resulting in elevations of plasma renin activities. This latter manifestation of increased renin release, increased plasma renin activity, appears to bear little relation to the actual pathophysiologic development of acute renal failure. Rather, the most important event related to the renin-angiotensin system activity is the generation of angiotensin at intrarenal sites which results in alterations in glomerular arteriolar resistance such that the following pattern of events ensues: decreased total renal blood flow, in association with a redistribution of intrarenal blood flow, characterized by marked cortical, preferential outer cortical ischemia; alterations in glomerular dynamics such that the net effective ultrafiltration pressure in glomeruli is diminished resulting in a net decrease in total kidney effective filtration pressure. These alterations in glomerular dynamics and renal hemodynamics result in the pattern of oliguria and azotemia characteristic of acute renal failure. It has also become evident that these hemodynamic and glomerular dynamic alterations may, in turn, in a self-perpetuating manner, result in further and/or additional alterations in nephron function to continue these pathophysiological phenomena. The object of the following study was to characterize the alterations in tubule epithelial function which resulted in the observed marked changes in electrolyte and water handling characteristic of acute renal failure. A renal micropuncture study of rats performed 6 hours (early) and 48 hours (late) after the administration of uranyl nitrate, a model known to produce acute renal failure, was performed in order to evaluate tubule epithelial function. Early after uranyl nitrate induced acute renal failure no abnormalities in absolute or fractional fluid absorption were observed in the proximal tubule, indicating maintenance of glomerular tubular balance. In contrast, absolute fluid absorption measured at sites between the proximal and distal tubule was significantly depressed, consistent with the demonstration of histopathological abnormalities in this segment of the nephron early in acute renal failure. Later in the course of uranyl nitrate induced acute renal failure, marked alterations in absolute and fractional water reabsorption by both the proximal and distal nephron segments were observed, consistent with progression of the lesion as a function of time. In order to further characterize the alterations in sodium, chloride and water movement in response to this known nephrotoxin, additional studies were performed using an isolated uro-epithelial membrane. The application of 5 mg% of uranyl nitrate to the mucosal surface of this isolated uro-epithelial surface resulted in a prompt and marked decrease in short-circuited current, active sodium and chloride transport. The lack of change in passive transport of sodium and chloride suggests that these alterations in active sodium and chloride transport occurred without loss of membrane integrity. In contrast, application of uranyl nitrate to the anti-luminal surface of this uro-epithelial membrane did not alter active or passive transport, consistent with the observation that it is filtered uranyl nitrate which results in renal abnormalities.

b. To further characterize the effect of alterations in active electrolyte transport and water absorption as stimuli for activation of the renin-angiotensin system an assay for the renin activity of single juxtaglomerular apparatuses was developed, in conjunction with Dr. Klaus Thurau, Physiology Institute, Munich, Germany. To this end, techniques for the identification of single superficial and deep juxtaglomerular apparatuses in situ using silicone rubber vascular injections were developed. The interaction of rat renin released from single juxtaglomerular apparatuses and the partial purification of sheep renin substrate was accomplished, revealing first order kinetics and non-substrate dependency of the renin substrate interaction. The angiotensin generated from the incubation of single juxtaglomerular apparatuses with substrate was assayed using a rat pressor bioassay for angiotensin II and confirmed using a radioimmunoassay for generated angiotensin I. In normal rats, injecting silicone rubber through the abdominal aorta, it was observed that renin activity in superficial nephron segments was 2½ times greater than that observed in deep nephron segments. The responsitivity of renin activity and its distribution was evaluated using carotid manipulation, extremes of sodium chloride intake, and during the early phases of uranyl nitrate induced acute renal failure. Acute carotid obstruction resulted in a marked increase in deep juxtaglomerular apparatus renin activity and eradication of the normal superficial to deep intracortical renin gradient. This effect was ascribed to stimulation of renal nerve sympathetic fibers and alterations in intrarenal renin synthesis. A high sodium chloride diet for a period of 2 weeks obliterated the intracortical renin gradient in juxtaglomerular apparatuses as a result of a more marked diminution in superficial juxtaglomerular apparatus activity as compared to that of deep activity. In contrast, a low sodium chloride diet resulted in more marked increases in deep juxtaglomerular apparatus renin activity than in superficial activity, although the gradient of intracortical renin activity tended to be maintained. Most importantly, increases in both superficial and deep juxtaglomerular renin activity were observed within 2 hours of the injection of uranyl nitrate, consistent with a primary and early role for activation of the renin-angiotensin system in the initial pathophysiology of acute renal failure. In order to interrelate the alterations in sodium and chloride transport by tubule epithelium and activation of the renin-angiotensin system additional in vivo, fusion studies as well as measurements of distal sodium and chloride concentrations in acute renal failure will be required. The exact mechanism of activation of the renin-angiotensin system during uranyl nitrate induced acute renal failure is not apparent from these studies and requires further delineation. In view of the important role which the renin-angiotensin system plays in the pathogenesis of acute renal failure, studies were designed to evaluate any alterations in the renin-angiotensin system that could modify the course of acute renal failure.

It is known that angiotensin I is not a potent renal vasoconstrictor and, therefore, it is presumed that the intrarenal conversion of angiotensin I to angiotensin II may significantly alter the renal hemodynamic abnormalities of acute renal failure. A nonapeptide inhibitor of angiotensin converting enzyme was evaluated in normal dogs. Although the intrarenal infusion of converting enzyme inhibitor abolished the pressor effect of angiotensin I, administered systemically, no significant alterations in glomerular filtration rate or renal hemodynamics were observed. To further evaluate the ability of infused converting the enzyme inhibitor to modify the renin-angiotensin system activity, additional studies were designed to examine the renin-angiotensin system's role in the autoregulatory ability of the kidney. The administration of angiotensin converting inhibitor neither extended nor diminished the ability of the kidney to maintain glomerular filtration rate in the face of marked diminutions in renal perfusion pressure analogous to that observed in acute renal failure. The lack of an effect of a converting enzyme inhibitor to alter these renal hemodynamic functions may be related to the inability to present required concentrations of the inhibitor at intrarenal sites of renin release and activation or, alternatively, may not be consistent with the roles of the renin-angiotensin system in the hemodynamic regulation of this phenomenon.

c. Hyperkalemia is a characteristic manifestation which appears in the clinical course of human acute renal failure. In view of the known effect of potassium concentration on renin activity, studies designed to evaluate the mechanism of potassium induced alterations in the renin-angiotensin system were performed. Potassium chloride, 12 mg/kg/min, was infused into a single renal artery of anesthetized dogs and plasma renin activity, bilateral renin secretory rate, aldosterone excretion, electrolyte excretion, systemic blood pressure, renal blood flow, glomerular filtration rate, and intrarenal blood flow distribution were studied. Prompt decreases in plasma renin activity and renin secretory rate were observed, greater on the infused side than non-infused side, after potassium administration. These alterations in renin-angiotensin system activity occurred without change in blood pressure, glomerular filtration rate, or total renal blood flow suggesting that potassium induces alterations in the renin-angiotensin system by an intrarenal mechanism. The observation that the intrarenal distribution of blood flow, as estimated by radiomicrosphere distribution, was not altered further suggests that potassium does not alter renin activity by inducing changes in renal hemodynamics, but, rather by a direct tubular or direct juxtaglomerular apparatus effect. The marked elevations in urinary sodium excretion observed after potassium infusion suggest that abnormalities in electrolyte transport may have resulted in an increased load of sodium chloride to the macula densa which did not stimulate renin activity because of a potassium induced inhibition of sodium transport across the macula

densa. Despite the marked diminution in renin secretory rate and plasma renin activity, there were marked decreases in aldosterone excretion, suggesting that under conditions of acute hyperkalemia aldosterone synthesis and secretion is more related to potassium levels than the previously proposed primary role for the renin-angiotensin system. These studies suggest that the hyperkalemia characteristic of acute renal failure may have a dual role in the course of acute renal failure: (1) as a consequence of elevated serum potassium concentrations, an absolute decrease or a buffering of increase in renin-angiotensin system activity would occur which may diminish the effect of renin on glomerular renal hemodynamics; (2) as a consequence of a potassium related increase in aldosterone synthesis and secretion, the enhanced reabsorption of sodium chloride in the distal segments of the nephron would tend to maintain or restore circulating blood volume and thus diminish the effect of any hypotension concomitant in the development of acute renal failure.

d. Characterizations of the renal hemodynamic abnormalities early in the course of acute renal failure were obtained as a result of studies performed during the initial 3 hours of the development of uranyl nitrate induced acute renal failure in the dog. The maintenance of systemic blood pressure, cardiac rate, and cardiac output indicate that, in the dog, uranyl nitrate induces acute renal failure without altering systemic cardiovascular parameters. The prompt fall in renal blood flow observed after the administration of uranyl nitrate in association with the prompt increase in plasma renin activity and decrease in sodium reabsorption confirms or parallels the postulated schema for the pathophysiology of the initiation of acute renal failure. At the end of 3 hours marked abnormalities in total renal blood flow as well as in the distribution of renal blood flow were observed. These were characterized by a preferential outer cortical ischemia using radiomicrosphere techniques. Previous studies of the isolated perfused dog kidney performed in these laboratories have demonstrated marked falls in filtration fraction consistent with a greater diminution in glomerular filtration rate than in renal blood flow. These abnormalities in glomerular filtration rate and total renal blood flow are similar to those observed later in the course of acute renal failure. In addition, the marked fall in urine osmolality in the isolated perfused dog kidney and diminutions in electrolyte transport also mimic the pathophysiologic findings of acute renal failure. To evaluate and define the mechanisms responsible for these abnormalities in the isolated perfused kidney, studies were undertaken to further characterize the alterations in renal blood flow using radio xenon washout and radiomicrospheres, and to examine the medullary role in urine concentrating ability by analyzing tissue solute concentration. Despite a progressive fall in total glomerular filtration rate total renal blood flow was relatively well maintained. Analysis of renal hemodynamics including intrarenal blood

flow distribution demonstrated that this maintenance of renal blood flow was at the expense of outer cortical flow. These alternations in renal hemodynamics occurred simultaneously with a washout of the normal corticomedullary gradient of osmotic concentration and an increase in medullary blood flow suggesting that the alteration of intrarenal hemodynamics was responsible for the observed diminution in urinary concentration. These studies further suggest that the marked abnormalities in renal hemodynamics characteristic of the maintained or later phases of acute renal failure are functional in nature and may not contribute to the continued diminutions in the glomerular filtration rate observed.

e. Marked abnormalities in calcium and phosphate homeostasis have been observed after acute renal failure. These abnormalities have been noted in clinical nephrology to contribute to the morbidity and mortality of this syndrome. In order to characterize and delineate the role of abnormalities in calcium and phosphate homeostasis in acute renal failure a detailed analysis of the effect of parathyroid hormone and the site of phosphate reabsorption was required.

Utilizing the isolated rabbit proximal tubule, the effect of parathyroid hormone on sodium and water absorption was evaluated. Segments of proximal tubules from superficial nephrons of the New Zealand rabbit were isolated and perfused with an ultrafiltrate of rabbit serum. Fluid absorption was measured by the addition of I^{125}-polyvinylpyrrollidone to the ultrafiltrate in trace amount and the volume collected was measured with a calibrated constant bore pipette, over timed intervals. The addition of parathyroid hormone to the bathing fluid in those proximal tubule segments with an initial attachment to their own glomerulus and defined as early proximal convoluted tubules caused a decrease in fluid absorption by approximately 25%. In order to determine if there were intrinsic differences along the length of the proximal tubule in the capacity to respond to parathyroid hormone the late proximal convoluted segment defined by its initial attachment to the pars recta, and the pars recta segments were studied. Addition of parathyroid hormone to the bathing fluid did not change net fluid absorption in either the late proximal convoluted tubule or the pars recta segments. In addition to demonstrating a heterogeneity of response along the nephron to parathyroid hormone it was also shown that there was a marked difference in baseline fluid absorption between the earlier and later segments. Mean baseline fluid absorption rate in the early proximal convoluted tubules was 1.41 nl/ml/min whereas the values obtained in the late proximal convoluted tubules was 0.63 nl/ml/min a value which was significantly different from the rate observed in the early proximal convoluted tubules. Fluid absorption in the pars recta was 0.52 nl/ml/min a rate which was not significantly

different from the late proximal convoluted tubule. Since the effect of parathyroid hormone on fluid absorption in the proximal tubule is thought to be mediated by the adenyl cyclase system, other agents which result in increased cyclic adenosine monophosphate activity should have a similar effect on net fluid absorption. In this regard previous studies have evaluated the effect of dibutyryl cyclic adenosine monophosphate on net fluid absorption in the proximal convoluted tubule and pars recta. The addition of 10^{-5} molar dibutyryl cyclic adenosine monophosphate to the bathing media decreased net fluid absorption approximately 20% in the proximal convoluted tubule. No response to this agent was noted in either the late proximal convoluted tubule or the pars recta segments. Therefore, there seems to be not only an intrinsic difference in the capacity of various segments of the proximal tubule to absorb fluid but also to respond to parathyroid hormone as well as dibutyryl cyclic adenosine monophosphate. Early segments have a high intrinsic capacity to absorb fluid and respond to parathyroid hormone and dibutyryl cyclic adenosine monophosphate whereas late segments of the convoluted tubule and pars recta have a relatively low rate of fluid absorption and do not respond to these agents. This functional heterogeneity along the length of the proximal tubule might help to explain why certain areas of the proximal tubule seem to be more susceptible to various nephrotoxic agents. Additional studies of the histology, and other functional parameters of the proximal tubule will be done to evaluate the cause of this nephron heterogeneity.

In order to further evaluate the role of parathyroid hormone in the renal handling of calcium and phosphate in normal and pathological situations, clearance experiments were performed on intact and acutely thyroparathyroidectomized dogs. Recent evidence in the literature has demonstrated that in acutely thyroparathyroidectomized dogs the phosphaturia that occurs normally with volume expansion is severely blunted after acute parathyroidectomy in the dog. The proposed mechanism of this blunted phosphaturia is significant phosphate reabsorption distal to the proximal convoluted tubule. Using the acute parathyroidectomized dog model affords the investigator an unique opportunity to evaluate the handling of phosphate in a pathological state. To delineate the site of distal nephron phosphate reabsorption studies were performed utilizing ethacrynic acid, a diuretic which is known to be a potent inhibitor of sodium and chloride reabsorption in the thick ascending limb of Henle. If the site of distal phosphate reabsorption following acute parathyroidectomy was the thick ascending limb of Henle the addition of ethacrynic acid should have caused a marked phosphaturia as well as a natriuresis. Preliminary studies to date indicate that the addition of ethacrynic acid to volume expanded or hydropenic, acutely parathyroidectomized dogs does not cause a phosphaturia, indicating that the distal site of phosphate reabsorption is probably not the thick ascending

limb of Henle or, if it is, the reabsorption is not coupled to sodium or chloride reabsorption. Current studies using this model are being performed to evaluate possible sites of phosphate reabsorption in the distal convoluted tubule and collecting duct. These studies may help to explain the cause of the hyperphosphatemia seen in acute and chronic renal failure and suggest possible therapeutic modalities.

f. In the course of acute renal failure marked abnormalities in acid basis homeostasis develop. Characteristically, both during the initiation and recovery phases, the kidney loses its ability to reabsorb the filtered bicarbonate via the assumed mechanism of hydrogen ion secretion. The mechanism and requirements of hydrogen ion transfer were investigated in the isolated turtle urinary bladder to characterize the effects of nephrotoxic agents, such as uranyl nitrate, on the capacity of renal epithelia to acidify. Hydrogen ion transport by this tissue has been shown to be independent of the active transport of sodium and potassium, energy requiring and electrogenic. Since the PCO_2 of the body is a major determinant of the rate of hydrogen ion transport the effects on this tissue induced by changes in the PCO_2 of the bathing solution were examined. Increasing CO_2 from 1 through 5% caused a prompt linear increase in the transport rate. When CO_2 was increased beyond 5% in the bathing solution there was no further increment in the rate of acidification. With the addition of carbonic anhydrase inhibitors at concentrations great enough to reduce the baseline rate of hydrogen ion secretion to the assumed uncatalyzed rate of CO_2 hydration in the cell ($5x10^{-5}$M acetazolamide), the response to CO_2 addition was such that the maximum effect of CO_2 occurred at 7-8% CO_2. The magnitude in the rate of acidification in the absence and in the presence of $5x10^{-5}$ M acetazolamide, however, did not differ with 7% CO_2 present. At still higher concentrations of acetazolamide, $5x10^{-4}$ M, the effect of CO_2 on the rate was severely blunted with only a minimal increase in H+ transport. The maximal rate of acid secretion under these conditions was 20% of the control rate. This latter effect of carbonic anhydrase inhibitors is more likely due to some primary inhibition of the transport mechanism than simply inhibition of carbonic anhydrase since elevation in CO_2 should have resulted in an uncatalyzed rate that would be more than adequate to support the maximum observed transport rate. Furthermore, these studies suggest that the rate of hydrogen ion secretion is in part CO_2 dependent but cannot be infinitely increased with increased PCO_2.

The energetic or metabolic requirements of the hydrogen ion secretory mechanism have also been investigated. At low rates of hydrogen ion transport de-oxygenation with nitrogen, addition of potassium cyanide or the addition of an uncoupler of oxidative phosphorylation such as dinitrophenol or salicylate have little or no effect on the transport rate. However, at maximum rates of hydrogen ion secretion in the presence of 5% or greater

CO_2 all of these maneuvers significantly inhibit the transport process suggesting that at high rates of transport aerobic metabolism is also required. After depletion of the tissue of substrate by incubating it for 20-24 hours in substrate free media addition of short chain fatty acids markedly enhance the rate of hydrogen ion transport whereas glucose does not. Similarly, in bladders inhibited with 2-deoxyglucose addition of exogenous short chain fatty acids reverses the inhibition of 2-deoxyglucose. This suggests that short chain fatty acids are the primary substrate for the transport mechanism rather than carbohydrate. Similar substrate requirements have been described for cortical collecting duct for which this tissue is a reasonable analogue.

With the addition of uranyl nitrate to the mucosal solution baseline hydrogen ion secretion was not appreciably changed. Addition of CO_2 caused a stimulation in the rate similar to that which occurred without uranyl nitrate present. However, if the rate of hydrogen ion transport was measured under conditions where there was a pH gradient of 2 or more pH units, uranyl nitrate produced a marked inhibition which was not responsive to CO_2 addition. This inhibition of uranyl nitrate under gradient conditions suggests that the passive conductance of the tissue to proton is markedly increased by the uranium salt. Other nephrotoxic agents such as amphotericin B and organic mercurials produced a similar defect in the capacity of the tissue to produce hydrogen ion gradients without affecting the transport rate in the absence of hydrogen ion gradients. This effect of uranyl nitrate on hydrogen ion transport is in contrast to its effect on sodium and chloride transport which was presented in an earlier section. For sodium, uranyl nitrate only inhibited the active component of transport without affecting the passive conductance for this cation or its accompanying anion chloride.

2. Chronic Renal Disease and Transplantation

a. Drug metabolism in chronic renal disease:

Patients with both acute and chronic renal failure, especially those undergoing hemodialysis treatment, frequently require modification of dosage of various therapeutic agents. Because of the difficulty in doing pharmokinetic studies in clinically ill and unstable acute renal failure patients, studies have been undertaken in patients with chronic renal failure. After establishing extraction and assay procedures for procainamide by both spectrophotometry and spectrophotofluorometry, pharmokinetic studies were done in patients and both in vitro and in vivo clearance studies were done using the artificial kidney. In chronic renal insufficiency the absorption of procainamide appears normal, while equilibration through volume of distribution may be prolonged and elimination rate is seriously

impaired, with serum half-life prolongation to approximately three times normal. The drug is dialyzable, with in vivo clearances of 67±4 cc/min. In collaboration with Temple University School of Medicine, quinidine elimination was also studied in patients with chronic renal failure. In contrast to procainamide, quinidine elimination in patients was not found to be impaired when compared to controls, using a double extraction technique and spectrophotofluorometry for measurements of plasma concentration. Propanolol, a beta adrenergic antagonist, is being used with increased frequency as an adjunctive agent in the therapy of hypertension in selected patients with chronic renal disease or hypertensives with elevated circulating plasma renin activities. Extraction techniques and a spectrophotofluorometric assay were established to study the pharmokinetics of propanolol in patients with abnormal renal function and to assess its clearance by hemodialysis. The studies have demonstrated normal absorption but suggest an impaired "first pass effect," whereby a greater fraction of absorbed drug is available to the systemic circulation, implying decreased hepatic extraction from portal circulation. Elimination rates in these patients, however, were not found to be impaired, correlating well with the clinical observation that they tolerated large total daily doses of propanolol without undue toxicity. Propanolol was found to be dialyzable both in vitro and in vivo, an unexpected observation suggesting that protein binding of the drug may be impaired in uremic patients. Since other investigators have suggested that the kidneys are largely responsible for the metabolism of salicylic acid to salicyluric acid, studies were undertaken in collaboration with the State University of New York at Buffalo to assess salicylate elimination in patients with end-stage renal failure. Patients with renal failure and patients after nephrectomy demonstrated normal salicylate elimination rates and normal formation rates of salicyluric acid. These observations suggest normal salicylate metabolism at the doses given. Current investigations include assessment of acetominophen and diazepam metabolism in renal failure patients, and investigations of the integrity of acetylation mechanisms for a variety of drugs in these patients.

b. Nutritional support:

Patients with both acute and chronic renal failure undergo varying degrees of catabolic stress with cellular starvation and negative nitrogen balance. Previous studies have demonstrated the need for at least 1.25 gm protein/kg body weight in the diet of clinically stable dialysis patients to maintain nitrogen balance. With additional stress and inadequate oral intake, for example, following major surgery, patients enter a period of marked negative nitrogen balance which may impair healing and prolong recovery. Studies utilizing parenteral nutrition have demonstrated the superior effectiveness of solutions with essential amino acids in addition to high dextrose

concentrations in blunting the catabolic stress of surgical, and presumably other forms of trauma. Investigations of the effectiveness of this treatment are being extended to patients with acute renal failure, and the usefulness of essential amino acid supplements in renal patients with failure to thrive syndromes are being evaluated.

c. Cellular immunity:

Early studies of cellular immunity in uremic patients suggested marked impairment and, therefore, perhaps increased susceptibility to infections. It is known that infection accounts for most of the mortality seen in acute renal failure patients. In addition, tests utilized for selection of donors for renal transplantation depend on lymphocyte responsiveness for their interpretation. It is important, therefore, to reassess the responsiveness of lymphocytes from renal failure patients under the circumstances of modern management and improved laboratory techniques. An assay technique was established whereby small numbers of lymphocytes could be cultured in small volumes and responsiveness to plant mitogens and histocompatibility antigens (on homologous lymphocytes) evaluated. The kinetics of lymphocyte responsiveness from renal failure patients within this system were compared to those of normal lymphocytes and optimal conditions (cell counts, mitogen concentration, etc.) were determined for identifying differences between groups. Studies have demonstrated that the vast majority of patients, managed so that clinical uremia is avoided, have lymphocyte responsiveness equal to that seen with normal controls. Thus, pre-transplant evaluation studies dependent on lymphocyte responsiveness (i.e. mixed lymphocyte cultures) can be interpreted with confidence in most patients.

d. Hemoperfusion:

There are endogenous and exogenous intoxications in which the responsible substances are not amendable to rapid elimination by either conservative medical treatment or renal dialysis techniques. Removal of these substances from the circulation can be achieved, however, by absorption to macro-reticular polystyrene resin with affinity for high molecular weight lipid soluble compounds. Biochemically thyrotoxic dogs were treated by hemoperfusion. Large amounts of T_4 were taken up by the resin and serum T_4 levels were decreased using hemoperfusion. This treatment will be evaluated in the management of patients with thyroid storm. Hemoperfusion will be evaluated for its efficacy in removing non-dialyzable in in vitro systems in experimental animals and in patients with life threatening intoxications.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 089 Pathogenesis of renal diseases of military importance

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6462	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71R	02	090
b. CONTRIBUTING				
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11. TITLE (Procede with Security Classification Code)

(U) Cellular Mechanisms of Disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

016200 Stress Physiology 017100 Weapons Effects

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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FISCAL YEAR PRECEDING 74	.5	227
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19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Adaptive Growth; (U) Cell Culture; (U) Cellular Respiration; (U) Energy Metabolism; (U) Functional Adaptation; (U) Oxygen Microenvironment; (U) Stress

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text of each with Security Classification Code.)

23. (U) Health impairment associated with the mission of the military is due to stress inflicted by the energy of weapons or exposure to adverse environments and pathogenic microorganisms. Survival is insured through increased function of the appropriate physiological systems, the elementary working units of which are specialized cells. The demand for increased function is met by activation of reserve cells and, depending on the intensity and duration of the stress, by increase of the cell population, i.e. adaptive growth which has far greater survival value and is the object of this study.
24. (U) The mechanisms controlling adaptive growth are studied by means of model cell culture systems with well defined phases of growth and differentiated function.
25. (U) 73 07 - 74 06 The essential sequence of adaptive growth, i.e. activation of DNA replication and mitosis with a concurrent decline of specialized function and the reversal of these changes upon restoration of the original cell population density was successfully reproduced in suspension cultures of fibroblastic cells. Transitions between the growth and functional phases of these cultures were associated with marked changes of the respiratory activity of the cells. To investigate the relevance of these findings to cultured cells attached on solid surfaces, approximating tissue cells in the body, the oxygen microenvironment of mono- and multilayer cultures was mapped by means of ultramicrocathodes. It was found that a) media pO_2 declined with depth at a rate proportional to cell density, b) at any given depth level pO_2 was not constant but oscillated between a set of maximum and minimum pO_2 values, c) oscillations exhibited density dependent amplitude and were abolished by respiratory inhibitors, with media pO_2 equilibrating with atmospheric oxygen. This is the first demonstration of rythmic respiratory activity in mammalian cells; its significance for the control of adaptive growth is under investigation. For technical reports see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 73 - 30 June 74.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A161102B71K RESEARCH IN BIOMEDICAL SCIENCES

Task 02 Internal Medicine

Work Unit 090 Cellular Mechanisms of Disease

Investigators.
Principal: Andre D. Glinos, M.D.
Associate: Robert J. Werrlein, M.S.; James M. Vail, Ph.D

Description

Health impairment associated with the mission of the military is due to stress and injury inflicted by the energy of weapons or exposure to adverse environments and pathogenic microorganisms. Survival is insured through the heightened functional activity of the appropriate physiological systems the elementary working units of which are specialized cells. The demand for increased function is met by activation of reserve resting cells and, depending on the intensity and duration of the stress, by increase of the cell population, i.e. adaptive growth. As the restoration of the soldier's health and combat capability depends on these processes, it is the objective of this study to uncover the underlying mechanisms and to develop means for increasing their effectiveness.

Progress and Results

The problem of the mechanisms controlling adaptive growth can be stated in the form of the following two questions: 1) What is the nature of the changes in the cellular environment which following injury first induce cells to proliferate and later limit cell division to the maintenance of a functionally active cell population? 2) What is the nature of intracellular molecular interactions which occur in response to these extracellular changes and result in the early phase of DNA replication and general protein synthesis followed later by a marked reduction of these activities while specialized cellular functions increase?

In the past, a great number of clinical and experimental studies have failed to provide satisfactory answers to these two questions because of the great complexities of the clinical situation in man and of the experimental conditions in the whole animal. Even the discovery that dense cultures of fibroblasts attached on glass or plastic surfaces respond to injury and cell loss by activation of DNA synthesis and cell division which terminates when the initial cell density is restored, i.e. adaptive growth, has failed to provide the desired answers. This is due to the fact that in attached cultures, it is impossible to distinguish between physical growth regulatory mechanisms necessitating cell-to-cell contact and humoral regulation

operating through decreased uptake of substances essential for growth, by dense cultures.

We resolved this difficulty by using suspension cultures of L-929 fibroblasts where cell-to-cell contact is minimal. We have previously shown[1] that maintenance of these cultures by daily media renewal without cell dilution results in population densities in excess of 6 X 10^6 cells/ml which are viable for extended periods and manifest the following characteristics: 1) mitosis and DNA synthesis are inhibited approximately 90 - 90% with the majority of cells either arrested or greatly retarded in the G_0 or early G_1 phase of the cell cycle[2]; 2) total protein synthesis is markedly depressed, while the fraction of protein synthesis represented by collagen is increased[3]; 3) respiration is decreased from 5.4 to 1.8 fmoles O_2 cell/min. with reoxygenation and nutrient implementation of the media capable of reversing only half of this decline the other half obviously being due to an adaptive change of the cells (1) and 4) cell loss is followed by a 24-hour latent period during which respiration, protein synthesis, DNA replication and cell division are activated and continue until the initial population density is restored (2).

The essential sequence of adaptive growth, represented by characteristics 1, 2 and 4 which the suspension culture model shares with attached tissue cells in the body or in solid surface cultures, was thus found to be associated with significant changes of the respiratory activity of the cells (characteristic # 3). To investigate the relevance of this association to attached cells, platinum microcathodes were constructed and utilized to map the oxygen microenvironment of attached cultures of WRL-10A mouse fibroblasts.

The cells were grown attached to the floor of optically flat petri dishes with a supporting medium of MEM supplemented with 20% horse serum, antibiotics and glutamine. The experimental dishes were placed on the stage of an inverted microscope inside an environmentally controlled chamber, and were maintained at a constant temperature of 35°C in a humidified atmosphere of 5% CO_2 and air. A Pt/Ir oxygen microcathode with a tip diameter ranging from 1-3 μ capable of resolving oxygen tension within a sphere 6-20 μ in diameter was secured in a microminipulator clamp placed in the center of the objective field so that the vertical descent of the fragile cathode could be observed microscopically, and positioned at desired depth levels until the establishment of contact with the attached cell sheet. An Ag/Agcl reference anode was placed in a saline well adjacent to the culture dish and was joined to the culture dish by an agar bridge. All experiments were started with the cathode just under the fluid air interface of the medium. The cathode was then lowered at measured intervals through the medium toward the attached cell layer and the oxygen partial pressure determined polarographically at selected depth levels.

Pilot experiments conducted in this fashion revealed the existence of pO_2 gradients within relatively short distances of the attached cell layer, but, in addition to the gradients, high density cultures exhibited regular and reproducible oscillations between low and high pO_2 readings. To explore further the significance of this unexpected finding a series of cultures with a population of approximately 10^7 cells/plate was set up and the electrode lowered into their media in discrete steps 500 μ apart. The current obtained at each depth level was recorded for a period of 30 minutes. A composite of the tracings thus obtained is shown in Fig. 1. It may be seen that the oscillations of environmental pO_2 at 500 μ are very shallow; at 1000 and 1500 μ there appears to be a mixture of large and small oscillations and at the level of the cell layer the oscillations appear almost uniformly large. It may also be seen that at progressively greater depth levels the oscillations range around progressively lower median pO_2 values. For example, at the 500 μ level the oscillations occur between 140 and 160 mmHg with a median pO_2 of approximately 150 mmHg, while at the cell level, the oscillations occur between 23 and 80 mmHg with a median pO_2 50 mmHg.

To determine whether these oscillations are related to the respiratory activity of the cells, rotenone, a known respiratory inhibitor which blocks electron transfer from NAD to cytochrome b, was prepared at a concentration of 10^{-5}M in ETOH. After recording the pO_2 oscillations at the cell layer for 30 minutes, 50 λ of the rotenone solution was added to the culture dish. This resulted in immediate cessation of the oscillations, followed by 9 minutes of maintained low pO_2, a last oscillatory rise and fall of the local O_2 tension and then a steady increase in the pO_2 bringing the microenvironmental oxygen tension back into sustained equilibrum with atmospheric oxygen at 160 mmHg pO_2.

Having thus conclusively demonstrated that the oscillations are in effect an expression of the respiratory activity of the cell population with O_2 depletion due to respiration and O_2 replacement due to diffusion, we proceeded to investigate the effect of population density on pO_2 gradients and on oscillation amplitude.

Accordingly, replicate cultures seeded with 7.0×10^5 cells per petri plate were grown with daily medium renewal and were polarographically analyzed at cell densities of approximately 5, 10 and 16 million cells/plate, i.e. at populations approximating 1, 2 and 3 monolayer equivalents, respectively. Instead of the oscillation median it was decided to use minimum oxygen tension values as more representative of the maximum respiratory activity of the cells and Fig. 2 shows the values thus obtained plotted as a function of medium depth. It may be seen that from the surface to a depth of 2000 μ, all three cell densities show oxygen tensions which are relatively high and nearly in equilibrium with the air of the environmental chamber. From 2000 μ down toward the cell layer, however, the oxygen tension decreases sharply as

a function of cell density, with the negative slope of the 5 X 10^6 cell population being the least steep and the slope of the 16 X 10^6 cell population being steepest. The recorded mean minimum pO_2 values at the cell level, i.e. in the immediate cellular microenvironment, appear to be directly proportional to the cell density, with the value for the 5 million cell population reaching approximately 100 mmHg, the 10 X 10^6 cell population about 60 mmHg, and the 16 X 10^6 cell population about 19 mmHg.

The relationship between microenvironmental oxygen and the respiratory activity of the cells was further investigated by comparing pO_2 oscillation amplitude in cultures of different cell densities. Figure 3 shows that as in the case of microenvironmental pO_2 gradients, the mean oscillation amplitude is a function of medium depth and population density. This together with sensitivity to inhibitors (Fig. 1) indicates that the oscillations of microenvironmental pO_2 observed in these experiments are a direct reflection of the respiratory activity of the cells.

Accordingly, these results are interpreted to indicate that, under the conditions used, mammalian cell respiration was not a continuous but a rythmic process. In addition, the results suggest that the entire cell population, or, at least very large portions of it must respire in synchromy. This is so, because environmental pO_2 oscillations due to individual cells respiring rythmically but out of phase would tend to cancel each other out.

Since these oscillations reflect partial microenvironmental oxygen depletion and renewal occurring at minimum pO_2 values above 15 mmHg, i.e. significantly greater than the critical pO_2 tension, they cannot be ascribed to mere periodic fluctuation of oxygen availability. Consequently, it must be postulated that cellular respiration in these cultures is controlled by environmental feedback[4] involving essential media components other than oxygen which alternately turns some key respiratory process on and off in a large number of cells. Such an oscillating system might involve the balance between AMP, ADP and ATP or a fluctuation of some oxidizable substrate pool such as NADH, or a matrix of interdependent variables such as pO_2, glucose, Na^+, K^+, NADH, and ATP/ADP ratios working in concert to control cellular respiration. Projected work plans aim at identification of the oscillating environmental feedback system and the assessment of its significance for the control of the production of cellular energy and its utilization in adaptive growth.

Summary and Conclusions

The essential sequence of adaptive growth, i.e. activation of DNA replication and mitosis with a concurrent decline of specialized function and the reversal of these changes upon restoration of the original cell population density was successfully reproduced in suspension cultures of fibroblastic cells. Transitions between the growth and functional phases of these cultures were associated with marked changes of the respiratory activity of the cells. To investigate the relevance of these findings to cultured cells attached on solid surfaces, approximating tissue cells in the body, the oxygen microenvironment of mono- and multilayer cultures was mapped by means of ultramicrocathodes. It was found that a) media pO_2 declined with depth at a rate proportional to cell density, b) at any given depth level pO_2 was not constant but oscillated between a set of maximum and minimum pO_2 values, c) oscillations exhibited density dependent amplitude and were abolished by respiratory inhibitors, with media pO_2 equilibrating with atmospheric oxygen. This is the first demonstration of rythmic respiratory activity in mammalian cells; its significance for the control of adaptive growth is under investigation.

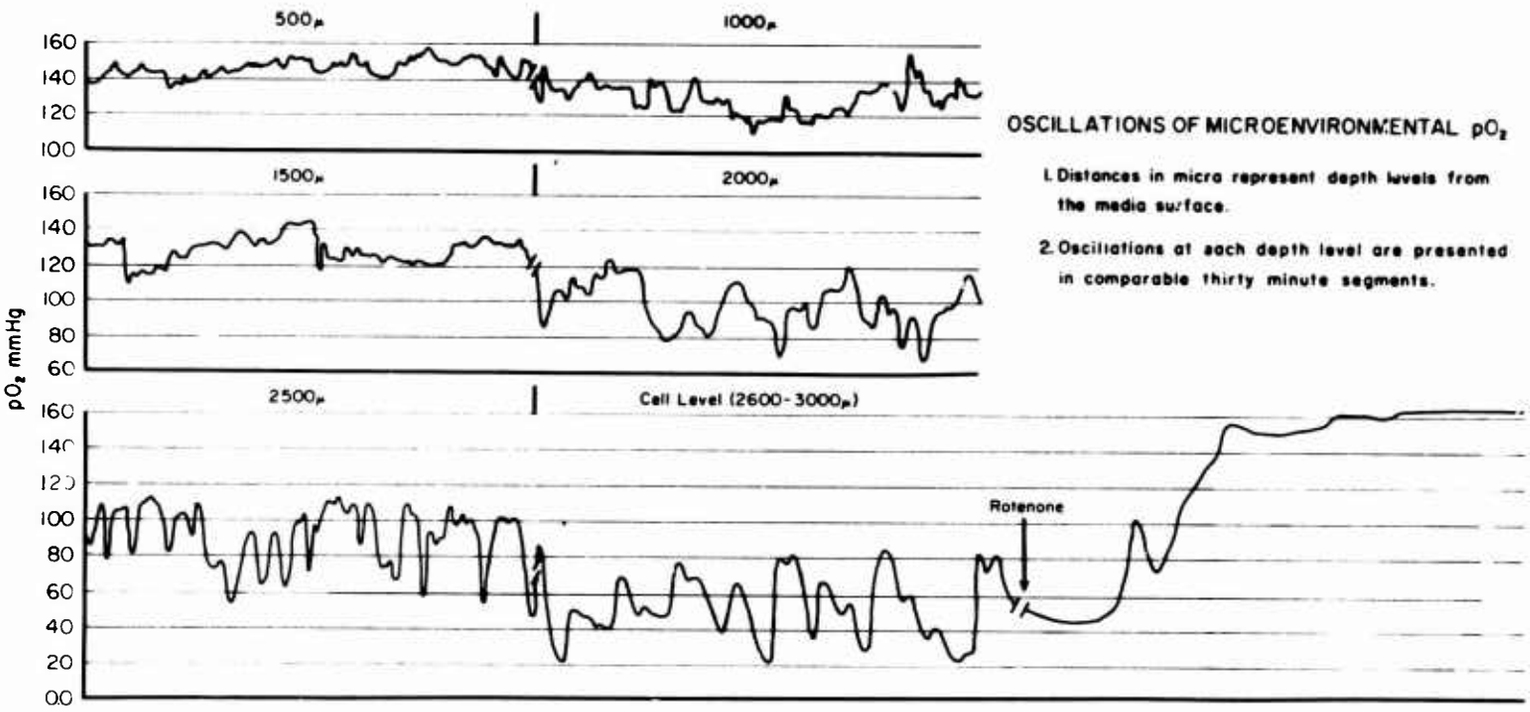
500μ
1000μ
1500μ
2000μ
2500μ
Cell Level (2600-3000μ)
Rotenone
pO2 mmHg
160
140
120
100
80
60
40
20
00
OSCILLATIONS OF MICROENVIRONMENTAL pO2
1. Distances in micra represent depth levels from the media surface.
2. Oscillations at each depth level are presented in comparable thirty minute segments.

Fig. 1

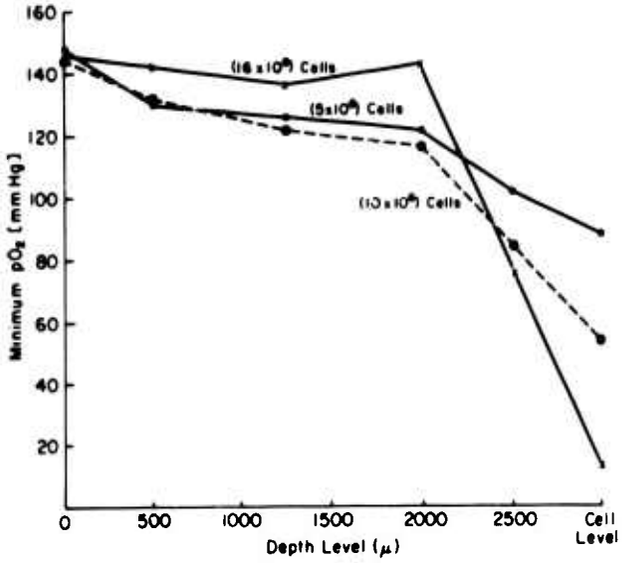

Fig. 2 Medium pO_2 gradients in cultures with varying cell densities. Depth level indicates distance from the fluid air interphase.

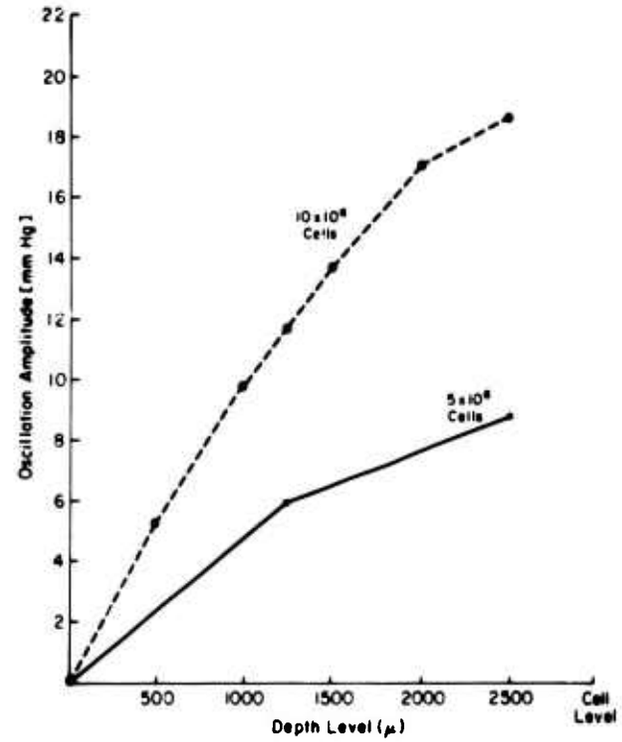

Fig. 3 Amplitude of pO_2 oscillations as a function of medium depth level in cultures with varying cell densities.

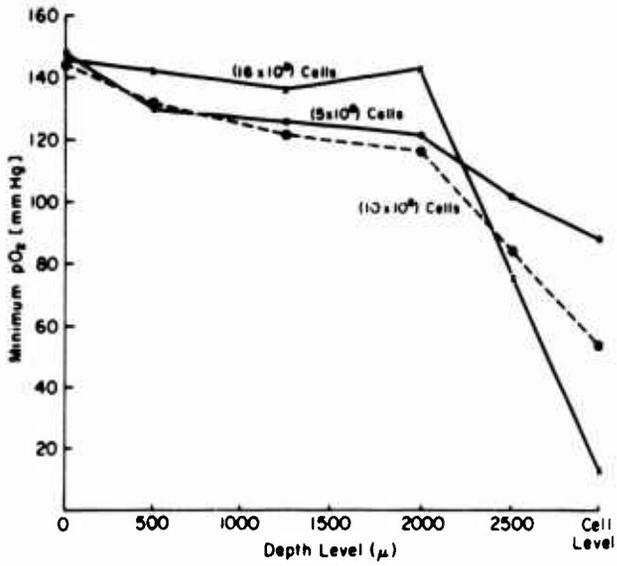

Fig. 2 Medium pO_2 gradients in cultures with varying cell densities. Depth level indicates distance from the fluid air interphase.

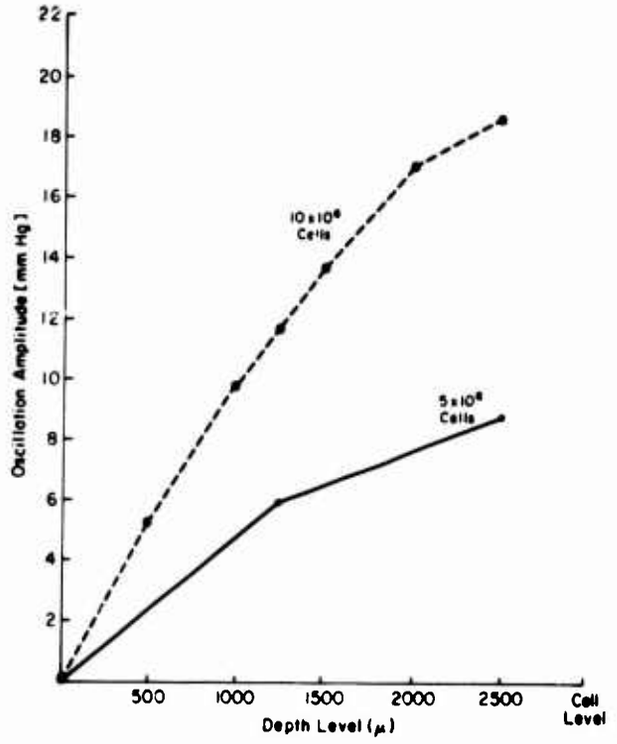

Fig. 3 Amplitude of pO_2 oscillations as a function of medium depth level in cultures with varying cell densities.

PROJECT 3A161102B71R
RESEARCH IN BIOMEDICAL SCIENCES

Task 03
Psychiatry

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6454	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D.CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A161102B71R	03	025
b. CONTRIBUTING				
c. [redacted]	CARDS 114 (f)			

11. TITLE (Precede with Security Classification Code)

(U) Analysis and Management of Behavior & Stress in Military Environments

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

013400 Psychology 012600 Pharmacology 012900 Physiology 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING 74	6.0	322
	CURRENT 75	6.0	322

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

ASSOCIATE INVESTIGATORS

NAME: Elsmore, T. F., Ph.D.

NAME: Manning, CPT F. J. DA

21. GENERAL USE

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22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Operant behavior; (U) Respondent Conditioning; (U) Reinforcement; (U) Conditioning; (U) Military Psychiatry

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Experimental analysis and development of complex behavioral models isolating variables likely to contribute to psychiatric decompensation or disease and subsequent ineffective performance in military environments. Functional relationships among physiological and behavioral variables governing the individual-environment interaction are defined and manipulated under controlled conditions to permit more precise specification of their role in the pathogenesis of behavioral and organic disorders.

24 (U) Techniques of experimental psychology, principally operant and respondent conditioning, combined with those of endocrinology, pharmacology, physiology and anatomical sciences are used to define variables that maintain and control both adaptive and dysfunctional behavior.

25 (U) 73 07 - 74 06 A study was initiated to determine the quantitative and qualitative features of stress which would be preferentially miminized by an organism given an opportunity to control its environment. A study of plasma cortisol levels and behavioral arousal suggests that cortisol levels may be of limited value as an index of stress. Modification of morphine self-administration can be effected by serotonin-depleting pretreatment but not by catecholaminergic or cholinergic mechanisms. Conditioned aversion techniques have been used to identify what may be morphine abstinence signs in the rat more than four weeks following their last exposure to the drug. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*Available to contractors upon originator's approval

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Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 03 Psychiatry

Work Unit 025 Analysis and management of behavior and stress in military environments

Investigators.
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Description.

During the reporting period, research directed specifically at the problem of drug abuse and psychosomatic disease, was subsumed under separate work units (see 102 and 115). Research managed within this work unit can be classified into two broad categories: (1) Experimental analysis of the individual-environment interaction as it relates to the maintenance and control of behavior; and (2) Experimental analysis of drug behavior relationships. It should be emphasized that this categorization is principally for purposes of organizing this report. The study of drug-behavior relationships is a specialized approach to the more general problem of the experimental analysis of the maintenance and control of behavior.

EXPERIMENTAL ANALYSIS OF THE INDIVIDUAL-ENVIRONMENT INTERACTION AS IT RELATES TO THE MAINTENANCE AND CONTROL OF BEHAVIOR.

The studies in this section are directed at description of functional relationships between environmental variables and the behavior and physiology of the organism. Their purpose is: (1) To develop, analyze and apply complex behavioral models which permit examination of the interaction of the organism with its environment under conditions likely to lead to psychiatric decompensation; (2) To specify general principles governing the maintenance and control of behavior; (3) To develop and maintain the technology required to objectively address questions of a behavioral nature arising from problems in military medicine and bearing on the health of the individual soldier.

Patterns of Hormone Secretion and Behavioral Stress. A series of experiments concerned with the pattern of hormonal secretion in the resting state and during the acquisition and performance of Sidman avoidance has been completed. After femoral vein catheterization and at least one month in the primate chair, plasma

samples were taken and monkey behavioral display scored by a motor activity scale every 20 min for a 6 hour experimental session. Similar to man, growth hormone was secreted episodically, i.e., plasma levels were at undetectable levels and then soared to spike-like peaks over 40 min. In contrast, plasma cortisol showed a sine wavelike oscillation that was different from the more striking fluctuating levels previously noted in man. In order to be sure that these oscillations about a mean represented secretory bursts, two experiments were performed in which radioactively tagged cortisol was injected; the specific activity of this label, an index of secretion of new cortisol, was then followed over time. In these experiments, it was learned that these oscillatory fluctuations represented true secretory episodes; either the gland secreted at varying rates, responsible either for relatively constant or ascending plasma levels, or did not secrete at all, responsible for decrementing plasma levels. A significant number ($p<0.05$) of the growth hormone secretory bursts were associated with illumination of the monkey booths each morning. Furthermore, behavioral score was significantly higher ($p<0.05$) during active secretion of growth hormone than during periods of time when growth hormone levels were falling or basal. By measuring glucose concurrently, it was learned that bursts of GH secretion were never related to hypoglycemia, but instead occasionally occurred at the same time as small hyperglycemic bursts. Because plasma glucose levels also related positively with behavioral score, it could be argued that these endocrine changes reflect a central excitatory state as evidenced by an animal's own behavioral display. No such correlations existed between behavioral arousal and plasma cortisol levels. This observation challenges the traditional concept that cortisol is a sensitive index of emotional or behavioral arousal. Analysis of the Sidman avoidance data is just beginning; however, there are several heretofore unreported findings. Plasma cortisol decreases over repeated Sidman sessions until it reaches either baseline or sub-baseline levels. This decrement in the usual stress response does not occur merely because animals learn to perform the task well and thus avoid delivery of the aversive stimulus, electric shock, because decrementing levels were found even in animals that performed inefficiently and continued taking large numbers of shocks. When monkeys were given unavoidable shock after 10 days of practice at avoiding shocks, they showed significant increases in response rate and in behavioral score with no change in plasma cortisol. This finding represents another challenge to the concept that cortisol is a sensitive visceral index of central arousal.

The role of reinforcers in choice behavior. Three studies are being conducted, addressed to the problem of how an organism distributes its behavior between various alternatives which produce different reinforcers. These studies can be described as investigations of the principles governing choice behavior. Previous researchers

have described all behavior as choice behavior in the sense that engaging in one response precludes many other responses and represents a choice to do one thing as opposed to another. Examination of the factors controlling such choices is fundamental to understanding the control of behavior; for example, the choice to self-administer drugs as opposed to emitting some other behavior. To date, however, nearly all studies of choice have avoided investigation of competing responses maintained by different reinforcers. Most of these early studies were concerned with factors which were best studied with behaviors maintained by similar reinforcers. As a consequence, little is known about the mechanisms which might govern a choice between qualitatively different reinforcers, heroin and money for example. Much that is known about choice remains tentative until it is extended to comparisons of different reinforcers. It is possible that such comparisons introduce factors which have until now been overlooked. The first study is a fundamental comparison of choice between similar and dissimilar reinforcers. The purpose is to discover those characteristics of choice which are unique to comparisons of dissimilar reinforcers. This can best be described as an attempt to define when two reinforcers are not alike in behavioral terms. The experiment is being conducted in primates given a choice between food and water obtained during daily 1.5 hr sessions. After stable baseline performance has been obtained, a third alternative will be provided consisting of food pellets incremented in amount over a period of several months. The animals will be able to choose between two similar reinforcers, one pellet vs several pellets, and two dissimilar reinforcers, several pellets vs water. As the number of pellets available is increased, the effect on choice for a similar reinforcer and a dissimilar reinforcer will be examined.

Parameters of safety. It is a well-known fact that avoidance of noxious stimulation will maintain performance. Usually the reinforcer in these experiments is the postponement of an aversive stimulus. If responding does not occur at some minimal frequency, the stimulus occurs; if the responding is rapid enough, it does not occur. In this setting, the reinforcer is present or not present. Yet, outside the laboratory setting, various degrees of safety are often available and an organism must often select what appears to be the safest alternative. Very little is known of the parameters of safety and what factors make one situation more safe (or less stressful) than another. These principles are of fundamental importance to an understanding of behavior under stress. Given several kinds of safety, what alternative will be chosen? A project is now underway to fashion a setting in which an animal can be stressed and then given several avoidance options. By manipulating the duration, the amount of aversive stimulus reduction, and the frequency of availability of safety, it is hoped that a systematic theory of avoidance behavior can be developed which is as complete as current theories of appetitive behavior.

Selective frontal cortex ablation and delayed-matching-to-sample performance. Monkeys were taught to make a choice response to one of two simultaneously presented visual stimuli. Reinforcements were delivered only for choice of the stimulus presented briefly in a third position 8 seconds previously. Ablation of the cortex in and around the principal sulcus produced a very mild, though lasting, deficit in such performance: preoperative performance 90%, postoperative 82%. Ablation of the cortex surrounding the inferior convexity of the lobe produced a much more serious deficit, reducing performance to chance levels for the full 1500 trial assessment period. Subsequent studies will attempt to relate this striking difference in the effects of two relatively small lesions in adjacent frontal cortex to the differing afferents and efferents characteristic of these areas.

Frontal cortex lesions and discrimination of interoceptive cues. Recent investigations of cortical function have pointed to increasingly sharper localization of function. The frontal lobes are now viewed as a collection of at least 3 functionally distinct areas. Several workers have proposed that the area in and immediately surrounding the principal sulcus is crucially involved in behaviors guided by interoceptive cues relating to the subject's position in space. This notion was tested by training monkeys on three tasks differing only in the degree to which interoceptive and spatial cues were critical for effective performance: two groups learned to make a simple choice response following an extended series of lever presses. One choice was reinforced only after a series of 64 presses, the other only after 32 presses. Response-produced interoceptive stimuli presumably guide this choice. For one group the choice responses involved left (64) and right (32) buttons (further interoceptive involvement); for the other, choices were between red (64) and white (32) buttons. Ablation of the dorsolateral frontal cortex surrounding the sulcus principalis indicated that while this cortex may well deal with interoceptive stimuli, this specialization is probably not limited to those interoceptive cues specifically related to position in space. Present work involves assessing the effects of the same lesion on a strictly visual discrimination task. Future work may well involve assessment of the effects of smaller frontal lesions on these tasks.

Behavioral effects of lesions in the dorsomedial thalamus. This nucleus is the major source of thalamic afferents to frontal cortex, as well as the major thalamic target of frontal efferents. Early work, done prior to the recognition of functionally distinct areas within frontal cortex, found that at least some of the behavioral effects of frontal cortex ablation could be mimicked by stereotaxic lesions of the dorsomedial nucleus. Anatomical studies have since confirmed that interconnections of orbital

frontal cortex and dorsomedial thalamus are restricted to the medial (magnocellular) portion of the nucleus, while the dorsolateral cortex is connected exclusively with the lateral (parvocellular) portions. The present studies examined the effects of frontal cortex lesions and medial and lateral lesions of the dorsomedial nucleus on a task designed to incorporate several features previously found diagnostic for orbital cortex damage. A second task, not sensitive to orbital cortical damage, is included to control for non-specific effects of thalamic damage. Several normal animals have been tested thus far, and the small variance among them in performance suggests the tasks should be sensitive ones.

Effects of lesions of the nucleus locus coeruleus. The nucleus locus coeruleus is a small group of cells situated in the brainstem beneath the floor of the fourth ventricle. It has been demonstrated that this group of cells is a major contributor of the neurotransmitter, norepinephrine in the forebrain and that there are anatomical connections between this structure and large portions of the cortex. These findings have stimulated a number of research efforts aimed at examining the functions of the locus coeruleus and its influence on behavior. Recently, it has been proposed that this area functions as part of a "reinforcement system" and that destruction of the area results in defective learning capacity. This conclusion was tested in a series of four experiments designed to evaluate the ability of rats with lesions in the locus coeruleus to acquire several different responses. Experiment 1 examined the ability of six lesioned rats to learn to run in an L-shaped runway for a food reward. The lesioned rats showed significantly longer latencies to emerge from the start box of the runway as compared with non-lesioned controls, as well as longer latencies to reach the food reward. Over 20 days of training, the lesioned animals gradually reduced their running time, but at a much slower rate than the controls. Experiment 2 was designed to test the abilities of the lesioned rats to acquire another appetitively motivated task. The lesioned animals showed no deficits in acquiring this response. Experiment 3 tested the ability of the lesioned animals to acquire a conditioned taste aversion to saccharin-flavored water, an avoidance response not primarily dependent upon performance. Both lesioned and control rats readily formed an aversion to saccharin solution after they were made ill by injections of a toxic drug, cyclophosphamide. Experiment 4 examined the acquisition of active and passive avoidance responses in a one-way shock avoidance task. No significant differences were observed in the performances of the lesioned and non-lesioned controls on either active or passive avoidance responding. At the completion of the experiments all rats were sacrificed and biochemical assays were conducted on the cortical tissues of all animals. The results showed that the lesioned animals had 30% less cortical norepinephrine than their controls,

confirming the effectiveness of the lesions. Since the lesioned animals showed no performance differences in three out of the four tasks examined, it was concluded that locus coeruleus lesions do not produce a general impairment of learning ability in rats and that the deficits seen in runway performance were due to factors specific to that task.

Limbic system structures and taste aversion learning. Several brain areas which are traditionally included in the classification of limbic system structures have been implicated as being functionally important in conditioned aversion learning, including the olfactory bulbs, the amygdala, the hippocampus and the septum. Studies investigating the effects of lesions of the hippocampus or the septal area in rats on aversion learning have produced somewhat conflicting results, with some studies finding deficits with lesions of these areas and others finding no effects. The hippocampal and septal areas are closely related anatomically, each receiving reciprocal connections from the other via the fornix, a thick fiber bundle connecting the two. Studies are presently under way investigating the hypothesis that the integrity of the hippocampal-septal connections is importantly involved in aversion learning.

Conditioned aversion technique for assessing effectiveness of radioprotective agents. The effectiveness of chemical agents in protecting against the effects of ionizing radiation is a matter of relevance to military as well as clinical applications. The use of the conditioned aversion technique may provide a simple and sensitive method for testing the effectiveness of chemical agents in protecting against the toxic side effects that patients often encounter during radiotherapy. In addition, the technique provides a sensitive test for toxicity of these drug agents themselves, before their use in human subjects. Studies are presently under way exploring the usefulness of this method. Preliminary results are available from a study investigating the effectiveness of S-2-(3-Aminopropylamino) ethylphosphorothioic acid (WR-2721) in protecting against the formation of radiation-induced taste aversions. Rats were injected with 0, 25, or 100 mg/kg of WR-2721 after ingesting a 0.1% saccharin solution. Half of each group of rats was then exposed to 50 rads of X-ray, delivered at a rate of 2.0 rads/min. The other half of each drug dose group was sham-irradiated. After three recovery days, the animals were given a preference test between saccharin solution and water. The results showed that the animals injected with WR-2721 and sham-irradiated developed aversions for the saccharin solution, suggesting that the effects of the drug itself were toxic and resulted in the formation of aversions. The animals given 25 mg/kg of WR-2721 and 50 rads of X-rays developed stronger aversions than either the animals given 25 mg/kg of the drug alone or radiation alone, suggesting that there was a cumulative toxic effect on both agents at this dose level. No cumulative effect was seen at the higher dose of 100 mg/kg, however, although the animals

given this dose of the drug and radiation did develop aversions. The level of aversion for this group was not as great as in the group which received 100 mg/kg WR-2721 alone, suggesting that the combination of drug and radiation at this level was not as toxic as the drug alone. This is the only evidence of any radioprotective effect seen in this study. Since both irradiated groups treated with WR-2721 developed aversions, it must be concluded that the doses used in this study do not protect rats against the toxic effects of low-level radiation responsible for the formation of conditioned aversions.

Behavioral contrast. A multiple schedule of reinforcement is one in which two or more schedules, or components, each associated with a distinct exteroceptive stimulus, are alternated regularly or randomly. It is often assumed that performance in a given component is essentially the same as the performance observed when the same schedule is programmed alone. In fact, the considerable interaction that occurs is easily observed when the schedule in one component changes in response to the new contingency, but responding in the other, unaltered component often changes as well. Behavioral contrast is the name given to the type of interaction where the change in responding in one component is in the opposite direction to the change in the other. Most demonstrations of contrast have employed the pigeon, so it is not surprising that recent analyses of the phenomemon have given a very important role to a newly discovered peculiarity of the pigeon: stimuli temporally paired with food delivery elicit pecking from the pigeon, quite apart from any consequences programmed by the experimenter and pecking. These analyses gain some support from the observation that contrast is not as readily demonstrated when rats are substituted for pigeons. Our observations led us to conclude that this may well mean only that optimal parameters for demonstrating contrast in the pigeon are not optimal parameters with the rat. Preliminary results indicate that reliable production of persistent contrast effects with the rat may require the use of separate manipulanda for responding during each component of the multiple schedule. A second line of investigation involves aversive control of behavior, a situation clearly unrelated to the pigeon's apparent innate propensity to peck stimuli paired with food delivery. Rats were trained to press a lever to avoid electric shocks delivered every 12 seconds. Each lever press postponed the next shock for 20 seconds. When responding stabilized on a multiple schedule involving 2 components with identical shock-shock and response shock intervals, extinction was programmed for one component. Since there is some debate over what constitutes extinction of avoidance responding, two different procedures are in use: In one, no shocks are delivered in one component; in the other, shocks are delivered every 12 seconds irrespective of the rat's behavior. Preliminary data indicate that only the

latter procedure results in an elevation of response rate in the unaltered component. These results have implications both for understanding the interactions among components of multiple schedules, and also for the controversy surrounding the definition of extinction of avoidance.

Circadian variations in single-key discrimination. Descriptions of circadian rhythms in behavior have been largely confined to reflexes and other types of unlearned behaviors (e.g., general activity). In an attempt to generalize these effects to more complex behaviors and relate circadian changes in behavior to more commonly studied behavioral changes, two baboons have been exposed to a discrimination procedure requiring completion of a number of key presses for food reinforcement in the presence of an intermittent tone of one duty cycle while allowing presses in the presence of another duty cycle to go unreinforced. Depending on several parameters of the procedure, notably the number of presses required for reinforcement, the overall level of deprivation of the animal, and the difficulty of the discrimination, circadian variations in the degree of discrimination can be observed. These variations can be characterized as an improvement in discrimination in the middle of the dark portion of the daily light cycle relative to performance during the day. Preliminary data analysis using signal detection techniques suggests that the change in performance might be indicative of a change in response bias rather than a change in the animal's ability to discriminate the stimuli in the task. These effects can be observed when several sessions are run on the same day or when only a single session is run per day at a different time each day.

Circadian variations in two-lever discrimination in rats. Four rats are being run on a procedure requiring responding on different levers in the presence of flashing lights of different duty cycles. Four to eight sessions are being run each day, with half in the light and half in the dark. This experiment should allow a more clear-cut signal detection analysis of circadian variability in discrimination performance.

Experimental analysis of drug-behavior relationships. For purposes of organization, studies of drug-behavior relationships are being presented as a separate subsection of this report. Studies in this category are conducted for two principal purposes: (1) The use of drugs in behavioral research is a particularly useful tool for exploring and defining principles which govern the interaction of the organism with its environment. As such, the study of drug-behavior relationships is an extension of the technology applied in the basic work unit; (2) Work unit 102 requires not only direct study of Biomedical Aspects of Drug Abuse, but also the development and critical analysis of an appropriate

behavioral technology. Therefore, studies in this work unit (025) yield data directly applicable to the drug abuse work units while, at the same time, they evaluate approaches to the drug problem, and assess the adequacy of the technology and feedback data on the generality of findings with respect to broader issues related to the maintenance and control of behavior. In this context, research in this section utilizing drugs in the study of behavior may be directly related, tangentially related, or unrelated to drug abuse. However, as a matter of policy, all studies in this section have employed drugs of abuse whenever these compounds would suffice in the examination of the issues being addressed.

Drugs as a discriminatory stimulus. Frequently, drug levels required to produce performance changes are relatively high compared to those reported to be associated with subjective experiences. This discrepancy in dose-level raises questions as to the generality of results obtained in studies directed at drug-related performance deficits. The argument has frequently been made that performance decrements are observed at doses considerably in excess of those used by man to produce subjective effects. Because most studies of drug effects on performance are conducted on laboratory animals and because it is known that species differ widely in their sensitivity to pharmacologic compounds, it was considered advisable to develop a procedure which would permit comparison of drug dose-levels producing only changes in subjective experience with those required to produce performance decrements. In the present study, laboratory primates are being used to develop this procedure. The drug employed in the study is heroin, administered intravenously. The procedure takes advantage of the fact that if an animal is working for food and a stimulus is presented indicating that electric shock is about to be delivered, there is an abrupt disruption in on-going performance. This procedure is known as conditioned suppression. In the present study, placebo, as well as varying doses of heroin, are being administered via chronic venous catheters. Placebos never signal that shock is to be presented, but an administration of heroin is always followed two minutes later by electric shock. If the animal can discriminate the difference between a placebo and a given dose of heroin, it can reliably anticipate the presentation of shock, and the characteristic anticipatory disruption of on-going behavior can be observed. To date, three animals have been prepared for use in this experiment and various dose-levels of heroin have been explored in order to determine dose-levels which will not alter on-going performance. The procedure described above will begin and should yield data as to the minimum discriminable dose of intravenously administered heroin and will, as well, provide a method for assessing changes in the discriminability of heroin attendant to the development of tolerance to

the drug. This same procedure will be utilized to assess the effects of spacing doses of heroin over time on the development of tolerance to its subjective effects. The data to date indicate that doses as high as 25 mg/kg have no direct effect on performance but are reliably discriminated by the organisms. Doses as low as 3.13 mg/kg (:02 mg total dose) are also reliably discriminated from saline. To date, no tolerance to the discriminative properties of the drug has been developed.

Acute and chronic effects of Delta-9-tetrahydrocannabinol, ethanol, and the combination of these two drugs on performance maintained under periodic schedules of reinforcement. Subjects (rats) were trained to respond on each of two levers. Reinforcement occurred for presses on the left lever after short periods of time (fixed-interval of 30 sec), and reinforcements occurred for presses on the right lever after long periods of time (fixed-interval of 150 sec). Only one schedule was in effect at a time. Animals typically press on the left lever for the first 30-50 sec, pause on both levers for 20-40 sec, and then press predominantly on the right lever for the balance of the 150-sec period. Of course, 50% of the time reinforcement is delivered for left presses after 30 sec, so the timer resets and starts again.

Four animals have received 14 acute doses of THC. Nine of these doses were in an ascending series (.25, .5, 1, 2, 4, 8, 32, and 64 mg/kg) with doses given once a week. The resulting dose-response at curves were fairly flat and showed slight response rate decreases at the large doses. The next three doses were administered 21 weeks later. In this series, doses were quadrupled rather than doubled (.5, 2, and 8 mg/kg) and given one week apart. Eight mg/kg produced a much greater response rate decrement under this dosing regime. indicating that the animals had developed tolerance to the drug in the first series. The final 2 doses were 4 and 8 mg/kg and were administered during the two weeks following the second series. This time the effects of 8 mg/kg were smaller and resembled the effects in the first series.

The same four subjects that served in the acute dosing series have been given two chronic series. In the first chronic series, two animals received 4 mg/kg of THC for 30 consecutive days. Neither animal recovered its predrug baseline rate of responding for either lever. However, both animals showed total recovery on the first nondrug day. After 20 days, a second chronic series (now in progress) was started. Neither animal has recovered baseline rates after 9 days of the drug. The other two animals received 1 mg/kg for 12 days during the first series. Neither animal showed any departure from predrug baselines so the dose was increased to 4 mg/kg for 18 days. Both animals showed some response rate decreases, but these were probably recovering by

the end of the series. During the current chronic series, these animals are receiving 4 mg/kg. One animal has shown little change from baseline, while the other animal has shown decrements that are not improving over the nine-day period.

Three of the four animals may have shown increased sensitivity to the THC (reverse tolerance) during the first chronic series: the decrement was progressive and the greatest decreases were seen after many doses. Because of the complex past history of acute THC doses that these animals had received, it is difficult to interpret this finding. Therefore, four additional drug-naive animals are currently being trained on this baseline and will receive a replication of the first chronic series.

Six additional animals are receiving acute doses of ethanol (.5, 1.0, 1.5, 2.0, and 2.5 g/kg--orally) under this procedure. Each dose is given 3 times with at least two non-drug vehicle days between each administration. Periods of maximal drug effect are being examined as well as dose parameters. Each subject has received 10 doses of ethanol to date. When the dose-response curves are completed, these animals will begin a chronic ethanol series. Small acute doses of THC will also be administered to these animals as they are becoming tolerant to the effects of ethanol. Likewise, the animals that have served in the THC experiments will be given another chronic series of THC and acute doses of ethanol will also be administered to them as they are becoming tolerant to the effects of the THC. To date, there has been little in the literature about the elucidation of the combined behavioral effects of these two socially used drugs.

Modification of morphine ingestion by rats with serotonin-depleting pretreatment. Individually caged albino rats were given a solution of morphine hydrochloride (0.5mg/cc) and sodium saccharin (0.1%) as their sole fluid for two days. On day three, two fluids were made available: a saccharin solution with morphine, and a similar solution without morphine. This 3-day cycle was then repeated until subjects reliably took 50% or more of their fluid from the morphine bottle on choice days. As reported previously, this percentage was highly correlated with weight loss in subsequent withdrawal from the drug. Injections of either parachlorophenylalanine or chloroamphetamine, but not methyl-p-tyrosine or scopolamine, produced a reliable decline in morphine intake and preference. The two effective pretreatments share the ability to selectively lower brain serotonin levels, while the two ineffective treatments primarily affect catecholamine and cholinergic systems respectively.

Secondary morphine abstinence symptoms in the rat revealed by the conditioned aversion technique. Morphine pellets were implanted subcutaneously in albino rats previously trained to take their daily fluid in a one-hour period. Three days later, these rats

were given a solution of saccharin for one hour instead of water, then injected with naloxone, a powerful morphine antagonist reported to have no agonist effects. The classical abstinence signs of writhing, teeth chattering, "wet dog shakes," and weight loss were observed significantly more often in these rats than in controls with placebo pellets implanted and/or saline injections. In addition, when subjects were given an opportunity, 48 hrs later, to drink either saccharin solution or tap water, the experimental animals drank significantly less saccharine than any of the three control groups, which did not differ. Thus, rats previously implanted with a morphine pellet, and only these, developed an aversion to a novel taste temporally paired with an injection of naloxone. This experiment was then repeated, allowing much longer intervals to elapse between pellet implantation and naloxone injection. Injections of the latter two, three, or four weeks after implantation failed to elicit the "classical" withdrawal signs noted above, even though RIA still revealed morphine in the serum. These rats still showed a significantly lower preference for saccharin than the controls. This simple and objective technique is thus apparently considerably more sensitive as a measure of withdrawal distress than any of the commonly used indices. The results also suggest that the qualitative distinction between "primary" and "secondary" abstinence phases may be primarly a function of the insensitivity of withdrawal measures rather than a reflection of a dichotomous underlying process.

Conditioned preference for morphine solution. The three-day cyclic drinking regimen outlined above allows study of morphine self-administration while eliminating the need for tedious and often traumatic injection regimens, surgical procedures, or elaborate instrumentation for venous cannulation. However, considerable exposure to this drinking schedule is necessary before rats begin to prefer morphine-saccharine solution to saccharin solution above. A small proportion of rats never come to prefer the drug solution. A study presently underway is attempting to speed this process substantially: after adapting naive rats to a 1-hr/day drinking schedule, daily intraperitoneal injections of morphine will be continued for 20 days. At this point, subjects will undergo repeated withdrawal. At the end of each of these periods, subjects will be offered a saccharin-morphine solution for an hour instead of water. A maintenance dose of morphine will be injected immediately after this ingestion period, thus insuring rapid relief from withdrawal distress soon after ingestion of the normally aversive saccharin-morphine solution. Previous work in other laboratories suggests that one or more such temporal pairings will markedly enhance the rat's preference for the drug solution. Affirmation of this hypothesis will not only speed subsequent research centered on morphine drinking, but will also support the contentions of many that conditioning plays a substantial role in drug self-administration.

Project 3A161102B71R RESEARCH IN BIOMEDICAL SCIENCES

Task 03 Psychiatry

Work Unit 025 Analysis and management of behavior and stress in military environments

Literature Cited.

Publications:

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2. Elsmore, T.F., and F.J. Manning.: Time course and dose-response effects of orally administered Δ-9-THC on interval schedule performance of rats. Bull. Psychonomic Soc. 2: 326-327, 1973.

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8. Natelson, B.H., G.P. Smith, P.E. Stokes and A.W. Root.: Plasma 17-hydroxycorticosteroids and growth hormone during defense reactions. Amer. J. Physiol. 226: 560-568, 1974.

PROJECT 3A762760A806
MILITARY PREVENTIVE MEDICINE

Task 00
Military Preventive Medicine

820

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6457	74 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM / A. WORK UNIT
73 07 01	H. Term.	U	U	NA	NL	☒ YES ☐ NO	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	6276A	3A762760A806	00	030
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Provide with Security Classification Code)

(U) Global Health Data

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

005100 Documentation 007800 Hygiene and Sanitation 007000 Geography

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
58 11	74 04	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 73	2	200
	CURRENT 74	2	19

19. RESPONSIBLE DOD ORGANIZATION

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NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Provide KEY with Security Classification Code) (U) Global Health Data; (U) Epidemiology; (U) Geography; (U)Potential; (U)Climate; (U)Ectoparasites; (U)Infectious Diseases

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Unclassified health and disease data on all foreign countries are accrued, analyzed, stored and made available to approved requestors. Consultation and assistance are provided for medical officers and to Tropical Medical Courses. Disease potential is calculated for all geographic areas and for all diseases.

24. (U) All available documents re diseases throughout the world are abstracted. These abstracts are evaluated as a basis for predicting incidence on a geographical and seasonal basis of diseases of military and civilian importance.

25. (U) 73 07 - 74 04 On 15 April 1974 the functions of this work unit were transferred to the United States Army Medical Intelligence and Information Agency, Washington DC 20314. During the life of this work unit more than 3,000 extracts were processed by the 3500 CDC computer through the cooperative system of acqu sition of data, evaluation of articles received and the coding of pertinent information from each article used by the geography programmer.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6489	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 1	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	6276A	3A762760A806	00	034
b. CONTRIBUTING				
c. [redacted]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Epidemiological Studies of Military Diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 005900 Environmental Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	4	36
FISCAL YEAR CURRENT 75	4	40

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

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NAME: Gaydos, Joel C., MAJ, MC

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Epidemiology; (U) Tuberculosis; (U) Serology; (U) Drug Abuse

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23.(U) To identify, define, and study known and potential causes of disability in military populations using relevant, existing epidemiologic techniques and developing appropriate new methodology. To apply this information to the prevention and control of disability in military populations.

24.(U) Contemporary epidemiologic methods are applied to causes of disability in military populations. Multidisciplinary collaborative approaches are utilized and new methods developed as required.

25.(U) 73 07-74 06 Study of the variability of skin tests for tuberculosis was completed. Investigation of an outbreak of tuberculosis in an experimental primate colony at Edgewood Arsenal was completed. Coding and keypunching of Fitzsimmons questionnaire and SAFA data was completed. Sera from the Fitzsimmons and Edgewood studies are being tested by a glycolipid antigen hemagglutination system in collaboration with University of Maryland investigators. Follow-up of personnel identified during a possible outbreak of toxic hepatitis at AFEES, Des Moines, was continued. An investigation of alleged excessive or unusual reactions to typhoid vaccination was completed. Abstracting of records from the testicular cancer study was completed in collaboration with the Clinical Research Service, WRAMC. Restudy of Yaviza Village, Republic of Panama, was completed; reduction and analysis of resulting data are in progress. Evaluation of epidemiologic sensitivity and specificity of urine tests for opiates was completed. A prospective study of drug users identified during BCT is reported elsewhere. (DA Project A833 - Work Unit 103) For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 73—30 Jun 74.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A762760A806 MILITARY PREVENTIVE MEDICINE

Task 00 Military Preventive Medicine

Work Unit 034 Epidemiologic studies of military diseases

Investigators:
Principal: MAJ Herbert E. Segal, MC
Associate: MAJ Alan S. Morrison, MC; MAJ Joel C. Gaydos, MC; MAJ Donald J. Balaban, MC; MAJ Frank L. O'Donnell, MC; SSG Michael C. Callahan; L. Charlene Evans

1. Variability in Skin Testing for Tuberculosis

Exposure of a group of 126 military personnel to a soldier with active pulmonary tuberculosis at Fitzsimmons Army Medical Center provided an opportunity to compare skin test results obtained with three different antigen preparations and with four observers. The purified protein derivative preparations used were: (1) stabilized with polysorbate 80 (Tween-80) (PPD-T); (2) a commercial tableted tuberculin (PPD-PD); (3) the Public Health Service standard (PPD-S).

All tests were administered and then read in a blind manner. The largest proportion of positive tests was obtained with PPD-T. The degree of agreement among the observers did not depend on the choice of antigens. The data indicate the usefulness of PPD with polysorbate 80 in tuberculosis screening.

2. Tuberculosis in an Experimental Primate Colony

An epidemiologic investigation of the occurrence of tuberculosis in an experimental rhesus monkey (Macaca mulatta) colony at the Biomedical Research Laboratory (BML), Edgewood Arsenal, was initiated in September, 1973. The objectives of the investigation were to define the prevalence of skin test positivity and disease in the colony and associated staff and to test the usefulness of the soluble antigen fluorescent antibody (SAFA) and passive hemagglutination tests in case finding.

All 89 monkeys were serially skin tested and bled, and were ultimately necropsied as specified in the original (surgical) protocol. SAFA testing of 203 specimens yielded 9 positive results. Testing with serologically active glycolipids is in progress. Correlation of skin test,

serologic, and necropsy results is in progress. No skin test conversions or cases of active disease were found in the staff members studied.

3. Serologic Tests for Human Tuberculosis

A two year serum collection for use in evaluating the SAFA test and, prospectively, other serologic tests for tuberculosis was completed at Fitzsimmons Army Medical Center. Subjects studied included all tuberculosis admissions both newly diagnosed and old, patients with diseases of the chest other than tuberculosis, tuberculin skin test converters, and normal persons skin tested as a result of case-finding efforts. 2,384 clinical histories and serum specimens were collected and resulting information coded and keypunched.

All sera have been studied using soluble antigen fluorescent antibody (SAFA) test. Analysis of the normal range, sensitivity, and specificity of the test antigen is in progress. A passive hemagglutination test employing serologically active glycolipids is also being evaluated in collaboration with University of Maryland investigators. (These collaborators are also testing non-human primate sera [see 2 above]). Serologic testing is in progress.

4. Toxic Hepatitis - Relationship of a Copying Process to Laboratory Results

Investigation of hepatitis at an Armed Forces Examining and Entrance Station (AFEES) in September 1972 resulted in identification of nine men with abnormal clinical and/or laboratory findings. Two were diagnosed as having viral hepatitis, while abnormalities recorded for the remaining seven did not fit any diagnostic entity. Epidemiologic studies suggested a relationship between these abnormalities and exposure to a developer powder used in a diazo copying machine. Toxicological studies on the developer powder done at the U.S. Army Environmental Hygiene Agency were unremarkable except for potential sensitization with skin testing.

The abnormalities noted on one man during the initial investigation persisted and in December 1973 his condition was diagnosed as primary sclerosing cholangitis. Follow-up of the other eight was begun in November 1973. Values outside the normal range were obtained in all eight, but only one had striking findings. This man has been diagnosed as having mild, chronic liver disease of undetermined etiology. At the present time the Office of The Surgeon General is

determining what additional evaluation is indicated for the personnel involved in the initial outbreak.

5. Adverse Reactions to Typhoid Vaccination

Between 2 April and 26 July 1973, eighty-eight people reportedly experienced reactions to typhoid vaccine. The investigation of these reactions sought to determine the incidence of reported reactions, the suitability of vaccine lots implicated in reactions for continued issue and use, and the circumstances surrounding reported reactions. Twelve reactions occurred among 440,000 people assumed to have received vaccine packaged in 20-dose vials and 76 reactions occurred among 500,000 assumed to have received vaccine packaged in 100-dose vials.

Specimens of all vaccine lots implicated in reactions were evaluated. Safety, sterility, and mouse toxicity were satisfactory. Investigation of the individual reactions revealed that the combined effects of typhoid immunization, heat, physical stress, and other concurrent immunizations may have accounted for many of the reported reactions. Only two of the reactions were considered atypical. In view of the low incidence of reported reactions and the satisfactory results obtained on laboratory testing of vaccine lots implicated in reactions, the significance of these atypical reactions is questionable. It was concluded that vaccine lots implicated in the reported reactions were suitable for continued use.

6. Clinical Epidemiologic Study of Testicular Malignancy in Army Personnel, 1950-1970

This case-control study performed in collaboration with the Clinical Research Service, WRAMC, seeks to define demographic and medical characteristics which are antecedent to the onset of testicular malignancy. Cases were ascertained from Class II Hospital Tumor Registries and Armed Forces Institute of Pathology records. Comparison individuals were selected from a 0.1% sample of personnel on active duty during the period in which the cases were diagnosed. The abstraction of personnel and medical records has been completed and the analysis begun.

7. Studies in Yaviza Village, Republic of Panama

During March and April, 1974, census and other demographic data were obtained for the entire population (1466) of the eastern Panamanian village of Yaviza. A random

sample comprising the occupants of 56% of the dwellings in the town were selected for venipuncture. Four hundred and sixty-six people (31.8% of the total population) actually provided specimens. Mean hemoglobin values were 12.6 gm/100 ml for females and 13.5 gm/100 ml for males, and mean hematocrit values were 37.9% and 40.0% respectively. Deficiency of the erythrocyte enzyme glucose-6-phosphate dehydrogenase was found in 9.5% of the males tested. Hemoglobin electrophoresis of all specimens showed the following prevalence of hemoglobin patterns: AA - 87.1%, AS - 5.8%, SS - 0.4%, AC - 6.2%, and AF - 0.4%. Plasma specimens showed an overall prevalence of antibody to hepatitis B antigen (HB_sAg) of 24.2%. Nine persons (1.9%) had HB_sAg in their plasma.

Further serologic testing and analysis of the data and correlation with demographic variables is in progress.

8. Epidemiologic Sensitivity and Specificity of Tests for Opiates

A study was performed to determine the epidemiologic sensitivity and specificity of biochemical tests used to detect drugs of abuse in urine samples. Negative samples were collected under controlled conditions by volunteers at Fort Detrick. Urines positive for morphine were collected from patients at Lexington who were receiving morphine as part of a research protocol. The urines were labelled with a random code number and analyzed for morphine by free radical assay technique (FRAT), thin layer chromatography (TLC), fluorometric technique of Kupferberg (FLUR), and gas liquid chromatography (GLC), with the following results:

	FRAT	TLC	FLUR	GLC
Sensitivity	.976	.686	.990	.995
Specificity	.962	.995	.941	.989

These calculated values were further analyzed to determine what the test sensitivity and specificity would be, if the test were arranged into a battery such that FRAT and TLC were in parallel (if either test is positive, the result is positive) and the resulting positives were tested by GLC in series. Assuming conditional independence between the tests, and using formulae for the composite sensitivity and specificity of tests in parallel and series, the overall sensitivity and specificity of the test battery would be 0.988 and 0.999 respectively. This particular arrangement of tests into a battery greatly increases the specificity beyond that of any of the component tests,

while the sensitivity is only slightly less than the sensitivity of the best test. This would be of particular importance in testing for low prevalence conditions, since the probability that a positive test is correct is directly proportional to the test specificity and the prevalence of the tested condition.

9. Personnel Identified as Drug Users During Basic Combat Training - Performance During First Military Tour

A prospective study of the military performance of drug users identified during Basic Combat Training is in progress. Results and discussion of this collaborative study are reported elsewhere (DA Project 3A762758A823, Work Unit 030, Military psychiatry, NP).

Project 3A762760A806 MILITARY PREVENTIVE MEDICINE

Task 00 Military Preventive Medicine

Work Unit 034 Epidemiologic studies of military diseases

Literature Cited.

Publication:

Erdtmann, F.J., Dixon, K.E., and Llewellyn, C.H.: Skin Testing for Tuberculosis, Antigen and Observer Variability. J.A.M.A. 228 (4): 479-481, 1974.

PROJECT 3A162110A821
COMBAT SURGERY

Task 00
Combat Surgery

828

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6466	74 06 30	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	H Term	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A162110A821	00	120
b. CONTRIBUTING				
c. [redacted]	CARDS 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Wound Healing

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	73 06 30	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 73	3.0	150
FISCAL YEAR CURRENT 74	0	0

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Immune Mechanisms; (U) Rejections; (U) Abscess; (U) Combat Injury

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

25. (U) 72 07 - 73 06. Summary dated 73 07 01 submitted in error. Project evaluated fiscal year 73 and support transferred to Operation and Maintenance Army Funds 73 06 30.

*Available to contractors upon originator's approval

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6467	74 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
73 07 01	D. CHG	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62110A	3A162110A821	00	121
b. CONTRIBUTING				
c. [redacted]	CARDS 114(f)			

11. TITLE (Precede with Security Classification Code)

(U) Gastrointestinal Responses to Trauma

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

008800 Life Support 016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 09	CONT	LA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 74	4.5	30
FISCAL YEAR CURRENT 75	4	30

19. RESPONSIBLE DOD ORGANIZATION

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PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede Each with Security Classification Code) (U) Splanchnic Hemodynamics; (U) Left Gastric Artery; (U) Right Gastric Artery; (U) Shock; (U) Vasoconstrictor Therapy

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To elucidate gastrointestinal responses to shock and evaluate their contribution to the pathogenesis of irreversibility of shock. To evaluate the pharmacologic effect of drugs currently used to control gastrointestinal hemorrhage. The studies relate to gastrointestinal pathology that frequently occurs in soldiers following trauma.

24 (U) Study gastrointestinal hemodynamic responses to experimental endotoxic and hemorrhagic shock. Evaluate the adrenergic mechanisms functional in the gastric circulation and the effect of vasopressin on that vasculature.

25 (U) 73 07 - 74 06. A lethal dose of endotoxin causes profound hypotension in the dog and a drastic reduction of gastric blood flow. At the same time oxygen extraction from the gastric arterial blood increases and tissue oxygen consumption increases. These studies are not yet completed. The subregional distribution of adrenergic receptors was studied in both the left and right gastric arterial circulations. The results suggest that the region of the stomach supplied by the left gastric artery is under a predominant beta adrenergic influence while that region served by the right gastric artery is primarily controlled by alpha adrenergic receptors. Thus, epinephrine, a drug often used to control bleeding from esophageal varices, causes a dilator response when infused into the left gastric artery. Since this response would be detrimental in the subject clinical setting, the data question the advisability of using epinephrine in this therapeutic modality. Vasopressin, when used in the same manner, caused sustained constriction of the gastric vasculature but caused greater changes in central hemodynamics than indicated in the clinical literature.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*Available to contractors upon originator's approval

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Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121 Gastrointestinal responses to trauma

Investigators.
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1. Adrenergic Mechanisms in the Gastric Circulation

a. Background and Statement of the Problem. Selective intraarterial infusion of vasoconstrictor drugs has become a frequently used means of controlling gastrointestinal hemorrhage (1-3). In addition to vasopressin, the adrenergic amines, epinephrine and norepinephrine, have recently been employed in this manner. Previous reports from this laboratory demonstrated autoregulatory escape in the mesenteric circulation when infused with catecholamines; a phenomenon that would argue against using these agents in the manner described (4). Since intraarterial epinephrine infusion is presently being used in hemorrhagic gastritis, a study was conducted to evaluate the effects of the common adrenergic amines on gastric blood flow.

b. Experimental Approach. The right and left gastric arteries in dogs were isolated and blood flow measured electromagnetically. The first branch of each artery was cannulated for intraarterial injection and infusion of the adrenergic amines, epinephrine, norepinephrine, and isoproterenol. Arterial and portal venous pressures were monitored and vascular resistance calculated. The effects of the catecholamines were measured before and after alpha or beta adrenergic blockade.

c. Results and Discussion. The intraarterial injection of epinephrine $[10^{-3} - 10^{0}$ μg (base) $kg^{-1}]$ produced a biphasic response in both arteries. In the left gastric artery it caused an initial constrictor response followed by a large amplitude increase in flow while in the right gastric artery the constrictor phase was greater and the dilation minimal. When infused into the left gastric artery for 10 minutes, epinephrine (0.05 μg kg^{-1} min^{-1}) caused a transient decrease in blood flow that rapidly increased to be significantly greater than control within three minutes. Right gastric arterial infusion caused a more sustained constriction which was, however, associated with autoregulatory escape returning flow to, but not exceeding, control. Alpha adrenergic blockade with phenoxybenzamine attenuated the constrictor and augmented the dilator responses and beta adrenergic blockade with propranolol exerted the opposite influence.

Norepinephrine injections in the same doses also produced biphasic responses in gastric blood flow. However, the response in each artery was predominantly constrictor in nature. Ten minute infusions

of the drug caused constriction accompanied by autoregulatory escape in both arteries. In this regard the effect of norepinephrine is similar to its effect in the canine mesenteric circulation (4). Alpha adrenergic blockade attenuated the constrictor response and beta adrenergic blockade enhanced it.

Isoproterenol caused vasodilation whether injected or infused. The magnitude of the responses in the left gastric circulation was significantly greater than the responses in the right gastric vasculature. Alpha adrenergic blockade did not affect the magnitude of these responses while beta adrenergic blockade significantly attenuated them.

These data suggest that the left gastric vasculature has a greater potential to undergo vasodilation than does the right gastric vasculature; possibly due to an unequal distribution of beta adrenergic receptors. The evidence thus argues against the use of intraarterial catecholamine infusions for control of hemorrhagic gastritis. It is feasible that one could employ infusion of epinephrine or norepinephrine in combination with beta adrenergic blockade for effective control of mucosal bleeding. However, an effective and safe method of delivering the proper amount of propranolol remains to be defined. These data are being prepared for publication.

2. Mesenteric Ischemia: A Therapeutic Approach

a. Background Problem. Acute mesenteric vascular insufficiency is a frequent clinical problem and is associated with a very high mortality. Since the early pathophysiologic changes include thrombi in the microcirculation and splanchnic vasoconstriction, a thrombolytic agent might prove useful in the management of this disorder. Of special value would be a thrombolytic agent that possesses "side" effects of vasodilation. Studies are nearing completion in which the vasomotor effect of three forms of urokinase were evaluated in both the mesenteric and femoral vasculature of dogs.

b. Experimental Approach. Superior mesenteric and femoral arterial blood flows were measured electromagnetically. Appropriate arterial and venous pressures were monitored in order to calculate mesenteric and femoral vascular resistances. The hemodynamic effects of intraarterial injections and infusions of the following three forms of urokinase were determined: (1) Ploug Urokinase (chemical grade, Calbiochem), (2) CTA Urokinase from urine sources (FDA approved, Abbott Labs), and (3) CTA Urokinase from tissue culture sources (not FDA approved, Abbott Labs). The urokinases were studied before and after treating the animals with the beta adrenergic antagonist, propranolol, or the antihistaminic, diphenhydramine.

c Results and Discussion. The CTA Urokinase of urine and tissue culture sources had no hemodynamic effect on mesenteric or femoral arterial flow. However, Ploug Urokinase exerted a dose dependent dilator influence on both vascular beds. Infusion of the drug caused sustained increases in flow without evidence of autoregulation. The dilator responses were not attenuated by either propranolol or diphenhydramine. The Ploug Urokinase thus appears to contain a vasodilator substance that acts by a mechanism other than beta adrenergic receptor stimulation or histamine release. This substance may be a contaminant such as isoenzymes of urokinase that are removed by purification to the CTA forms of urokinase. Since the Ploug Urokinase represents, in principle, a potentially beneficial drug, efforts will be made to identify the contaminant.

3. Adrenergic Mechanisms in the Hepatic Arterial Circulation

a. Background and Problem. Autoregulatory escape has been proposed as a mechanism of protecting the liver from hypoxia when exposed to vasoconstrictor drugs for prolonged periods. The effects of intraarterial catecholamines on hepatic arterial blood flow was studied and compared in dogs and baboons.

b. Experimental Approach. Hepatic arterial blood flow was measured electromagnetically and appropriate central pressures were monitored. The adrenergic amines, epinephrine, norepinephrine, and isoproterenol, were studied as intraarterial injections and infusions in the same concentrations noted above.

c. Results and Discussion. Intraarterial injections of epinephrine and norepinephrine caused dose dependent decreases in hepatic arterial blood flow in both species. The constrictor responses were not accompanied by the "overshoot" phenomenon which is characteristic of the action of the drugs in the canine mesenteric circulation. The "overshoot" has been related to the mechanism of autoregulatory escape and the relationship has been described in a manuscript to appear in the Proceedings of a recent Symposium on Gastrointestinal Physiology. This relationship was supported by the observation that both drugs, when infused into the hepatic artery, caused sustained constriction. Neither species demonstrated evidence of autoregulatory escape. The data thus contradict the concept of autoregulatory escape serving as a mechanism to prevent hepatic ischemia.

The beta adrenergic agonist, isoproterenol, stimulated small magnitude increases in hepatic arterial blood flow in the dog and five times that dose was required to elicit equal responses in the subhuman primate's hepatic circulation. These data suggest that there are few beta adrenergic receptors in the hepatic arterial vasculature of both dogs and baboons.

Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 121 Gastrointestinal responses to trauma

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8A. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
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(U) Pathophysiology of Systemic Responses to Trauma

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19. RESPONSIBLE DOD ORGANIZATION
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23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) a. To correlate environmental oxygen tension with cellular and tissue oxygen consumption under various conditions; b. to characterize the formation of microaggregate in fresh and stored blood and evaluate methods for their removal. These studies are of military relevance.

24 (U) a. Oxygen consumption of rabbit liver slices under several experimental conditions has been determined in an in vitro system employing an oxygen electrode. Oxygen consumption of an isolated vascular bed (canine hindlimb) was studied with the aid of a membrane lung. b. The presence of microaggregates in fresh and stored blood was determined with an electronic particle size analyzer and the effectiveness of several commonly used blood filters in removing the debris was evaluated.

25 (U) 73 07 - 74 06.a. The oxygen consumption of rabbit liver slices correlates directly with the oxygen tension, hemoglobin content and osmolarity of the bathing electrolyte solution. Small amounts of endotoxin significantly reduce the consumption. In the isolated canine hindlimb, oxygen consumption is independent of oxygen tension (above 25 mm Hg) and the presence of endotoxin but is directly correlated with carbon dioxide tension and pH. b. A significant volume of microaggregate debris is present in stored whole blood. Studies with four commercially available filters used during blood transfusion to remove the debris suggest that the filters are only effective in filtering two units of blood and should be replaced if further blood transfusion is required.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 73-30 Jun 74.

*Available to contractors upon originator's approval

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Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 124 Pathophysiology of systemic responses to trauma

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1. Microaggregate Formation in Stored Human Blood.

a. Statement of the Problem and Background. Recent studies have suggested microaggregates composed of degenerating platelets and white blood cells in a fibrin matrix are formed during the storage of human blood at 4° C. Indirect evidence suggest that these microaggregates, when infused into the pulmonary circulation, may produce microembolic pulmonary dysfunction. The results of studies in animals demonstrate that the passage of blood through specialized filters prior to infusion can prevent the postembolic pulmonary dysfunction. Routine transfusion filters composed of 160 micron grid filters are inadequate to remove these microaggregates which have a size range from 13 to approximately 120 microns. Thus, specialized ultrapore filters with filtration capacities in this range have been devised. Studies have been undertaken to determine the factors involved in the formation of microaggregates, test the effectiveness of various commercially available filters, and to ascertain alternate ways for deletion of these microaggregates from stored human blood.

b. Experimental approach. Microaggregate levels were determined in human blood using the Model T Coulter Multichannel Particle Size Analyzer. This instrument can instantaneously analyze the volumes of microaggregates at differential aggregate diameters. Seven experiments have been completed: (1) The levels of microaggregates in human blood anticoagulated in acid citrate dextrose (ACD) and citrate phosphate dextrose (CPD) were analyzed and compared. The effects of passage of these bloods through four commercial filters (Dacron wool, 40 micron grid, urethane, and nylon wool) were determined so that the most efficient filter could be identified. (2) The effects on microaggregate removal of the passage of multiple units of blood through each of the given filters was performed so that the filtration effectiveness of a given filter might be determined. This study had significance in ascertaining the number of units that could be safely administered through one filter. (3) Using differential centrifugation techniques, all components of blood were analyzed for microaggregates. It was determined that the great majority of microaggregates occur in the buffy coat fraction although a significant volume of microaggregates is present

in the packed red cell fraction. (4) Studies were undertaken to determine factors that might prevent the formation of microaggregates during the storage of human blood. The removal of platelets and white blood cells prior to storage is associated with a significant decrease in the formation of microaggregates. Treatment of blood with aspirin (100 micromolar or 200 micromolar), a drug known to inhibit platelet aggregation, caused no significant decrease in microaggregate formation although treatment of blood with prostaglandin (PG E_1) significantly decreased microaggregate levels. Constant agitation of blood during storage decreased microaggregate levels although this technique was traumatic to red blood cells. (5) Studies were completed to determine methods for deletion of microaggregates from stored blood following their formation without the employment of expensive ultrapore filters. After 21 days of storage, the treatment of blood with urokinase or streptokinase (100 units per ml) significantly decreased microaggregate formation. Centrifugation of stored blood with high microaggregate levels at 6000 G for 5 minutes caused coalescence of the microaggregates to form larger particles which were easily removed during passage through standard 140 micron transfusion filters. (6) Microaggregate levels were determined in various forms of component therapy. Commercially prepared packed red cells and plasma preparations were virtually free of microaggregates. Commercially prepared albumin fractions were also free of microaggregates. (7) A study was undertaken to determine the levels of microaggregates in glycerol frozen red cells. Red cells frozen in glycerin in three systems (leutramatric, IBM, and hemanetics) were studied. Microaggregate levels in the leutramatric and hemanetic preparations were low although significant population of microaggregates were present in the IBM prepared blood.

c. Results and Discussion. Although data is inconclusive that the infusion of microaggregates into the human is indeed responsible for post-traumatic pulmonary insufficiency, this cause and effect relationship has been well documented in the canine. Our studies have demonstrated that microaggregates are composed of platelets and of white blood cells although a small volume of microaggregates of unknown origin are contained in the packed red cell fractions. Blood anticoagulated in ACD and CPD contain equal volumes of microaggregates. However, this volume was surprisingly low amounting to 1500 cubic microns x $10^3/mm^3$ of stored blood. Thus, these particles are probably inadequate to produce mechanical occlusion of the pulmonary vasculature and this might suggest that vasoactive-humoral mechanisms are involved in posttransfusion pulmonary dysnction. Ultrapore filters are expensive and may add as much as 10 to to the cost of blood transfusions. Our filtration effectiveness ies have demonstrated that only one of the four commercially available filters is capable of effectively filtering more than 2 units of od. This is the 40 micron grid filter which functioned well during the infusion of 5 units of blood - the limitations of the study. Component therapy has been demonstrated to be free of microaggregates. Thus, washed red cells, packed red cells, plasma and albumin can be administered

without use of filters. However, if these red cells are prepared in the IBM system and large volumes administered, then filters should be employed. The problem of microaggregates can be circumvented by pretreatment of blood before storage with PG E_1 or treatment of blood after microaggregate formation with urokinase or, most simply, by centrifuging the blood so that a coalescence of microaggregates occurs.

2. Effect of Trauma on the Fibrinolytic Activator.

a. Statement of the Problem and Background. Fibrinolytic activator is found in various tissues of mesothelial origin and in vascular endothelium. Although the importance of this system has not been rigorously defined, it is felt that the system by nature of its fibrinolytic (clot dissolving) properties has significance in the maintenance of vascular patency by the removal of deposited fibrin. In addition, this system may play a role in the etiology of postoperative and post-traumatic fibrin adhesions. Studies have been undertaken to determine the effects of trauma on fibrinolytic activator activity in both venous intima and thoracic pericardium and to attempt to determine ways to augment this activity using activators of the fibrinolytic system so that the post-traumatic problems such as thrombosis or adhesion formation might be circumvented.

b. Experimental Approach. Fibrinolytic activator activity was studied by the fibrin slide and fibrin plate techniques of Astrup and associates. Initial experiments were designed to standardize the fibrin plate analysis. The effects of incubation time, incubation temperature and handling of tissues prior to incubation were rigorously studied and standardized. The effects of altering the size of the tissue placed on the fibrin plates were investigated. Shelf life of the fibrin plates was determined by storage of plates for various times and temperatures and then determining sensitivity of plates to standard concentration to urokinase.

(1) The presence of fibrinolytic activator activity in canine pericardium was established. This activity was noted to significantly decrease during various forms of surgical trauma including abrasion, cautery, exposure to hot and cold solutions, exposure to hot lights and desiccation. A study is now in progress to determine the relationship of decreases in activity produced through various forms of trauma to adhesion formation.

(2) Pharmacologic agents (glucose, Keflin, KCl, etc.) known to produce thrombophlebitis, significantly decrease fibrnolytic activator activity when placed in venous segments. Control drugs produced no decrease in activity. The passage of a Fogarty catheter through an arterial bed significantly decreased arterial activity. Decreases in activity paralleled the number of passes of this catheter. In another group of experiments the time course of return of arterial fibrinolytic activity after injury was investigated. Activity was determined in

the femoral arteries at intervals from 1 day to 21 days after passage of a Fogarty catheter. Activity was noted to return to levels of normal approximately 7 days after trauma - although fibrin slides showed that intimal activity never reappeared.

(3) In another set of experiments traumatized vessels were treated with activators of the fibrinolytic system to prevent the decrease of fibrinolytic activity. Jugular veins were crushed (known to decrease activity) or distended with saline in a fashion similar to techniques for the preparation of veins for arterial bypass surgery. Following this trauma, the vessels were incubated in vitro with solutions of urokinase, streptokinase, and heparin. Using a solution of 50 units of UK per cc of saline, activity decreased 20% less than saline controls. Studies investigating the use of larger doses of these drugs are now in effect.

(4) A study was undertaken to determine the rate at which fibrinolytic activator activity appeared on the intima of synthetic grafts after placement in the arterial system. Fibrinolytic activity appeared in the aortic graft as early as 6 hours after insertion, reached a maximum activity in 4 days and then returned to normal by 28 days. Thus, for 6-8 hours after insertion of Dacron graft, the fibrinolytic system is not present to aid in removal of clots and the use of adjunctive anti-coagulation of fibrinolytic therapy might therefore prevent early graft thrombosis.

c. Results and Discussion. Fibrinolytic activator activity is present in pericardium and is decreased following multiple forms of surgical trauma. These decreases may be involved in the formation of postoperative intra-thoracic adhesions - a significant source of morbidity to the patient. This mechanism achieves added significance in light of recent reports that a cause of failure of coronary artery - saphenous vein bypass may be obstruction with intrathoracic adhesions following bypass. Fibrnolytic activator activity is decreased following simple passage of the Fogarty catheter, following this decrease activity is slow to return. Although activity by 28 days has achieved gross normal levels, the activity of the intima never returns to normal and is permanently impaired. The significance of this awaits further definition. Drug therapy, trauma and distention - all common in the surgical treatment of patients - significantly decreases activity. This loss of activity can be partially prevented by treatment of vessels with activators of the fibrinolytic system. Further studies are now in progress to investigate the use of larger doses of urokinase. Finally early loss of patency following the insertion of grafts may be related to the poor fibrinolytic activator activity. For 6-8 hours adequate activity is present to remove the small thrombin and small fibrin and platelet aggregates which are known to adhere to the graft. The use of postoperative heparin to prevent thrombosis or fibrinolytic therapy to aid in the removal of fibrin clots may well seem clinically indicated.

3. Pathophysiology of the Intraarterial Injection of Abused Drugs

a. Statement of the Problem and Background. Since 1942 there has been continuing awareness of the ischemic complications in extremities following the intraarterial injection of a variety of drugs. Most reports have dealt with a few cases following injection of a specific drug. A few experimental studies have suggested either vasospasm or microembolism as possible etiologies. Since some of these drugs involved do not exist in crystalline form and the hemodynamic responses to these drugs are unclear, investigation into the possible induction of vasospasm by the common drugs of abuse when injected intraarterially has been carried out. The experimental approach in groups of dogs and baboons has been to measure arterial flow, arterial and venous pressure and construct dose responses to intraarterial narcotics, non-narcotic analgesics, barbiturates, and amphetamines.

b. Experimental Approach. The experiments were conducted in both dogs and baboons. Femoral arterial blood flow and arterial and venous pressures were monitored. Narcotic, non-narcotic analgesics, barbiturates, and amphetamines were injected intraarterially over a wide dose range and the data constructed as dose/response curves.

c. Results and Discussion. Thiopental, secobarbital, pentobarbital, methadone, morphine, heroin, Talwin (pentazocine) and Darvon (propoxyphene), all produce dose dependent vasodilation when injected into the femoral artery. Dextroamphetamine, a mixed adrenergic drug, produced a biphasic response but no significant reduction in flow occurred. From these data vasospasm as an etiology for the ischemic syndrome seemed unlikely. Subsequently excipients used in the preparation of tablet forms of the drugs and cutting agents used by addicts were administered intraarterially and sequential angiograms were performed. Corn starch and talc each reduced femoral arterial flow and, in fact, produced plugging of the microcirculation with retrograde thrombosis of the vessels. It appeared that microembolism certainly can produce the ischemic syndrome. Induction of platelet aggregates was studied with the Coulter T Particle Analyzer and no significant platelet aggregation in response to comparable doses of the drugs could be achieved.

4. Hemodynamics of Venous Occlusion

a. Statement of the problem and Background. Despite major advances in the treatment of arterial and combined arteriovenous injuries, controversy remains as to the most efficacious management of the isolated venous injury or the venous injury combined with a major arterial injury. Clinical experience in Vietnam suggested that major peripheral venous injuries, particularly in the superficial femoral or popliteal vein, should be repaired when possible. However, earlier reports of thromboembolism following venous repair, coupled with the technical difficulty in repairing veins, has served as a deterrent to this practice.

Previous data from our laboratory have demonstrated the deleterious hemodynamic effects of femoral venous occlusion in the dog and have elucidated the time course and response to sympathectomy during femoral venous occlusion in the dog. Since there are major anatomic differences between the dog and man, it was elected to repeat some of the studies in the subhuman primate, baboon, which is anatomically and phylogenetically closer to man.

b. Experimental Procedures. Femoral arterial blood flow was measured electromagnetically and arterial and peripheral venous pressures were monitored. The effect of femoral venous occlusion on these parameters was monitored before, during (for up to five days of occlusion), and following release of the occlusion. In separate studies the effect of lumbar sympathectomy on these responses was determined.

c. Results and Discussion. The data in the subhuman primate demonstrate similar reductions in femoral arterial flow during femoral venous occlusion as seen in the dog. During chronic venous occlusion the reductions in flow persist for up to 72 hours, thus correlating clinical observations of a "critical" interval for venostasis. Lumbar sympathectomy improves the femoral arterial flow in the presence of venous occlusion without significant increases in the distal venous pressure or alterations in systemic arterial pressure. In conclusion, it has been suggested that major peripheral venous injuries should be repaired if at all feacible. Although collateral veins appear angiographically, these are functionally inadequate to maintain normal flow to the injured extremity. In the presence of an irreparable venous injury, sympathetic denervation by epidural blockade or lumbar sympathectomy may allow an increased flow to the limb with relief of ischemia without deleterious alterations in peripheral venous pressure. The mechanism for this is thought to be reduction of alpha adrenergic stimulation to the venous collaterals allowing further dilation.

5. Venous Substitutes

a. Statement of the Problem and Background. In light of the adverse hemodynamic effects outlined in the previous section and recognizing that suitable autogenous veins are not available,for all sizes of injured veins, evaluation of various potential vein substitute materials have been carried out. In the past, venous prostheses have been studied in the dog's superior vena cava with the azygos vein ligated; in the dog's portal vein, or in the dog's suprarenal inferior vena cava with good results with a variety of materials. In general, the most difficult area for venous replacement has been the femoral vein. Prostheses which have remained patent in the other areas have routinely failed when studied in the femoral area. For this reason our studies have all been carried out in the femoral venous circulation of dogs, the area most likely to yield data on a nonthrombogenic, clinically useful prosthesis. In current clinical use for arterial substitutions are collagen tubes, bovine heterograft carotid arteries (Artegraft-Johnson and Johnson), knitted Dacron tubular grafts,

and microporous teflon (Gore-Tex). Each of these prostheses has been advocated for difficult vascular replacement substitutions but they have not been studied in the venous circuit in the difficult femoral region. Previous observations in this laboratory using autogenous canine vein placed in the femoral vein as autografts or homografts have demonstrated our ability to maintain 75 to 80% patency with autografts and a 40% patency with homografts and the present studies contrasted the other materials with the previous data.

b. Experimental Approach. Each of the above named materials was implanted with a suitable control in the femoral vein of dogs. Venograms were performed at 3 days, 7 days, 3 weeks and 6 weeks following the replacement. This allows the evaluation of thrombosis and/or recanalization as well as continuous patency of the prosthetic sample.

Graft Patency

Material	N	Post-Operative	Day 3	Week 3	Week 6
Autologous Vein*	42	42	35	39	41
Homologous Vein (Fresh)	12	12	5	5	5
Collagen Tube	10	10	0	0	0
Bovine Heterograft	10	2	2	2	2
Gore-Tex®	4	4	0	0	0
Dacron, Hydron coated	27	27	13	9	12
Dacron, plain	17	17	6	3	6

*Significantly ($p < 0.05$) better patency than other graft materials.

From these data, it is concluded that there is presently no venous substitute which favorably compares with an autogenous vein. However, results in mesocaval veno-veno shunting using knitted dacron have been encouraging. In a recent editorial in Surgery, Gynecology and Obstetrics it was stated that all future vascular prostheses should be evaluated either in small arteries or in the venous circuit prior to any statements regarding their thrombogenicity. We certainly concur with this editorial comment and will continue to study new materials for their potential use as venous substitutes.

6. Miscellaneous Studies

Studies were completed that described the effect of adrenergic stimulation on ureteral motility, canine femoral blood flow, and cerebral blood flow. Alpha adrenergic stimulation increased the frequency of ureteral contraction while beta adrenergic receptors mediated inhibition. Both epinephrine and norepinephrine dilated the canine femoral circulation at low dosage and constricted it at higher dosage. The cerebral circulation as supplied by the internal carotid artery is non-responsive to intra-arterial injection of adrenergic amines.

Project 3A162110A821 COMBAT SURGERY

Task 00 Combat Surgery

Work Unit 124 Pathophysiology of systemic responses to trauma

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