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RCS MEDDH · 288 (RI)

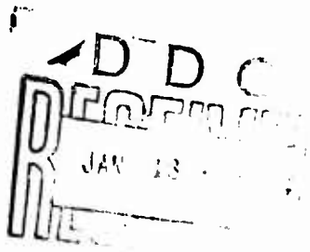
RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

Including

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

ANNUAL PROGRESS REPORT

1 July 1970 - 30 June 1971



VOLUME I

WALTER REED ARMY INSTITUTE OF RESEARCH
WALTER REED ARMY MEDICAL CENTER
WASHINGTON, D.C. 20012

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RCS MEDDH-288 (R1)

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES, INCLUDING BIOCHEMISTRY, COMMUNICABLE DISEASES 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 1970 - 30 June 1971

Volume I

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

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.

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.

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VOLUME I

PROJECT 3A061101A91C
IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00
In-House Laboratory Independent Research

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

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NAME:

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

23(U) To obtain knowledge of the role of immediate and delayed hypersensitivity in the immunopathology of helminthic infections.

24(U) After careful study of pertinent literature and consultations with immunochemists and pathologists, both classical and new methods will be used to obtain data from controlled experiments.

25(U) 70 11 - 71 06 A radioactive iodine labeled microprecipitin assay was developed for measuring binding of antigen by antibody in schistosomiasis. This assay reliably measures antibodies of several Ig classes, but primarily those of IgE. Using 5 different helminth antigens there was specific in vitro leukocyte-mediated histamine release in rabbits infected with Schistosoma mansoni or Trichinella spiralis. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

(Applicable 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 010, Hypersensitivity in the immunopathology of helminthic infections

Investigators
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1. A radioactive antigen microprecipitin assay for schistosomiasis.

Many useful techniques are currently available to detect and measure antibodies developing as a result of parasitic infections. In vitro tests such as the precipitin, agglutination and complement fixation tests have been classified by Minden as secondary reactions. These tests frequently fail to detect significant amounts of antibody demonstrable by primary binding tests. The primary antigen-antibody interaction is the first step in a series of biochemical and biologic processes which may or may not subsequently result in a secondary manifestation. This interaction can be detected by qualitative procedures such as radio immunoelectrophoresis and immunofluorescence or quantitative tests such as equilibrium dialysis, fluorescence quenching or antigen binding capacity of antiserum by the use of radioactive labeled antigens.

Procedures measuring the primary antigen-antibody interaction were first used only with haptens and later with relatively pure protein antigens. Despite the sensitivity and precision of quantitative procedures to detect the primary binding and precipitation of antigen by antibody, these methods have been neglected by those studying resistance to parasitic infection probably in part because it is difficult to iodinate relatively labile antigens and partly because there are so many antigens in crude extracts of animal parasites. Although the immune response to schistosomes has been measured by a variety of tests, a test system which measures a primary antigen-antibody interaction is not available. The present investigations were designed to develop a radioactive antigen micro-precipitin assay and to compare the results with those in the soluble antigen fluorescent antibody and passive cutaneous anaphylaxis tests. The results demonstrate that a radioactive antigen micro-precipitin assay which measures binding of antigen by antibody is a sensitive indicator of the immune response to schistosomiasis in a number of hosts.

I. Antigens

Lipids were removed from lyophylized Schistosoma mansoni cercariae and the lipid-free antigen was fractionated by gel-filtration chromatography. Ten ml of the lipid-free preparation containing 44.4 mg of

protein were placed on a 2.5 cm x 100 cm Sephadex G-200 column and eluted with 0.14 M phosphate buffered saline.

Based on optical density units, two distinct protein fractions were obtained, the first fraction contained 32.4% of the protein and fraction 2 contained 62.6% of the protein. Each fraction was reduced to a 10 ml volume by passing it through an Amicon Cell using a PE 20 membrane. The effluent from the concentration was then reconcentrated by positive pressure using cellulose acetate tubing, and the amount of protein in each was determined by the Lowry method. Fraction 1 contained 1.25 mg protein/ml, fraction 2 contained 2.33 mg protein/ml and a trace amount of protein was recovered from the effluent from the Amicon Cell.

Lipid-free antigen, fraction 1 and fraction 2 were labeled with radioactive iodine (^{125}I) according to the technic of McConahay and Dixon. The ^{125}I labeled antigens were divided into aliquots sufficient for each experiment and stored at -70°C until used. Titrations were done with each new lot of ^{125}I labeled antigen to determine the desired dilution of antigen to obtain the optimal reactivity.

II. Serum

a. Human Source

A total of 104 serum specimens from individuals infected with schistosomiasis living in endemic areas was studied. All diagnoses were confirmed by the presence of eggs in the stools. Of these 48 were obtained in areas of low endemicity (Saint Lucia, BWI) and 56 in areas of high endemicity (Egypt, UAR). In addition, 90 specimens from healthy individuals from nonendemic areas, 85 specimens from persons with known parasitic infections other than schistosomiasis and 50 specimens from persons with viral, bacterial or mycotic infections were used as specificity controls.

b. Animal Source

All sera used in these experiments were obtained from young adult animals. Rabbits were immunized by inoculating 10 mg of the lipid-free cercarial antigen in 1 ml of complete Freund's adjuvant into the foot pads. The animals were challenged with 10 mg lipid-free cercarial antigen in saline intradermally 10 and 17 days later and bled at the appropriate times.

Rhesus monkeys were exposed to either 100, 500, 800 or 1,000 cercariae and bled at weekly intervals throughout the experiment.

Some chimpanzees were exposed once to either 500, 1,000 or 2,000 cercariae while others were exposed monthly to 250 cercariae. All animals were bled at 2 week intervals throughout the experiment.

Mice were exposed to [illegible] S. mansoni cercariae each and bled at weekly intervals.

c. Fractionation

Pooled human anti-S. mansoni sera were precipitated with [illegible]% saturated ammonium sulfate and dialyzed against several changes of 0.1 M phosphate buffered (pH 7.[illegible]) saline. Column fractionation on DEAE A-[illegible] was performed essentially as described by Ishizaka. Of the [illegible] fractions collected from the DEAE column, one was designated as fraction I and the other 4 were pooled and concentrated by positive pressure dialysis. The concentrated pooled fraction was then refractionated by chromatography on Sephadex G-200 essentially as described by Cweifler and Robinson. The four fractions thus obtained were designated as Fractions II, III, IV, and V. These, as well as the first fraction from DEAE chromatography (Fraction I) were then individually concentrated before testing for antibody activity.

All sera used in these experiments were obtained from blood which was allowed to clot at room temperature for approximately one hour and then placed in a refrigerator (4°C) to complete retraction of the clot. The serum was separated within 24 hours after collection and stored at -20°C.

d. Reduction and Alkylation and Heat Treatment

Aliquots of antiserum (0.5 ml) were dialyzed against 250 ml of 0.1 M 2-mercaptoethanol for 4 hours at room temperature and then dialyzed in 500 ml 0.02 M iodoacetamide for 4 hours. These sera and their controls were then dialyzed against several changes of 0.14 M phosphate buffered saline pH 7.2 at 4°C. Samples of antiserum were heated at 56° in a water bath for 4 hours. Unheated samples served as controls.

e. Absorption Experiments

Antisera were absorbed with S. mansoni antigen by diluting the nonlabeled lipid-free antigen 1:10 and then preparing two-fold dilutions in triethanolamine buffered saline (TBS), pH 7.2. The immune and normal serum samples were absorbed with the antigen dilutions as follows: 300 µl of immune or normal serum was placed in a 10 x 75 mm glass test tube and 300 µl of the dilution of antigen was added, mixed well and incubated at 4°C for 18 hours, after which they were centrifuged at 250 x G and the supernatant fluid was removed for testing in the RAMP assay.

Immune rabbit serum was absorbed by adding 0.1 ml of goat anti-rabbit IgE serum to 1.0 ml of the immune serum and incubating the mixture at 37°C for 30 minutes. The samples were then centrifuged at 250 x G and the supernatant portion was transferred to a clean test tube and reabsorbed two more times. The final supernatant fluid was removed and divided into aliquots for testing.

III. Antibody Determination

a. Soluble Antigen Fluorescent Antibody

The SAFA test was conducted as described for schistosomiasis. The titers were expressed as the reciprocal of the highest dilution giving a positive reading.

b. Passive Cutaneous Anaphylaxis (PCA)

PCA tests in monkeys were performed as described by Sadun, in rabbits as described by Zvairler and Becker and in mice as described by Mota. In all three species of animals PCA reactions were induced 72 hours after sensitization, and the reactions were recorded 30 minutes after injecting antigen. A positive reaction was recorded when the area of cutaneous bluing was greater than 5 mm in diameter.

c. Immunodiffusion

Immunodiffusion of antisera against the lipid-free antigen and both of the fractions collected from G-200 chromatography was carried out by the method of Ouchterlony with minor modifications. Ouchterlony plates were prepared on microscope slides using 5 ml of a 1% agar. A number one cork borer was used to prepare the wells which were spaced one diameter apart. All wells were refilled once, 24 hours after the first filling. The plates were incubated for 48 hours at room temperature, then photographed so that the precipitin bands could be compared.

d. Radioactive Antigen Micro-precipitin Assay (RAMP)

The test serum antigens were diluted in a 1:100 homologous normal serum in TBS (0.15 M-TBS). A 300 µl aliquot of each serum dilution was transferred to a 0.5 µl polypropylene micro-test tube. Twenty µl of ^{125}I tagged antigen (I* Ag) dilution were added to the serum using an Eppendorf micropipette. The tubes were then capped and mixed well with a Beckman model 164 micro-mixer. After 18 to 24 hours incubation at 4°C, the samples were centrifuged in a Beckman microfuge model 152 at 15,000 rpm (approximately 11,000 x G's) for 5 minutes at room temperature. The supernatant fluid was carefully removed and transferred to another clean micro-test tube. The amounts of I* Ag in the precipitate and in the supernatant fluid were determined by means of ^{125}I activity in a Baird-Atomic Model 711 well-type gamma counter. Washing the precipitates did not significantly reduce the radioactivity of the precipitate.

Results were expressed as a percent of the total precipitable radioactivity obtained by adding 100 µl of 20% trichloroacetic acid to 20 µl of antigen in 280 µl of 1:10 normal serum diluted with 1:100 M-TBS. Sixty to sixty-five percent of the radioactive antigen was precipitated with 20% trichloroacetic acid; a serum sample which precipitated greater than 10% of the radioactive antigen was considered positive.

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

Immunoprecipitin tests were conducted on the lipid-free antigen and fractions 1 and 2 obtained after passing through a Sephadex G-200 column. Three distinct bands formed between the immune sera and the lipid-free antigen, whereas only 1 band was produced against fraction 1 and 2 bands against fraction 2.

IV. RAMP Assay in Schistosomiasis

a. Effect of Different Antigenic Preparations

Preliminary studies indicated that the RAMP assay with lipid-free extracts of S. mansoni as a antigen could differentiate between serum specimens obtained from immunized or infected animals and their normal controls. A series of experiments was set up to compare the relative effectiveness of several antigenic preparations. Lipid-free S. mansoni somatial extract and the 2 fractions obtained after separation in a Sephadex G-200 column (Fig. 1) were iodinated and reacted against serum from immunized, infected and uninfected animals. The results obtained in five different experiments were essentially similar and have been summarized in Table 1. Differentiation between infected and normal sera was observed with two of the antigens tested. Best results were obtained when the first fraction collected from the Sephadex G-200 column was used as antigen. This is shown by the high ratio of antigen precipitated by schistosomiasis antisera as compared to that precipitated by normal sera. A greater percentage of antigen precipitation was observed with the serum from immunized animals. Based on the results obtained by immunodiffusion and in testing different preparations in the RAMP assay, fraction 1 was used as the antigen of choice throughout the following experiments.

To determine the effect of various serum and antigen dilutents in the assay procedure, serum specimens from infected and normal monkeys were diluted 1:10, 1:20 and 1:40 with TBS alone, TBS plus 0.5% bovine serum albumin or TBS plus normal monkey serum in different concentrations. As indicated in Table 2, best results (ratio 7.1) were obtained when the serum was diluted in TBS plus 1:100 normal monkey serum.

Optimal results were obtained when test sera were initially diluted 1:10 with 1:100 NMS-TBS. Two-fold serial dilutions were made to determine end point reactions.

Table 1 *Relative efficiency of different radioactive iodinated antigenic preparations of* S. mansoni *cercarial antigen in the RAMP assay.*

	Lipid-free extract		Sephadex G-200 fractionation			
			First fraction		Second fraction	
Serum source	% ppt	Ratio*	% ppt	Ratio	% ppt	Ratio
Rabbit immunized	63.4	7.5	97.9	13.4	6.4	1.1
Rabbit normal control	8.5		7.3		5.9	
Rabbit infected	34.8	4.1	93.4	12.8	ND	ND
Rabbit normal control	8.5		7.3		ND	
Monkey infected	40.4	2.2	72.0	6.7	8.4	1.5
Monkey normal control	18.5		10.7		5.7	

* Infected/control.
ND = Not done.

Table 2 *Effect of serum and antigen diluent with serum dilutions in the RAMP assay.*

	Per cent antigen precipitated with monkey serum at given dilution:								
	1:10			1:20			1:40		
Diluent	Infected	Normal	Ratio*	Infected	Normal	Ratio	Infected	Normal	Ratio
TBS + 1:100 Normal monkey serum	68.6	9.6	7.1	34.6	10.6	3.3	16.1	9.3	1.7
TBS + 1:10 Normal monkey serum	55.2	12.5	4.4	21.2	11.0	1.9	6.1	7.8	0.8
TBS + 0.5% BSA	40.0	9.6	4.2	35.1	10.9	3.2	16.7	9.4	1.8
TBS only	35.7	10.0	3.6	33.3	10.3	3.2	19.0	11.1	1.7

* Infected/control.

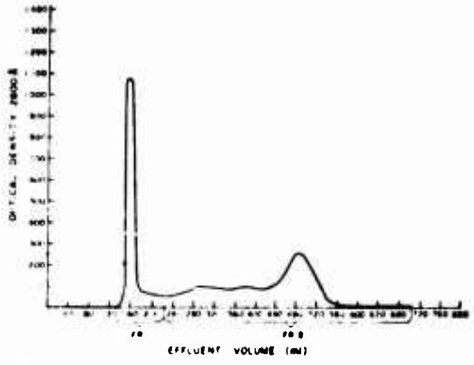


Figure 1 Elution pattern of lipid-free antigen from *S. mansoni* cercariae, using Sephadex G-200 column.

b. Effect of Specific Antibody Absorption

To determine whether the RAMP assay is due to a specific antigen-antibody reaction, attempts were made to remove antibody by absorption with two-fold dilutions of the non-labeled antigen prior to testing. Aliquots of the same sample of antiserum were mixed with increasing amounts of antigen and then assayed against I* Ag for their ability to react. The results of testing antisera after various degrees of absorption are shown in Fig. 2. The percentage of radioactive antigen precipitated by the antiserum decreased in direct proportion to the concentration of antigen used in absorbing it before testing in the RAMP assay. Incubation of immune serum in TBS alone failed to reduce its ability to precipitate radioactive antigen.

c. Sensitivity and Specificity of the RAMP Assay with the Sera from Different Species of Infected Animals.

To study the sensitivity and specificity of the RAMP assay with S. mansoni antigen, serum from animals of different species experimentally infected with S. mansoni and S. haematobium, sera from immunized rabbits and normal serum controls were tested at a constant dilution of 1:10 (Table 3). Except for mice, positive results were obtained in all specimens from infected or immunized animals regardless of the schistosome species with which they were infected. Of the 90 specimens from infected mice 17 showed borderline reactivity. None of 30 specimens from noninfected mice reacted in this assay.

d. Sensitivity and Specificity of the RAMP Assay with Human Sera.

Serum samples from patients with proven schistosomiasis, from patients with a variety of infections other than schistosomiasis and from patients with degenerative diseases were assayed for their ability to react in the RAMP assay. Of the 104 schistosomiasis patients, 48 were from areas of low endemicity and 56 from areas of high endemicity. All samples were diluted 1:10. As shown in Table 4, positive reactions were obtained in 81 percent of the serum samples from schistosomiasis patients. Conversely, no reactions were observed with 90 sera from healthy persons. A few positive reactions were obtained among the sera from individuals with various parasitic, bacterial, mycotic or viral diseases. Of these, most occurred in persons with syphilis.

e. Reproducibility

In order to obtain some information on the reproducibility of results, serum pools from infected and normal rhesus monkeys and from immunized and normal rabbits were divided into aliquots and tested at different times with different lots of radioactive antigen. As indicated in Table 5, the results were highly reproducible. All of the specimens from infected or immunized animals reacted and none of the

specimens from normal controls reacted. The labeled antigen appeared to be stable for at least 4 months when frozen at -70°C.

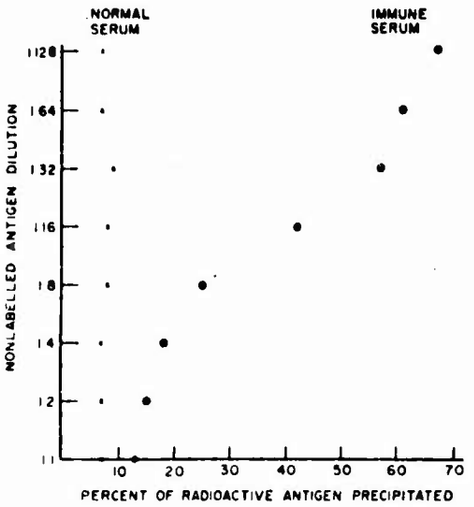


FIGURE 2. Per cent of radioactive antigen precipitated with antiserum after absorption with various nonlabeled antigen concentrations.

Table 3 *Sensitivity of RAMP assay using* S. mansoni *antigen in sera of immunized and experimentally infected animals.*

Serum source	Number specimens	Number reactive	Mean % antigen ppt	Ratio*
Rabbits immunized lipid-free antigen	14	14	95.6	8.9
Rabbits normal controls	14	0	10.8	
Monkeys infected with *S. haematobium*	71	71	69.5	6.5
Monkeys normal controls	23	0	10.7	
Monkeys infected with *S. mansoni*	158	158	62.9	5.9
Monkeys normal controls	23	0	10.7	
Chimpanzees infected with *S. haematobium*	53	53	38.8	3.3
Chimpanzees normal controls	14	0	11.7	
Chimpanzees infected with *S. mansoni*	28	28	41.6	3.6
Chimpanzees normal controls	14	0	11.7	
Mice infected with *S. mansoni*	90	17	11.1	1.7
Mice normal controls	30	0	6.5	

* Infected/controls.

f. Effect of Hydrogen Ion Concentration

To determine the possible effect of hydrogen ion concentration on the antigen-antibody reaction, experiments were conducted with sera in which the pH varied from 6.0 to 8.5 by adjusting it with NaOH or HCl. The results indicated that although the RAMP assay could be performed successfully at all the hydrogen ion concentrations tested, a pH of 7.0-7.2 gave optimal antigen-antibody interaction.

g. Effect of Nonspecific Proteins

The effect of nonspecific albumin and gamma globulin on the results of the RAMP assay was studied. Varying amounts of normal rabbit gamma globulin or bovine serum albumin were added to a constant dilution of sera from infected and immunized animals and their controls, and assayed. The presence of albumin or gamma globulin in the amount up to 8 mg/ml had no demonstrable effect on the reactivity of the various sera tested.

Table 4 *Sensitivity and specificity of RAMP assay using* S. mansoni *antigen with human sera.*

Diagnosis	Number samples	Reactive			Nonreactive		
		Number samples	Mean % ppt	Ratio*	Number samples	Mean % ppt	Ratio
Healthy	90	0			90	9.5	
Schistosomiasis	104	84	28.0	2.9	20	12.9	1.4
Low endemicity	48	30	25.3	2.7	18	12.8	1.3
High endemicity	56	54	29.5	3.1	2	14.0	1.5
Other parasitic infections	85	13	17.7	1.9	72	11.4	1.2
Nonparasitic infections	59	8	17.6	1.9	51	11.9	1.3
Syphilis	14	7	17.0	1.8	7	11.2	1.2

* Infected/controls.

Table 5 *Reproducibility of results with different lots of* S. mansoni *antigen.*

Serum source	Number times tested	Nonreactive	Number times reactive at 1:10 dilution: expressed in per cent precipitated Ag				
			15–34	35–54	55–74	75–94	> 95
S. *mansoni* infected monkey	14	0	—	10	4	—	—
Normal monkey	14	14	—	—	—	—	—
S. *mansoni* immunized rabbit	18	0	—	—	7	11	—
Normal rabbit	18	18	—	—	—	—	—

V. Characterization of Antibodies in RAMP Assay

A series of investigations was conducted to define some of the properties of the antibodies which reacted in this assay.

a. Serum Fractionation

Ion-exchange chromatography and molecular sieving were employed in an attempt to separate some of the antibodies present in sera from infected human patients. The pooled serum was separated into 5 fractions by DEAE chromatography. Fraction I contained most of the protein and was tested as such. The other 4 fractions were pooled and passed through a G-200 Sephadex column. Four fractions were eluted and designated as Fractions II III, IV and V (Fig. 3). Immunoelectrophoresis showed that Fraction I contained most of the IgG and IgA immunoglobulins; Fractions II and III contained a trace amount of IgG and Fractions IV and V had no precipitin lines against any of the immunoglobulin classes. All of the SAFA activity was found in Fraction I. PCA activity was found only in Fractions I and V with the strongest reactions in Fraction V. All five fractions reacted in the RAMP assay and Fraction V produced the strongest reactions (Table 6, Fig. 3).

Table 6 *Results of fractionation of immune* S. mansoni *human sera (pooled).*

	RAMP % ppt	PCA activity	SAFA titer
Immune serum	43.7	4 +	1:128
Immune globulin	55.0	4 +	1:128
Fraction I	21.8	2 +	1:128
Fraction II	22.1	—	—
Fraction III	17.2	—	—
Fraction IV	19.8	—	—
Fraction V	37.4	4 +	—

II. Characterization of antibodies in RAMP assay

A series of investigations was conducted to define some of the properties of the antibodies which reacted in this assay.

b. Effect of Heat, Reduction and Alkylation

The effect of heat, reduction and alkylation was studied with specimens from an immunized rabbit, experimentally infected chimpanzees and naturally infected human patients. The results (Table 7) show that heat and treatment of serum with 0.1 M 2-mercaptoethanol followed by alkylation with 0.02 M iodoacetamide destroyed the reaginic

antibodies as detected by PCA and reduced the reactivity with the RAMP assay. Conversely, similar treatment failed to decrease the reactivity of the fluorescent antibodies in any of the sera.

Similar results were obtained with 20 serum samples from infected patients and 6 from uninfected controls tested nonheated and heated for 4 hours at 56°C. Before heating, all of the specimens from the infected patients and none from the controls reacted in this assay (28.3 and 10.0% precipitate, respectively). After heating all 26 samples gave negative results (13.0 and 9.0% respectively). The addition of guinea pig serum as a source of complement after heating failed to restore the reactivity of the sera.

c. Serum Absorption with Monospecific Antiglobulin

Absorption of rabbit antiserum with goat anti-rabbit IgE serum markedly reduced the reactivity of the whole serum in the RAMP assay (from 82.1 to 53.2 percent) and eliminated its reactivity in the PCA test (from 4+ to negative), but the titers in the SAFA test were not reduced.

d. Correlation of Results with PCA Activity in Human Sera

PCA activity was determined in 85 SAFA reactive human schistosomiasis sera using rhesus monkeys, and the results were compared with the antigen binding of RAMP assays. As shown in Table 8, 48 specimens were positive in both the RAMP and PCA tests. Whereas only 3 sera in which reaginic antibodies had been detected failed to react in the RAMP assay, 17 which reacted in the RAMP assay, gave negative results in the PCA test.

Table 7 *Effect of heat, reduction, and alkylation on immune sera in the RAMP, PCA, and SAFA tests.*

Serum treatment	Immune rabbit			Infected chimpanzees			Infected patients		
	RAMP	PCA	SAFA	RAMP	PCA	SAFA	RAMP	PCA	SAFA
Untreated	82.1	4+	1:32	47.7	4+	1:512	43.7	4+	1:128
56 C—4 hr	25.1	—	1:32	16.3	—	1:512	10.8	—	1:128
2 Mercaptoethanol + iodoacetamide	47.6	—	1:32	36.3	—	1:512	13.7	—	1:128
Iodoacetamide	69.7	4+	1:32	46.8	4+	1:512	49.4	4+	1:128
PBS dialysis (only)	73.8	4+	1:32	39.9	4+	1:512	36.6	4+	1:128

Table 8 *Correlation of results obtained with PCA and RAMP tests in sera from infected patients reactive in the SAFA test.*

		RAMP assay reactivity		
		Positive	Negative	Total
PCA activity	Pos.	48	3	51
	Neg.	17	17	34
	Total	65	20	85

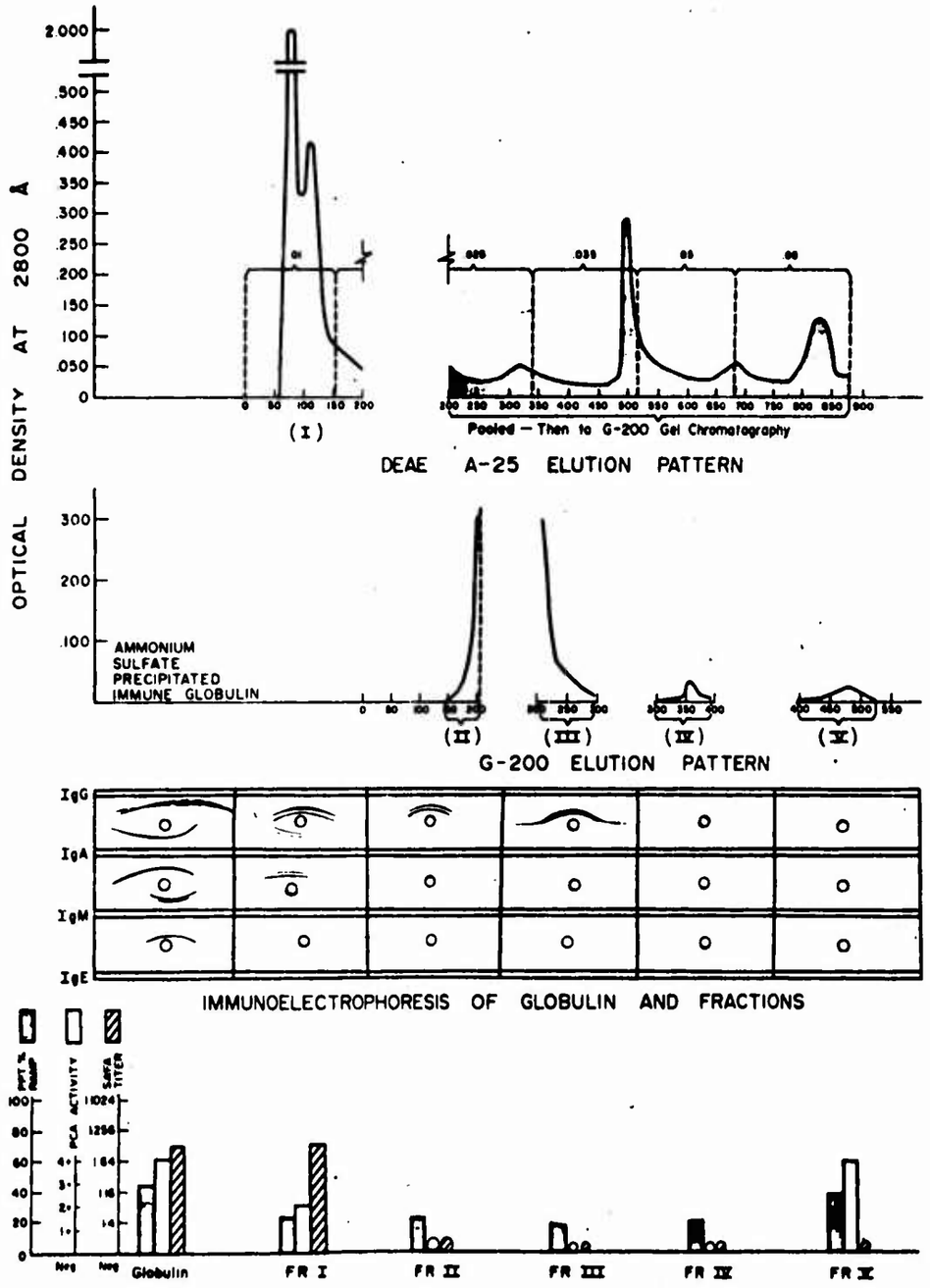


FIGURE 3. RAMP, PCA, SAFA, and immunoelectrophoretic activities of fractions of human anti-S. *mansoni* serum. Ammonium sulfate-precipitated immune globulin was fractionated on DEAE A-25. Of the 5 fractions collected 4 were pooled (top right shaded area), concentrated, and refractionated on Sephadex G-200. The first fraction obtained on DEAE A-25 (I) and the 4 fractions obtained on Sephadex G-200 (II, III, IV, and V) were analyzed by immunoelectrophoresis and for RAMP, PCA, and SAFA activity.

VI. RAMP Assays with Sera from Experimentally Infected Animals

To determine the time-course development of antibodies detected by the RAMP assay, chimpanzees, monkeys and mice were infected with either S. mansoni or S. haematobium. Sera taken before exposure to infection and at regular intervals afterwards, were tested by the RAMP assay and the results were compared with those obtained in the passive cutaneous anaphylactic and fluorescent antibody tests.

In the first experiment, 4 chimpanzees were exposed once to 500 or 2,000 S. mansoni cercariae each. Serum specimens from 3 of these animals gave positive PCA reactions within 3 to 4 months and remained elevated through 18 months. Fluorescent antibodies were observed in all of the animals within 2 months after exposure to infection, reached a peak in 6 to 10 months and remained elevated throughout the study. Antibodies were detected by the RAMP assay in all the animals as early as one month after infection, reached a peak after approximately one year and then decreased as the infection progressed. The pattern obtained on one infected chimpanzee is typical of the group and shows the three curves of antibody development (Fig. 4).

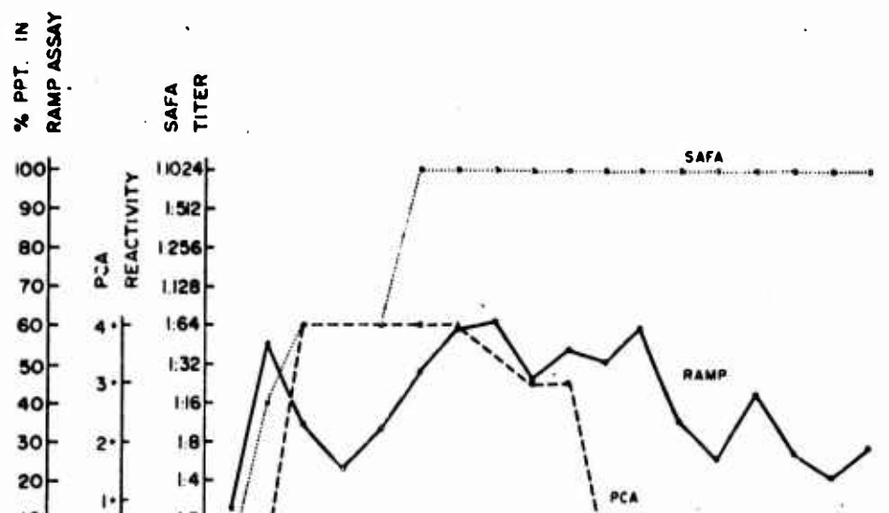


FIGURE 4. Representative time-course development of antibodies in one chimpanzee exposed to 200 cercariae of *S. mansoni*.

In a subsequent experiment, 4 rhesus monkeys were exposed to 800 S. mansoni cercariae and challenged with 5,000 cercariae ten months later. The PCA antibody response following the primary exposure varied. PCA antibodies were detected in 2 of the 4 animals as early as 2 months after exposure. Fluorescent antibodies were demonstrable in all of the infected animals 2 months after infection, reaching a peak almost immediately and remaining elevated throughout the study. Antibodies were detected by the RAMP assay in all of the animals as early as one month after infection and reached a peak almost immediately afterwards. As the infection progressed, there was a diminution of reactivity in these sera and no indication of an anamnestic response in the SAFA and RAMP assay was detected following reinfection. A representative pattern of time-course development of antibodies in these animals is shown in Figure 5.

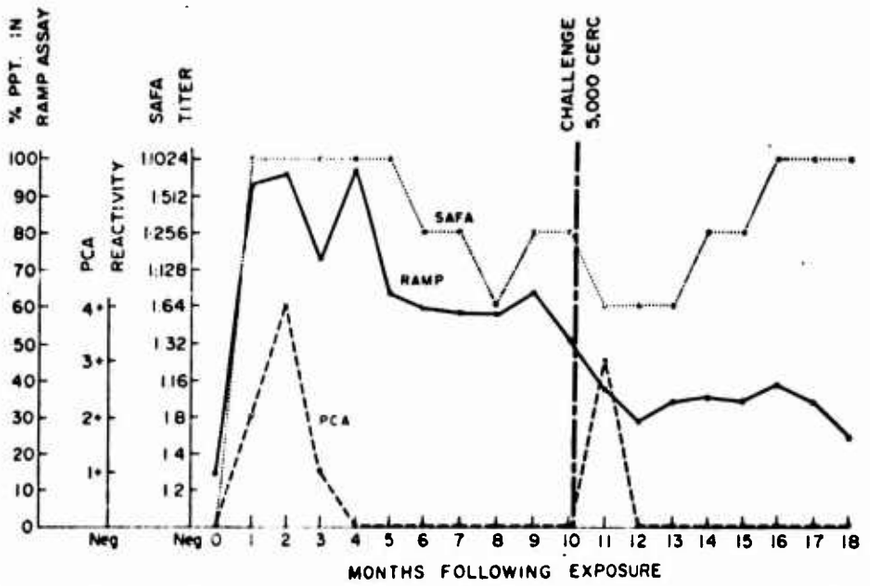


FIGURE 5. Representative time-course development of antibodies in one rhesus monkey exposed to 800 *S. mansoni* cercariae and challenged with 5,000 cercariae after 10 months.

In a third experiment, 4 rhesus monkeys and 8 chimpanzees were exposed to S. haematobium cercariae. As shown in Figure 6, 2 monkeys were exposed to 100 cercariae, 2 to 500 cercariae and 2 to 1,000 cercariae. Of the 8 chimpanzees, 4 received a single exposure of 1,000 cercariae and 4 received monthly exposures of 250 cercariae each. RAMP antibodies were detected as early as one month following exposure in all of the monkeys, reached a peak shortly afterwards and decreased gradually for the duration of the experiment. A higher percentage of precipitation was detected at all times in the monkeys with the heaviest infections.

These antibodies developed slower in chimpanzees than in monkeys and a lower percentage of precipitation was obtained. In all animals with single exposures there was a reduced reactivity as the infection progressed. In animals with multiple exposures antibody activity remained elevated throughout the experiment.

Similar results were obtained in 2 chimpanzees exposed once to 2,000 S. haematobium cercariae. The time-course development of fluorescent, reaginic and RAMP antibodies in one of these 2 chimpanzees is shown in Figure 7.

Similar experiments were conducted with mice exposed to 200 S. mansoni cercariae each. The mice were bled at weekly intervals after exposure to infection. Ten individual sera of each weekly bleeding were pooled and used in the RAMP assay as well as in tests for fluorescent antibodies and PCA reactions. Although fluorescent antibodies (Fig. 8) appeared relatively early during the course of the infection, no PCA activity nor significant RAMP reactivity could be demonstrated.

Schistosoma mansoni antigen labeled with radioactive iodine combines with antibody in the serum from infected patients and animals. The technic which is relatively rapid and highly reproducible, requires very little antigen because of its extreme sensitivity. Our findings agree with observations reported by Minden et al., who compared primary binding tests to detect the primary binding of antisera directed against bovine serum albumin.

The percentage of antigen bound by antibody was remarkably consistent for various serum dilutions at a given antigen concentration. Beyond a certain dilution, however, the reactivity decreased greatly. A pH of 7.0-7.2 was found to be optimal for the RAMP assay which is in agreement with Gleich and Stankievic in their radio-immune precipitation. However, contrary to their results, heating of immune sera resulted in marked decreased in binding activity. Antigen binding did not depend on

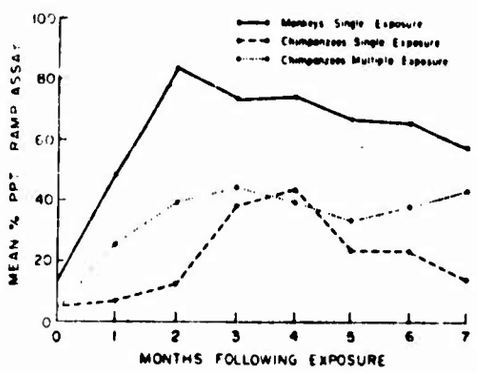


FIGURE 6. Time-course development of RAMP antibodies in monkeys and chimpanzees exposed to varying numbers of S. *haematobium* cercariae.

complement since fresh complement added to heated sera failed to restore RAMP reactivity. Reduction and alkylation also lowered considerably the reactivity of sera in this test. Increasing the albumin or gamma globulin concentration neither reduced nor increased binding significantly. This assay, however, differs from other serological tests for parasitic infections both because it may measure the primary binding of antigen by antibody and because the end points can be determined on the basis of measured radioactive antigen content rather than on subjective visual determinations. Semi-quantitative tests expressed in terms of a serum dilution end point do not provide precise data concerning the quantity of antigen bound by antibody as does the test described here.

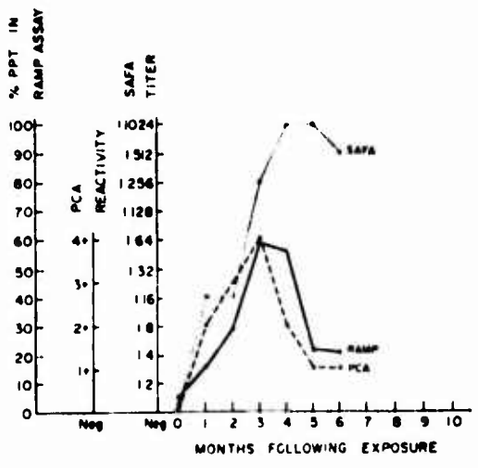


FIGURE 7. Time-course development of antibodies in one chimpanzee exposed to 2,000 *S. haematobium* cercariae.

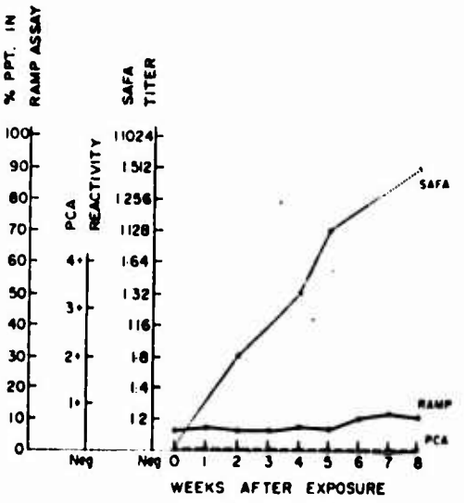


FIGURE 8. Time-course development of antibodies in mice exposed to 200 *S. mansoni* cercariae each.

The value of this assay lies not only in its sensitivity and its usefulness in making accurate quantitative determinations, but also in its capability of providing a means of measuring antibodies in different immunoglobulin classes. Comparison of the results of the RAMP assay with those obtained with the SAFA test indicate that there are many features distinguishing these antibodies in schistosomiasis. Evidence that this test measures antibodies in all immunoglobulin classes and particularly in IgE was obtained indirectly by noting differences in the curves of time-course development of antibodies of the RAMP assay from those of the SAFA test. Moreover, fluorescent antibodies were heat stable, not sensitive to 2-mercaptoethanol and appeared in the IgG fraction, whereas heating, reduction and alkylation eliminated or considerably reduced the antigen binding capacities of various sera in this test. A remarkable similarity of results was observed with the RAMP assay and the passive cutaneous anaphylactic tests

with sera from human patients and experimental animals. However, antigen binding activity in the RAMP assay appeared earlier and persisted longer than PCA activity.

Absorption of immune rabbit serum with anti-rabbit IgE serum completely removed PCA activity and reduced the antigen binding activity of the RAMP assay, but failed to decrease the SAFA titer.

Radioimmune precipitin tests have been employed successfully in patients with atopic diseases by Hansen and Gleich and by Newcomb and Ishizaka to examine the immune response to diphtheria toxoid. The allergen binding activity of antibodies associated with different immunoglobulin classes in serum from atopic patients was studied by Ishizaka et al. They also found that essentially all skin sensitizing activity in the serum specimens was removed by absorbing with anti-IgE sera and that their skin sensitizing activity correlated with antigen binding values of IgE but not with those of other immunoglobulins. Bandilla and McDuffie indicated that IgM and IgA predominate in the primary response while IgG predominates in the secondary response. Our studies indicate that the RAMP assay can be used effectively to measure antibodies in all immunoglobulin classes and particularly in IgE, and is a means of demonstrating primary binding of antigen by antibody in schistosomiasis.

2. Specificity of in vitro leukocyte-mediated histamine release with helminth antigens in rabbit trichinosis and schistosomiasis.

Peripheral leukocytes obtained from rabbits infected with Schistosoma mansoni have been shown to be actively sensitized, as measured by an in vitro antigen induced histamine release from rabbit platelets. This interaction of leukocytes from sensitized rabbits with platelets has been confirmed by Henson as well as Siraganiam and Osler using an entirely different antigen-antibody system. Subsequent investigations by Barbaro and Schoenbechler on the mechanism of this immune reaction indicate that antigen is bound to specific receptor sites on the sensitized leukocytes, resulting in "activation" of the leukocytes. The release of histamine from platelets results from the interaction of activated leukocytes with platelets, either directly by a cell to cell contact or indirectly as reported by Henson, through the release of a soluble factor from the leukocytes.

The purpose of this investigation was to examine the specificity of this in vitro leukocyte-mediated histamine release in two rabbit helminth infections and to compare these results with the specificity of passive cutaneous anaphylaxis (PCA), complement fixation (CF) and the soluble antigen fluorescent antibody (SAFA) reactions.

Experimental infections. Albino rabbits were infected with S. mansoni by cutaneous exposure to 25,000 viable cercariae, or with Trichinella spiralis by the oral administration of pepsin-digests of infected rat muscle containing 15,000 larvae. Trichinella and Schistosoma infected rabbits were tested 3-4 weeks and 13-15 weeks post infection, respectively, for in vitro histamine release and PCA reactions. The "Guide for Laboratory Facilities and Care" as promulgated by the Committee of the National Academy of Science - National Research Council were observed.

Antigens. Antigens consisted of lipid-free extracts of S. mansoni (cercariae), S. japonicum (adults), T. spiralis (larvae) and Dirofilaria immitis (adults) prepared according to Chaffee et al. In addition, an adult Melcher's extract of Paragonimus westermani and a freeze-dried preparation of scolices of Echinococcus granulosus were employed. Each antigen preparation was standardized at 10 mg of dry weight per ml.

PCA reactions. Intradermal injections of 0.2 ml of serially diluted sera obtained from the infected animals were administered to the backs of freshly shaven normal rabbits. After a latent period of 72 hours, one ml (10 mg protein) of antigen mixed with 1.5 ml of a 5% pontamine sky blue solution was intravenously injected. Thirty minutes later, the reactions were observed and a positive reaction was defined as the highest dilution associated with cutaneous bluing of at least 5 mm diameter.

Complement fixation reaction. These tests were performed as described by Kent and Fife.

Soluble Antigen Fluorescent Antibody Reaction. These tests were conducted as described by Dalun and Gore.

In vitro histamine assay. The procedures for this assay are similar to those previously reported, and are summarized as follows. Peripheral blood from infected rabbits was collected into siliconized glass syringes containing 0.1 M ethylenediaminetetraacetate (EDTA) and centrifuged in the cold at 17[illegible] x g for 15 minutes. The supernatant was discarded and the loosely packed cells were resuspended to the initial volume with Tyrode's solution containing 0.005 M EDTA. The cell suspension was then added to 4 volumes of a 1% dextran (MW 235,000) and mixed well. Two parts of the dextran-blood mixture was gently layered over one part of [illegible]% Ficoll. After the agglomerated red cells had settled in the Ficoll layer, the supernatant was aspirated and centrifuged three times at 17[illegible] x g for 10 minutes, each time discarding the supernatant and resuspending with Tyrode's-EDTA solution. The cells were then suspended in plain Tyrode's solution without EDTA, counted with the Coulter electronic particle counter and standardized at [illegible] x [illegible]^{6} leucocytes per ml.

For the preparation of purified platelet suspensions, blood from normal rabbits was collected by intracardiac puncture into siliconized syringes containing EDTA and centrifuged at 175 g for 15 minutes. The platelet-rich, leukocyte-poor supernatant was aspirated with plastic pipettes, added to an equal volume of Tyrode's-EDTA solution and washed twice. The platelet preparations were counted by phase microscopy and suspended in Tyrode's solution to contain 0.6 x 10^9 platelets per ml.

One half ml aliquots of both the standardized leukocyte and platelet suspensions were incubated with 0.2 ml of a 1:2 dilution of the initial homologous or heterologous antigen preparation for 5 minutes at 37°C for histamine release. After incubation, the reaction tubes were centrifuged in the cold and the histamine content of the supernatant was assayed by the fluorometric method of Shore et al., employing a known histamine standard. The total histamine content of the platelet and/or leukocyte preparations used in these experiments were also determined. The histamine release was expressed as a percent, relative to the total platelet histamine content. Any release above 15 percent was considered significant.

Controls included leukocytes obtained from normal rabbits prior to infection with T. spiralis, which were incubated with normal platelets and the various helminth antigens.

Antigen Induced Histamine Release

The degree of leukocyte-mediated histamine release from normal rabbit platelets prior to infection was determined with leukocytes obtained from non-infected rabbits. These leukocytes were tested with T. spiralis, S. mansoni, D. immitis, E. granulosus and I. westermani antigens, and only one demonstrated little, but significant histamine release (less than 20%) with the T. spiralis. S. mansoni and P. westermani antigens. However, it should be noted that the leukocytes from this rabbit also caused an unusually high spontaneous release from platelets without the addition of antigen.

All rabbits infected with T. spiralis and S. mansoni demonstrated significant leukocyte-mediated histamine release when tested with the homologous antigen. Table 1 lists the percent release obtained in the Trichinella group with the homologous antigen as well as the release obtained with the heterologous helminthic antigens. Three of the rabbits infected with T. spiralis showed cross reactivity with S. mansoni antigens. In addition, leukocytes from two of these rabbits also reacted with either I. immitis or E. granulosus. Leukocytes from a rabbit that did not cross-react with S. mansoni did react with D. immitis and the percent histamine release was comparable to that obtained with the homologous antigen.

The results obtained from rabbits infected with S. mansoni are listed in Table 10. Similar to the Trichinella group, all 10 Schistosoma infected rabbits demonstrated significant histamine release with the homologous antigen. The histamine release ranged from 34 to 82%. Eight of the 10 also showed cross reactions with S. japonicum antigen and the magnitude of release was similar to that obtained with the homologous antigen. However, in contrast to the Trichinella infected group, the Schistosoma infected rabbits showed no cross reactions with T. spiralis antigen. Two of the Schistosoma infected rabbits demonstrated cross reactivity with P. westermani antigen.

Table 9

In Vitro *Histamine Release with Homologous and Heterologous Antigens in Rabbits Infected with* Trichinella spiralis

Rabbit no.	*T. spiralis*	Histamine release with indicated antigen				
		S. mansoni	*S. japonicum*	*D. immitis*	*E. granulosus*	*P. westermani*
T1	76[a]	6	ND[b]	2	0	0
T2	65	17	ND	0	0	0
T3	63	0	3	61	2	3
T4	60	16	0	4	21	2
T5	55	14	ND	16	1	0
T6	55	6	0	0	0	1
T7	33	0	0	3	0	0
T8	32	5	0	1	0	0
T9	18	0	0	0	0	0
T10	15	0	0	3	0	0

[a] Percentage of release (10% or greater considered significant release).
[b] Not done.

Table 10

In vitro *Histamine Release with Homologous and Heterologous Antigens in Rabbits Infected with* Schistosoma mansoni

Rabbit no	*S. mansoni*	Histamine release with indicated antigen				
		S. japonicum	*T. spiralis*	*P. westermani*	*D. immitis*	*E. granulosus*
S1	82[a]	81	0	0	0	0
S2	78	54	0	0	0	0
S3	75	68	5	25	2	3
S4	59	48	10	44	6	0
S5	58	56	0	0	0	2
S6	58	62	0	0	4	2
S7	55	49	8	5	10	1
S8	54	49	0	3	1	0
S9	48	0	1	0	0	0
S10	34	1	0	0	0	0

[a] Percentage of release

Passive Cutaneous Anaphylaxis, Complement Fixation and Double Antigen Fluorescent Antibody Tests:

Table 11 shows the results obtained with PCA, CF and FAFA tests on sera from the Trichinella infected rabbits. As can be seen, only the homologous antigen was capable of eliciting a positive PCA reaction and then only in 50% of the rabbits. Sera from the Trichinella infected rabbits reacted in the CF test with all the antigens. The FAFA test was somewhat more specific than the CF test. None of the rabbit sera reacted with F. granulosum antigen and one rabbit reacted with F. japonicum antigen. Seven reacted with F. japonicum antigen. Seven reacted with F. manconi antigen. However, in these tests the titers were considerably lower than those obtained with the homologous F. spiralis antigen.

Table 12 lists the results obtained in the PCA reaction of rabbits infected with F. manconi. These results are similar to those obtained with rabbits infected with F. spiralis. The PCA reaction demonstrated the greatest specificity, while the CF reaction was the least specific. As with the Trichinella infected rabbits, only 50 percent of the Schistosoma infected animals gave a positive PCA reaction with the homologous antigen. Only one rabbit demonstrated a cross reaction with the closely related F. japonicum antigen. The FAFA reaction with the Schistosoma infected rabbits was somewhat more specific than the Trichinella infected rabbits. Eight rabbits reacted with F. japonicum antigen, 7 reacted with F. spiralis antigen and 5 reacted with F. granulosum antigen. The CF results were essentially the same as obtained with the Trichinella infected rabbits.

The specificity of the anti-human IgE mediated histamine release from rabbits infected with F. spiralis and F. manconi was compared with the specificity of various serological tests. As can be readily seen from the results listed in Table 13, the passive cutaneous anaphylaxis reaction is the most specific. Previous investigators have shown that many animals with parasitic infections produce circulating antibody which can sensitize tissues of the same species. These antibodies may exhibit many of the same properties exhibited by human reagin and have been termed skin-fixing. A major drawback in the use of passive cutaneous anaphylaxis for the detection of these skin-fixing antibodies is the fact that the antibody is present in the serum for only a short time after the infected animals. This is in agreement with the observations of other investigators. For this reason, the PCA reaction, while quite specific, is not suitable for use as an immunodiagnostic test for parasitic infections.

It is also of interest to note that the titers obtained in the rabbit sera in the FAFA test were much higher than either the CF or PCA tests with rabbit sera. It is possible that the greater sensitivity in the FAFA test is due to the fact that the Schistosoma

Table 11

Comparison of PCA, SAFA, and CF Reactions in Rabbits Infected with Trichinella spiralis

Rabbit No.	*T. spiralis*			Titers with indicated antigens											
				N. americanus			*N. japonicum*			*D. immitis*			*E. granulosa*		
	PCA	SAFA	CF	PCA	SAFA	CF	PCA	SAFA	CF	PCA	SAFA	CF	PCA	SAFA	CF
T1		256	128		4	32		4	1		ND	32			4
T2	10	256	256			64			4		ND	2			8
T5	10	64	256		16	32			8		ND	8			
T4	20	64	AC		4	AC			AC		ND	AC			AC
T5	160	256	256		64	64					ND	32			4
T6		256	256		4	64		4	1		ND	32			16
T7		256	128		4	16			4		ND	16			4
T8		256	ND		4	ND			ND		ND	ND			ND
T9		256	128		64	32		4	4		ND	16			4
T10	10	64	32		4	32					ND	16			

Reciprocal of highest dilution showing positive reaction; dash indicates negative reaction

Table 12

Comparison of PCA, SAFA, and CF Reactions in Rabbits Infected with Schistosoma haematobium

Rabbit No.	*S. haematobium*			Titers with indicated antigens											
				S. japonicum			*T. spiralis*			*D. immitis*			*E. granulosa*		
	PCA	SAFA	CF	PCA	SAFA	CF	PCA	SAFA	CF	PCA	SAFA	CF	PCA	SAFA	CF
S1	400	16	256		4	16	–	–	64	–	ND	8	–	16	4
S2		4	8		–	4			16	–	ND	16	–	–	2
S3		64	256		16	32		16	32	–	ND	64	–	–	16
S4	200	16	128	200	4	32	–	–	64	–	ND	64	–	–	32
S5		16	256	–	–	8	–	16	64	–	ND	32	–	–	2
S6	100	256	AC	–	64	AC	–	64	AC	–	ND	AC	–	4	AC
S7	10	4	128	–	4	32	–	16	–	–	ND	1	–	16	4
S8	70	256	256		64	32	–	16	64	–	ND	64	–	–	16
S9	–	64	256	–	16	16	–	16	32	–	ND	64	–	–	16
S10	–	64	256	–	16	32	–	16	64	–	ND	32	–	–	–

Reciprocal of highest titer showing positive reaction (dash indicates negative reaction)

infected rabbits would be only 5% if compared according to genus rather than species. In this study, positive CF reactions with heterologous antigens were observed in almost all the serum specimens obtained from infected rabbits. The few remaining sera were anti-complementary. Previous reports by Kidd and Friedewald have indicated that the CF test with rabbit sera is unreliable because of frequent non-specific positive reactions. In the latter studies, it was demonstrated that normal adult rabbit sera contained natural antibodies which could fix complement when mixed with saline extracts of normal homologous rabbit tissues.

Table 13

Composite of the Results of the Histamine Release, Passive Cutaneous Anaphylaxis, Complement Fixation and Soluble Antigen Fluorescent Antibody Reactions with Various Antigens

Antigens	*S. mansoni* infected				*T. spiralis* infected			
	HR	PCA	CF	SAFA	HR	PCA	CF	SAFA
S. mansoni	10/10*	5/10	10/10	10/10	3/10	0/10	8/8	10/10
S. japonicum	8/10	1/10	9/9	8/10	0/7	0/10	6/9	3/10
T. spiralis	0/10	0/10	8/9	7/10	10/10	5/10	8/8	10/10
E. granulosus	0/10	0/10	9/10	3/10	1/10	0/10	6/8	0/10
D. immitis	0/10	0/10	9/9	ND	2/10	0/10	8/8	ND
P. westermani	2/10	0/10	ND	ND	0/10	0/10	ND	ND
Total heterologous reactions	10/50	1/50	35/37	18/30	6/47	0/50	28/33	13/30
Percent	20	2	95	60	13	0	85	43

* Positive reactions no. tested.

The phenomenon of non-reciprocal cross-reactivity between T. spiralis and S. mansoni has been reported in previous serological studies and in the cross adsorption studies of Anderson et al. The lack of reciprocal cross-reactivity between these helminth antigens in the in vitro histamine assays of the present study supports and extends the earlier observations.

Although the specificity of the in vtiro leukocyte-mediated antigen-induced histamine release is not absolute, the frequency and magnitude of positive reaction with heterologous antigens is far less than that obtained with current serological tests now available. This assay procedure, in addition to increasing our understanding of the immunological aspects of helminth infections, may be of practical interest in several respects. The leukocyte-mediated response requires minute quantities of antigen to trigger histamine release from platelets. Schoenbechler and Sadun showed that antigen concentration in nanogram amounts were sufficient to induce significant histamine release from rabbits infected with S. mansoni. This extremely sensitive technique might prove useful in detecting small amounts of circulating antigen in helminth infections. Finally, this procedure might be applicable to the standardization of antigens prepared by different physicochemical methods.

5. Interaction of rabbit platelets and leukocytes for release of histamine: Electron microscopic observations

Several investigators have studied the mode of allergic histamine release from the platelets of rabbits which had been immunized against several different antigens. These studies on release of histamine required the presence of complement. Schoenbechler and Sadun were the first to demonstrate that antigen-induced histamine release from well-washed platelets of rabbits infected with Schistosoma mansoni necessitated only the inclusion of leukocytes from the infected animals. Recently similar platelet histamine release from the rabbit immunized against protein antigens such as bovine serum albumin and horse spleen ferritin has been accomplished by the addition of these sensitized leukocytes. The exact mode of histamine release from the platelets mediated either by the production of a soluble factor from the activated leukocytes or by direct cell to cell interaction remains unsettled.

This communication describes the interaction between the platelets and activated leukocytes by electron microscopy in an attempt to shed light on the mechanism involved in histamine release from the former.

The preparation of leukocytes and of platelets from rabbits were described previously. The procedures are briefly outlined as follows. The leukocytes from schistosome infected rabbits were obtained relatively free of red blood cells by means of dextran agglutination. The major portion of the platelets from the leukocyte preparation were removed by differential centrifugation. Pure lymphocyte suspensions were also made employing a glass bead column. The platelets used in this experiment were obtained from normal rabbits, and were essentially free of leukocytes. All experiments were conducted with leukocytes (sensitized) from infected rabbits and normal rabbit platelets washed three times with Tyrode's buffer so as to ensure the absence of free plasma. The buffer used throughout this experiment was Tyrode's solution. Four different combinations of the various cell suspensions of these platelets, leukocytes and antigen were made as follows:

Group 1: Normal platelets + sensitized leukocytes (control group)
Group 2: Normal platelets + sensitized leukocytes + antigen
Group 3: Normal platelets + antigen-activated leukocytes
Group 4: Normal platelets + antigen-activated leukocytes + antigen

The activated leukocytes were made by incubating sensitized leukocytes with an equal volume of antigen. After incubation the activated leukocytes were washed twice with Tyrode's buffer.

These four types of cell suspensions were fixed in 1.25% glutaraldehyde, 4% sucrose in 0.05 M phosphate buffer (pH 7.4). The fixed material was washed in 0.05 M phosphate buffer and were postfixed in 1% O_sO_4. The preparations were dehydrated in the series of ascending ethyl alcohols and propylene oxide and were finally embedded in Epon 812. The resulting blocks were cut with a Porter-Blum MT-2 ultramicrotome and were stained

with 1% uranyl acetate and lead citrate. Sections from 10 blocks of each group were examined with a Siemens Elmiskop 101 electron microscope.

Morphology of rabbit platelets and leukocytes

The morphology of the normal rabbit platelets will be described briefly since their ultrastructural characteristics are pertinent to this study. Rabbit platelets are elongated or ovoid in shape and possess several cytoplasmic organelles (Figs. 9 and 10). They contain mitochondria, large round to oval electron opaque alpha-granules, small "very dense" granules, vesicles, microtubules, glycogen particles, endoplasmic reticulum and ribosomes. The alpha-granules are abundant and measure 200-300 mμ in diameter. Each granule is surrounded by a unit membrane; the matrix in general is uniformly electron opaque and occasionally is separated from the surrounding unit membrane by a narrow translucent zone (Fig. 10). The "very dense" granules are smaller than the alpha-granules and measure about 200 mμ in diameter. They often appear as round vesicles in which a very dense particle of 100 mμ is situated eccentrically. On rare occasion the entire matrix ix completely occupied with very electron dense material.

The vesicles vary in shape and are limited by a unit membrane. The matrix is electron transparent and does not contain any stainable substance (Fig. 10). The microtubules are usually located near the plasma membrane (Fig. 10) and measure about 200 A in diameter.

The leukocytes seen in these preparations are small lymphocytes, monocytes, neutrophils and eosinophils. The small lymphocytes are round and contain a large round nucleus which occupies about 80% of the cell body (Fig 11). A narrow rim of cytoplasm surrounds the nucleus and contains several mitochondria, numerous ribosomes, microtubules and infrequent smooth endoplasmic reticulum. No rough endoplasmic reticulum is evident. The monocytes are larger than the small lymphocytes. The abundant cytoplasm nad the presence of the rough endoplasmic reticulum together with larger cell size helps to differentiate the monocytes from the small lymphocytes. The granulocytes are round with irregular microvilli extending from their surface (Fig. 9). The granulocyte cytoplasm contains a large number of electron dense granules of various size and shape as well as several nuclei.

Interaction of platelets and leukocytes

In the suspension of platelets and sensitized leukocytes, in Tyrode's solution without antigen (Group 1) numerous platelets, leukocytes and erythrocytes are randomly distributed in the thin sections (Fig. 9). There is no apparent interrelationship or close contact between the leukocytes and the platelets. No morphological changes are observed in these cells.

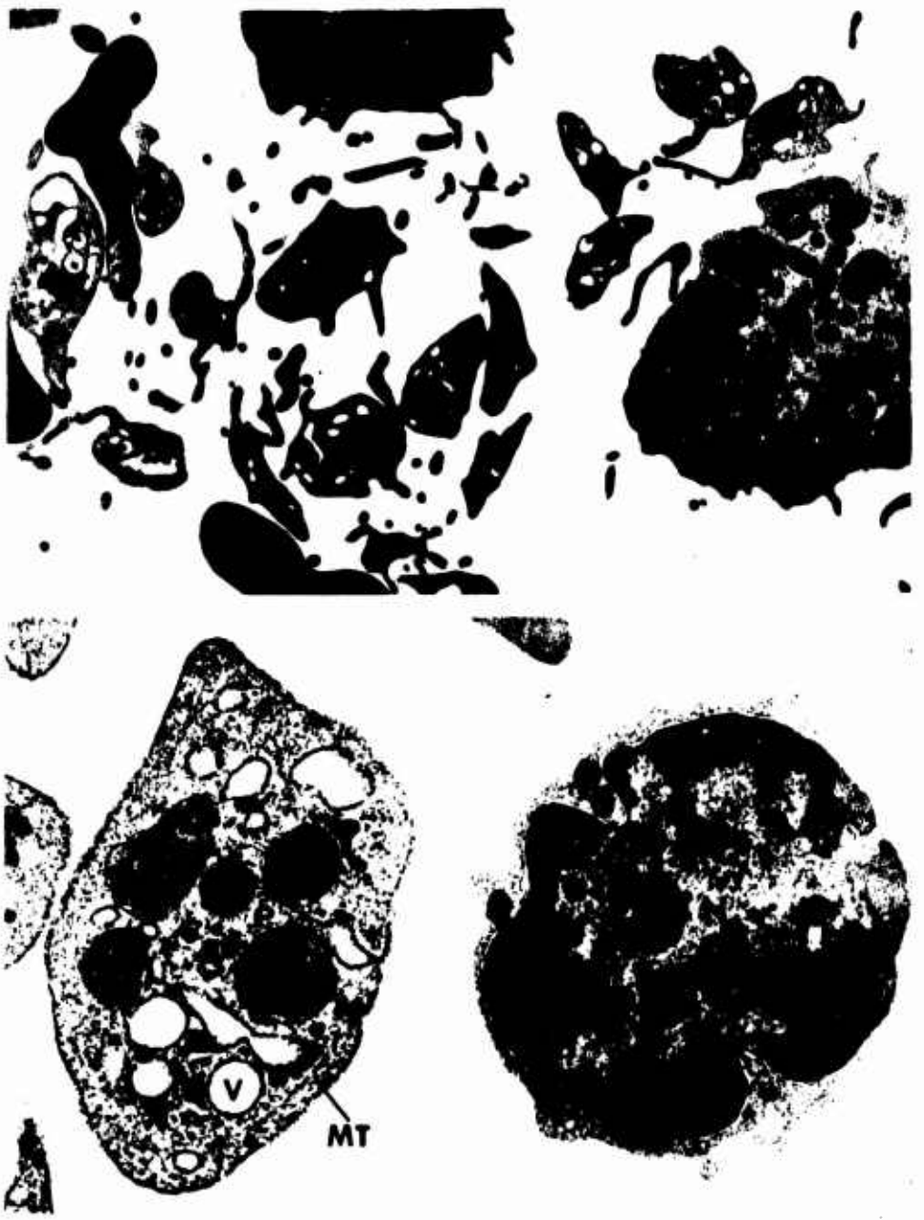


Fig. 9—Cell suspension composed of platelets, and sensitized leukocytes of rabbits without the presence of antigen (group 1). Small lymphocyte (*L*), granulocyte (*G*) and erythrocytes (*R*) are intermingled with platelets (*P*). However, there is no physical interaction between these cells (× 12,000).

Fig. 10—Higher magnification electron micrograph of platelet from control group. It possesses several electron opaque α granules (*AG*), mitochondria (*M*), vesicles (*V*) and microtubules (*MT*). Many dense granules are not observed in this platelet (× 43,000).

Fig. 11—Small lymphocyte from group 1 containing large nucleus (*N*) surrounded by narrow rim of cytoplasm (*C*). Cytoplasm contains a few mitochondria and ribosomes. Endoplasmic reticulum is sparse (× 15,000).

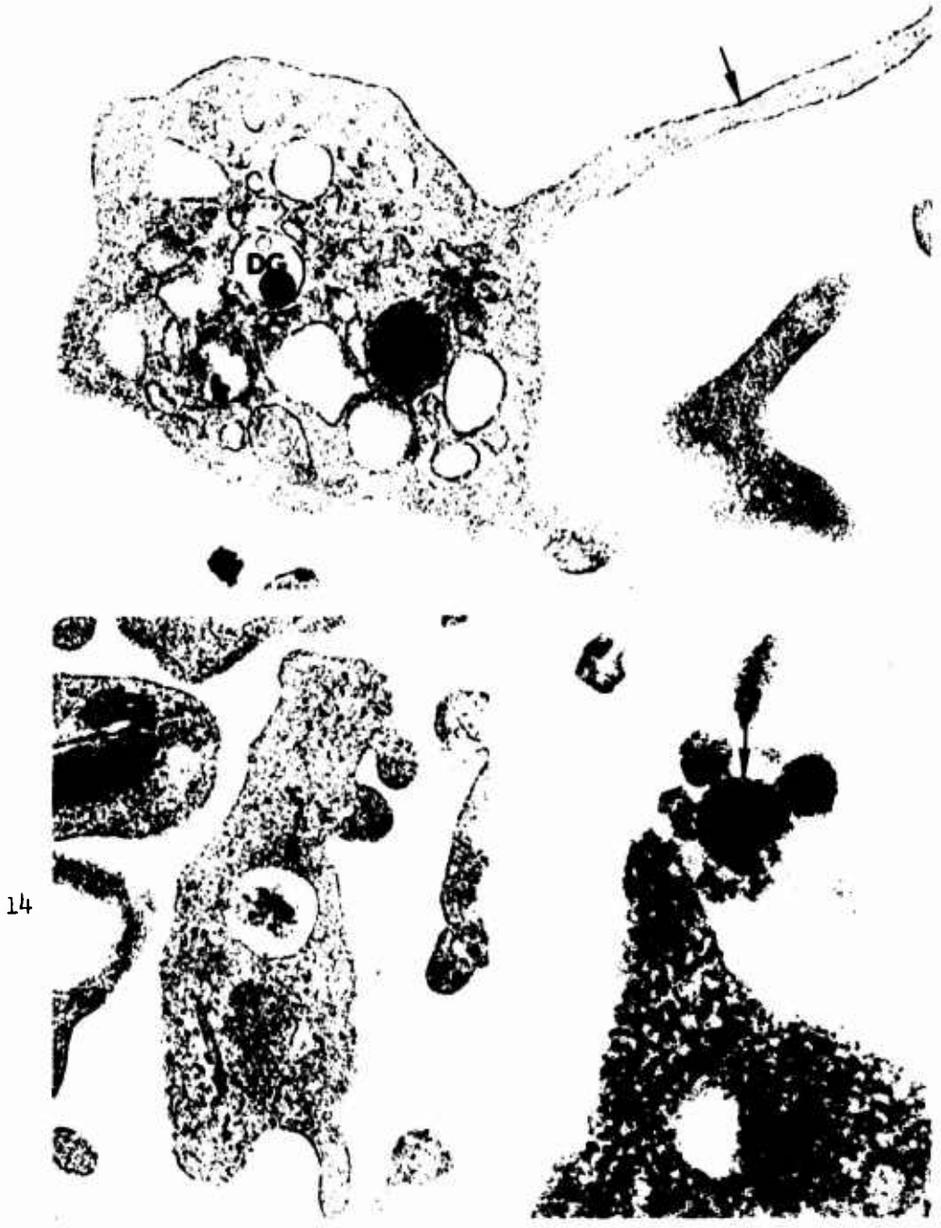
Combinations of the platelets and sensitized leukocytes with the addition of antigen (Group 2) or activated leukocytes and platelets without antigen (Group 3) or with antigen (Group 4) demonstrate remarkable changes in their physical relationship as well as their morphology. These changes are most pronounced in Group 4 (Fig. 12). There are many aggregates of platelets intermingled with leukocytes and cellular debris (Fig. 12). The centers of these aggregates are composed of a few small

Fig. 12—Cell suspension consisting of platelets and activated leukocytes with added antigen (group 4). Aggregation of platelets (*P*) surrounds small lymphocyte (*L*). These platelets are intermingled with cell debris. Each platelet extends long pseudopods and some of them are in direct contact with small lymphocyte (*arrow*). A few granulocytes (*G*) are seen in periphery of this aggregate (× 10,000).

lymphocytes and monocytes which are surrounded by numerous platelets. Many of these platelets are irregular in shape and possess pseudopods (Fig. 13) extending toward the centrally located lymphocytes and monocytes. Often these pseudopods are in close contact with these leukocytes and some protrude into the leukocyte cytoplasm at the base of the microvilli (Figs. 16 and 17). Thus, the pseudopods of the platelets and microvilli of the lymphocytes and monocytes are interdigitated. The tips of the pseudopods of the platelets are often inserted into the cytoplasm of these leukocytes, forming a cytoplasmic anastomosis (Fig. 18). No limiting membrane can be seen in these areas. These platelets demonstrate further morphological changes.

The cytoplasmic matrix of a platelet located near the leukocytes become more electron opaque than the control group (Figs. 16-19). The alpha-granules are decreased in number with the appearance of increased numbers of vesicles. The remaining alpha-granules occasionally undergo changes in which their matrices become partially electron translucent (Fig. 14). It would appear as if the matrix is in the process of discharging its contents. The very dense granules are also decreased in number. The cell debris intermingled with these platelets often consists of electron opaque granules similar to the alpha-granules and very dense granules (Figs. 11 and 15). These changes appear to be more prominent in the platelets which are in contact with the lymphocytes and monocytes than those situated at the periphery of the cell aggregates. However, there are no lysed platelets observed in our preparations. The lymphocytes and monocytes appear to maintain their morphology with the exception of an increased number of microvilli. Although granulocytes are always present in the vicinity of these aggregates, their intermingling with platelets is minimal.

It has been shown by several investigators that a suspension of sensitized leukocytes and platelets of rabbits release histamine from the plaetlets when antigen is added to the suspension of these cells. Sensitized platelets of rabbits alone were unable to release histamine, even in the presence of antigen. On the other hand, the activated leukocytes are capable of causing histamine release from rabbit platelets in the presence or absence of antigen. These findings indicated that activated leukocytes in some way interact with the rabbit platelets causing the release of histamine irregardless of prior activation. The present study clearly demonstrated that a physical interaction occurs between the activated rabbit leukocytes and normal rabbit platelets. Of particular interest is the formation of platelet pseudopods and the insertion of these pseudopods into the leukocyte cytoplasm with the concomitant decrease in number of platelet granular inclusions.



13

14

15

Fig. 13—Higher magnification photograph of platelet (from Group 2) with an extended long pseudopod (*arrow*). Platelet has many vesicles and vacuoles with two α granules (*AG*) and a very dense granule (*DG*) (× 46,000).

Fig. 14—Platelet with a vacuole (*arrow*) partially filled with electron-opaque material, the density of which is similar to that of the α granule (from group 3) (× 42,000).

Fig. 15—Globule (*arrow*) similar to α granule is observed outside platelet (from group 2) (× 60,000).

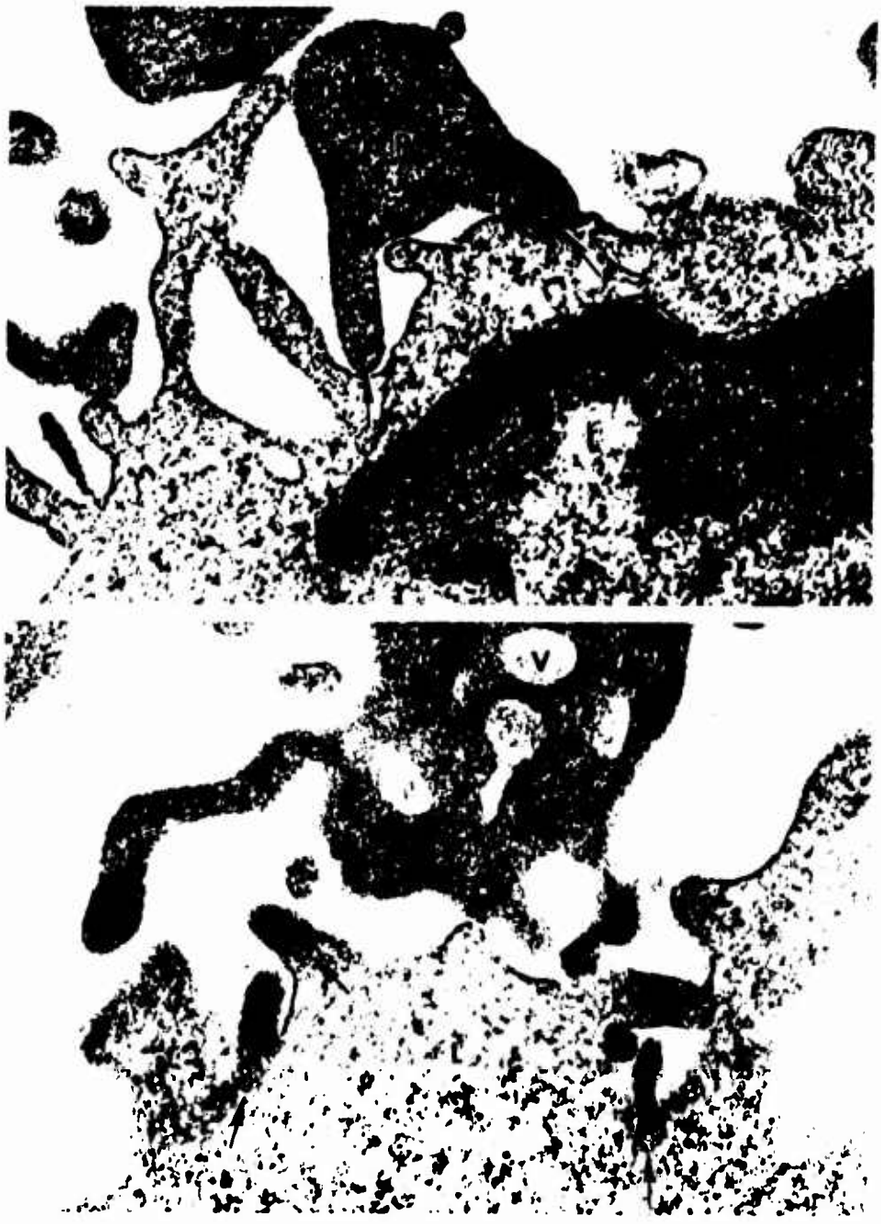


Fig. 16—Another example (group 2) of physical interaction between pseudopods of platelets (*P*) and lymphocytic cytoplasm (*L*). Tips of platelet pseudopods insert into cytoplasm of lymphocyte (*arrow*). Cytoplasm of both cells merge, and that of platelet appears to vacuolate (*V*) (× 55,000).

Fig. 17—Preparation consisted of platelets, activated leukocytes and antigen (group 4). Platelet (*P*) with two extended pseudopods is in direct contact (*arrow*) with cytoplasm of lymphocyte (*L*). Cytoplasmic organelles are absent within platelet (× 50,000).

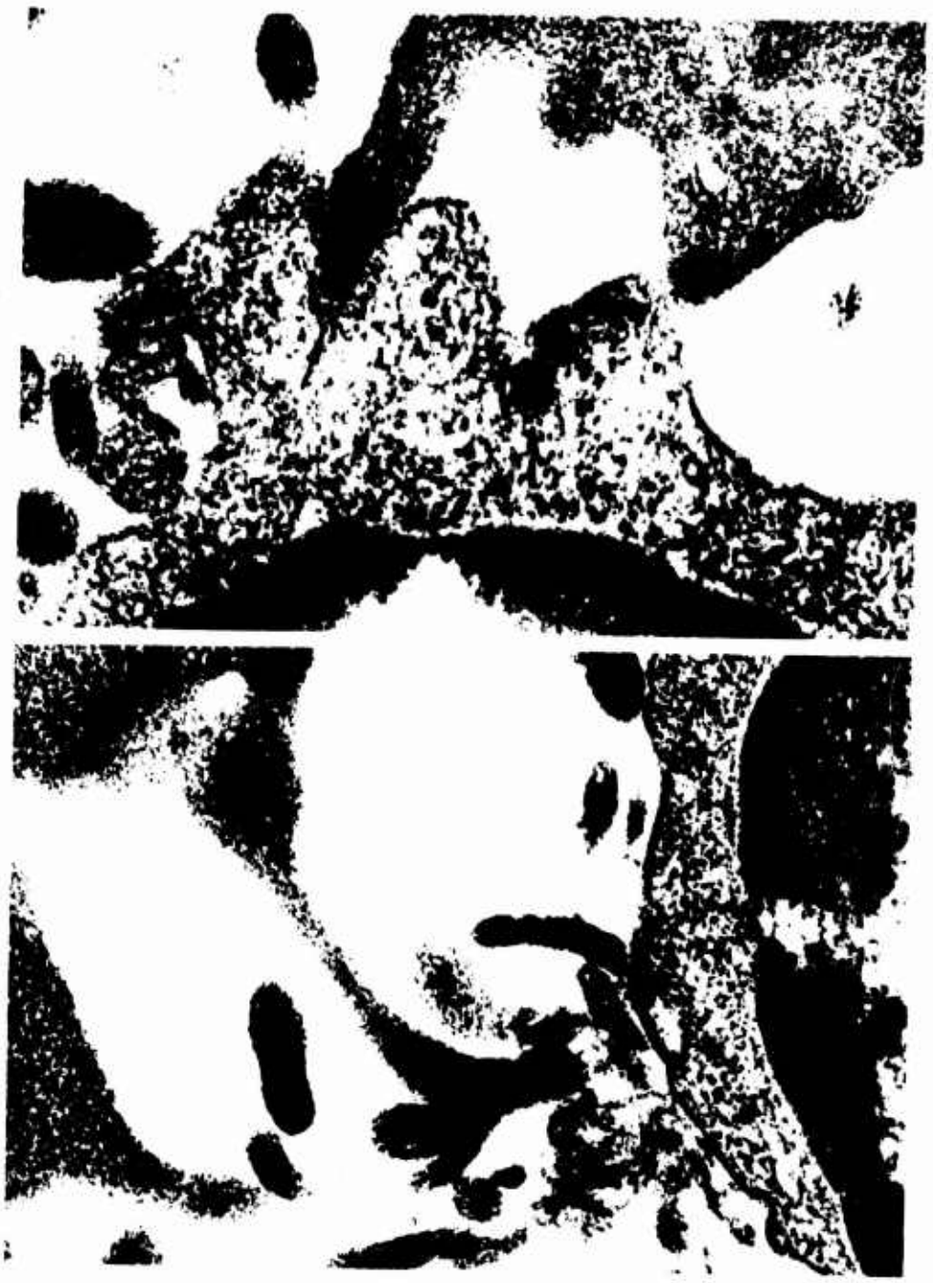


Fig. 18.—Higher magnification micrograph demonstrating physical interaction between platelet (*P*) and small lymphocyte (*L*) (from group 4). Pseudopod tip of platelet inserts into the lymphocytic cytoplasm (*arrow*). Plasma membrane of both cells is not apparent at junction (× 71,000).

Fig. 19.—Another example of interaction (*arrow*) of platelet (*P*) pseudopods and lymphocyte (*L*) (from group 3) (× 52,000).

Although the fine structure of the rabbit platelets under various experimental conditions has been described by many workers, the formation of pseudopods by the platelets during antigenic reaction has not been reported. Pseudopod formation and its insertion into leukocytes indicates that cytotaxis of the platelets toward the activated leukocytes occurs in the presence of antigen. The factors controlling the cytotaxis of the platelets is not obvious.

The close physical interaction between the sensitized leukocytes and the platelets noted in this study is not entirely in agreement with the findings of Henson. He rarely observed an interplay between the sensitized leukocytes and the platelets, when he studied preparations of rabbit cells which had been sensitized to bovine serum albumin and horse spleen ferritin. Since he did not regularly discern the association of the leukocytes and the platelets, he suggested that this contact was not necessary for the release of platelet histamine. Instead, he obtained a soluble factor from the reaction of platelets, antigen and sensitized leukocytes which he found caused histamine release. Although Barbaro and Schoenbechler were unable to obtain evidence for the production of a soluble factor, they have not ruled out the possibility of its existence. It is possible that both physical interaction of the platelets and sensitized leukocytes and a soluble factor from these leukocytes may be involved in the release of histamine.

Release of the platelet constituents under various conditions has been described. Virus, bacteria, antigen-antibody complexes and non-biological particles coated with gamma-globulin are reported to cause release of platelet contents. Similarly the release of cytoplasmic organelles of platelets after interaction with sensitized leukocytes were reported by Henson, and are likewise presently observed. Packham et al. reported that the mechanism by which platelets lose their constituents involved the formation of holes in the platelet membrane. They demonstrated lysed platelets by electron microscopy. Though we did not observe lysed platelets, the loss of the plasma membrane at the tip of the pseudopods of the platelets after its insertion into the leukocyte cytoplasm may be the site from which platelet components leak out.

Although serotonin has been shown to be localized in the "very dense" granules, a specific organelle site for the storage of histamine has not conclusively been established for the rabbit platelets. DePrada and his co-workers fractionated rabbit platelets and separated the organelles by differential centrifugation. By this method they found high histamine activity in the layer which is predominantely composed of the "very dense" granules and vesicles. Thus, they suggested that the histamine is present either in the very dense granules in association with serotonin or in the vesicles. On the other hand since the morphology of the rabbit platelets, which is unique in that they contain large amounts of histamine, is not radically different from those animals which contain small amounts of histamine, it is

difficult to relegate histamine to any particular granules. The decreased number of granular inclusions and the presence of empty vesicles seen in this study may indicate that under these experimental conditions the platelet releases other constituents such as hydrolytic enzymes, coagulation factor 3 (both possibly contained in alpha-granules) and serotonin which is located in the very dense granules. The increased number of empty vesicles observed in our study indicates that some of these may have originated from alpha-granules and very dense granules, the matrices of which have been released. This is supported by the observation that free electron dense granules similar to alpha-granules and very dense granules are often interspersed with the platelets.

The cell types which directly interact with platelets for histamine release have been disputed. Benson suggested that the large mononuclear cells, possibly monocytes, are involved in the release of histamine from the platelets, while Schoenbechler and Barbara reported that small lymphocytes are determined by light microscopy to be responsible for the release. In the present electron microscopic study, both small lymphocytes and monocytes are closely associated with platelets. Although the suspension containing platelets and small lymphocytes without monocytes showed physical interaction, we could not eliminate the possibility of interaction between the platelets and monocytes leading to histamine release.

The interaction between normal rabbit platelets and sensitized rabbit leukocytes activated with an iron was studied by electron microscopy in an attempt to elucidate the mode of histamine release from the platelets.

In the suspension of platelets and sensitized leukocytes, not activated with antigen (control), there was no physical interaction between them. Combinations of the platelets and sensitized leukocytes activated with antigen demonstrated remarkable changes in their physical and morphological relationship. Many aggregates of platelets surround small lymphocytes and occasional monocytes. Many of these platelets are irregular in shape with pseudopods extending toward the lymphocytes and monocytes. Often the pseudopods are in close contact with these leukocytes and their tips are often inserted into the cytoplasm of these cells forming an anastomosis. The platelets appear to be vacuolated and lose some of their granular inclusions.

These findings indicate that the physical interaction between the platelets and activated leukocytes, particularly with lymphocytes play an important role for release of histamine from the rabbit platelets.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 010, Hypersensitivity in the immunopathology of helminthic infections

Literature Cited.

Publications:

1. Briggs, N. T. and DeGiusti, D. L.: Increased sensitivity of Trichinella-infected mice to serotinin and histamine. Life Sci. (Part I) 9:797-802, 1970.

2. Sadun, E. H. and Gore, R. W.: Schistosoma mansoni and S. haematobium; Homocytotropic Reagin-Like Antibodies in Infections of Man and Experimental Animals. Exper. Parasit. 28 (No. 3): 435-449, 1970.

3. Barbaro, J. F. and Schoenbechler, M. J.: The Nature of the Reaction of Antigen with Lymphocytes from Rabbits Infected with Schistosoma mansoni on the Release of Histamine from Rabbit Platelets. The Jour. of Immun. 104 (No. 5): 1124-1131, 1970.

4. Colwell, E. J., Ortaldo, R., Schoenbechler, M. J., Barbaro, J. F. and Fife, E. H.: Trichinella spiralis and Schistosoma mansoni: Specificity of in vitro, Leukocyte-Mediated Histamine Release from Rabbit Platelets. Exper. Parasit. 29:263-270, 1971.

5. Williams, J. S., Sadun, E. H. and Gore, R. W.: A Radioactive Antigen Microprecipitin (RAMP) Assay for Schistosomiasis. The Jour. of Parasit. 57(No.2):220-232, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0r 0473	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCT	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
	A. NEW	C	U	NA	XL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	211
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Immunoprophylaxis of protozoan infections (90)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002000 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 11	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 71		5
CURRENT FISCAL YEAR 72		70

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME: MOON, A. P. DA

NAME

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Immunize; (U) Irradiate; (U) Parasite; (U) Trypanosome

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

23(U) Technical objective: To develop reliable serological tests for determining the degree of host response to infection and to develop means for inducing acquired immunity to protozoan infections.

24(U) Conventional and experimental serological methods will be used to evaluate host reactions to infection following treatment with gamma irradiated parasites. Data will be used to device effective means of diagnosis, treatment, and/or immunoprophylaxis of infection.

25(U) 70 11 - 71 06 In 5 experiments cattle were immunized with either irradiated Trypanosoma rhodesiense, T. brucei or T. congolense and then challenged with unirradiated parasites. Studies showed that immunity can be induced with irradiated trypanosomes. Best results were with T. rhodesiense, but the level of immunity was lower than that obtained previously in rodents. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

* 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 68 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project [illegible] IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 011, Immunoprophylaxis of protozoan infections

Investigators:
Principal: E. H. Lebun, Sr.L., Lit. Doc.
Associate: CPT J. F. Anderson, VC; E. E. Duxbury; B. T. Wellde

1. Immunization of cattle against African trypanosomiasis using irradiated parasites.

After showing that a strong immunity is produced in rodents against Trypanosoma rhodesiense by immunizing them with irradiated blood forms, experiments were initiated to determine whether or not a similar technique could be applied to immunizing cattle against various species of African trypanosomes.

A series of experiments comprising a total of 52 cattle was conducted in Kenya and in Maryland, USA. In the first experiment, 3 Hereford steers were given 2 inoculations of irradiated T. brucei at one week intervals and then challenged with 500,000 unirradiated trypanosomes one week after completion of the immunizing inoculation. The three nonimmunized steers became infected 5 days after challenge whereas the immunized steers became positive on days 8, 9 and 13 after challenge. Better results were obtained in a second experiment in which T. rhodesiense was used. Of three steers inoculated with irradiated trypanosomes, one remained free of parasites after challenge and two showed a parasitemia 6 and 17 days after challenge. All three nonimmunized controls became positive 4 days after challenge. Results with T. congolense showed that the nonimmunized control animals became patent 5-6 days after challenge whereas the immunized animals became patent 7-10 days after challenge. The experiments with T. rhodesiense and T. congolense were repeated with similar results. These studies indicate that immunity can be induced in cattle with irradiated trypanosomes. Best results were obtained with T. rhodesiense.

Project [illegible]

[illegible]

[illegible]

Literature Cited

Publications.

1. Duxbury, [illegible] African trypanosomiasis by [illegible] International Atomic Energy Agency, Vienna. (IAEA - [illegible]), [illegible].

2. Roberts, [illegible] Immunity in man to Trypanosoma rhodesiense [illegible] by fluorescent antibody test. [illegible]. 1(No. 4): [illegible], 1970.

PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION: DA 0B 6474	2. DATE OF SUMMARY: 71 07 01		REPORT CONTROL SYMBOL: DD-DR&E(AR)636
3. DATE PREV SUM'RY: 70 11 15	4. KIND OF SUMMARY: D. Change	5. SUMMARY SCTY: U	6. WORK SECURITY: U	7. REGRADING: NA	8a. DISP'N INSTR'N: NL	8b. SPECIFIC DATA CONTRACTOR ACCESS: ☒ YES ☐ NO	9. LEVEL OF SUM: A. WORK UNIT

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	012
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code):
(U) Enzyme Activity Measurements by Centrifugal Chemistry (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS:
002300 Biochemistry 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 11	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.	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FY 71	10	250
YEAR 72	10	250

19. RESPONSIBLE DOD ORGANIZATION
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ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Buescher, COL E. L.
TELEPHONE: 202-576-3551

21. GENERAL USE
Foreign Intelligence Not Considered

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
Division of Biochemistry
ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Angel, LTC C. R.
TELEPHONE: 202-576-2211
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Beach, LTC D. J.
NAME: Powell, MAJ J. B. DA

22. KEYWORDS (Procede EACH with Security Classification Code):
(U) Enzyme Measurements; (U) Intermediary Metabolism

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

23. (U) The technical objective of the work unit is to define, categorize and evaluate enzyme methodologies as applied to centrifugal chemistry.

24. (U) Utilizing a centrifugal chemistry unit, develop methodologies and apply the standardized methodologies to enzyme levels in physiological fluids.

25. (U) 70 11 - 71 06 The centrifugal chemistry unit has been leased and placed in operation. A number of enzymatic procedures have been applied to the system. Notable among these are lactic acid dehydrogenase (LDH), serum glutamic oxaloacetic transaminase (SGOT) and glucose-6-phosphate dehydrogenase (G-6-PD). Results show that enzymes such as LDH and SGOT have a low temperature dependence. This allows for reliable measurement without controlling temperature on the rotor. Enzymes like G-6-PD, MHb reductase, glutathione reductase and pyruvic kinase require strict temperature control. The rotor on the test unit has been jacketed and controlled with a precision temperature control unit. An information output unit in terms of optical density per unit time has been completed. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

* 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 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 012, Enzyme activity measurements by centrifugal chemistry

Investigators.
Principal: LTC Charles R. Angel, MSC
Associate: LTC Douglas J. Beach, MSC; LTC Ting-Kai Li, MC;
MAJ Lawrence Lumeng, MC; MAJ James B. Powell, MC.

DESCRIPTION.

The technical objective of this work unit is to define, categorize and evaluate enzyme methodologies as applied to centrifugal chemistry.

PROGRESS.

The concept that centrifugal force can be employed to mix and transfer reagents in cuvettes and to measure, simultaneously, the sequential reaction of multiple samples represents the first new departure in automatic chemical analysis in the past 15 years. A prototype instrument, called the GeMSAEC Fast Analyzer, was built by Dr. Norman Anderson at the Oak Ridge National Laboratory in 1969, and it was the general impression that such an instrument can be readily developed for routine clinical use. The obvious advantages are: 1) microsamples, 2) simultaneous measurement of multiple samples for volume work or profiling, 3) multiple-point rate measurements, 4) automatic blank correction, 5) automatic operation with a minimum of technical training, 6) computer compatibility, 7) small size and mobility.

To date, three commercial companies are building GeMSAEC instruments, and much progress has been made toward a reliable, accurate, and automatic system. The Division of Biochemistry has assembled one such instrument with the specific mission of further developing the instrumentation and methodologies such that the centrifugal chemistry system can be reliably utilized in the diverse Army medical laboratory environments.

The following goals have thus far been attained: 1) the precision of the instrument has been improved to ± 0.001 absorbance units by the use of a time averaging device, 2) proper mixing of the samples after transfer of reagent and serum into the cuvette by means of a vacuum pulse, 3) installation of UV optics to allow measurements into the near ultraviolet region, 4) control of the temperature of the cuvette. The development of an automatic sampler-diluter and reagent dispenser is in progress.

Methods for the assay of glucose-6-phosphate dehydrogenase, methemoglobin reductase, and glutathione reductase were developed. Other enzyme assays found compatible with the instrument are lactic dehydrogenase, glutamic-oxacetic transaminase and glutamic-pyruvic transaminase.

SUMMARY.

Using centrifugal chemistry techniques, assays for G6PD, MHb reductase and glutathione reductase have been developed. The experiments are in progress to incorporate the assays for other enzymes.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

REPORT CONTROL SYMBOL: DD-DR&E-AR(SA

3. DATE PREPARED	4. KIND OF SUMMARY	5. SUMMARY LEVEL	6. WORK UNIT
70 07 01	D. Change		

10. PROGRAM ELEMENT	PROJECT NUMBER	WORK UNIT NUMBER
A. PRIMARY: 61101A	3A061102A810	00
B. CONTRIBUTING		
C. CONTRIBUTING		

11. TITLE (Precede with security classification code)

(U) Biochemical Measurements in the Field Interfaced with Small Computers (09)

12. SCIENTIFIC AND TECHNOLOGY AREAS

002300 Biochemistry 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. PRINCIPAL INVESTIGATOR
70 11	Cont	In House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE	NA	EXPIRATION	b. FUNDS (In thousands)
b. NUMBER			
c. TYPE		d. AMOUNT	286
e. KIND OF AWARD		f. LEVEL OF EFFORT	286

16. RESPONSIBLE DOD ORGANIZATION

a. NAME: Walter Reed Army Institute of Research

b. ADDRESS: Washington, D. C. 20012

18. PERFORMING ORGANIZATION

a. NAME: Walter Reed Army Institute of Research

b. ADDRESS: Washington, D. C. 20012

19. RESPONSIBLE INDIVIDUAL

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TELEPHONE: 202-576-3561

[redacted] PII Redacted

20. GENERAL USE

Foreign Intelligence Not Considered

DA

22. KEYWORDS (Precede each with security classification code)

(U) Biochemistry; (U) Clinical Chemistry; (U) Computer Programming; (U) Field Teams

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Furnish individual paragraphs identified by item numbers, with security classification codes)

23. (U) The technical objective of this work unit is to interface small computers with biochemical instrumentation and evaluate the system in the field.

24. (U) Mini-computers that have proven reliable in the laboratory will be interfaced with standardized laboratory instruments such as wet chemistry analyzers, gas chromatographs, UV and other laboratory analytical systems. Emphasis will be placed upon the utilization of the developed systems for field use.

25. (U) 70 07 - 71 06 During the reporting period two systems have been studied for their applicability to field use. One, the Electronic Associates Digital Automatic Computer (EADAC) has been extensively studied. The EADAC system is poorly documented thus software development has been pushed to the machine level. Subroutines for all major operations have been written and tested. Peripheral interfacing has been completed and the gas chromatograph has been linked to the EADAC. On line operations have been demonstrated to be feasible. The other small computer, the D-17, is not extensively documented. Thus a major effort has been put into accomplishing this goal. Interface design for the D-17 has been completed with the intent to link the system to the AutoAnalyzer. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE AND WILL NOT BE USED AFTER 30 JUNE 1968

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 013, Biochemical measurements in the field interfaced with Mini-computers

Investigators.
Principal: LTC Charles R. Angel, MSC
Associate: LTC Douglas J. Beach, MSC; B. G. Bass, M.S.

DESCRIPTION.

The technical objective of this work unit is to develop interface methods for utilizing mini-computers with biochemical instrumentation systems for application in the field and the analytical laboratory. Included in this objective is the modification of excess DOD surplus computers for use as instrument controllers and the development of techniques such as time sharing of digital computers for use in the clinical laboratory.

PROGRESS.

A total of nine computers have been obtained from DOD surplus stores. Three of these computers were obtained from the Frankford Arsenal when the IDEEA Project (Information Data Exchange Experimental Activities) was phased out. Six of the computers are the guidance control devices from the U.S. Air Force Minuteman ICBM that have been replaced by newer models and are now available as surplus.

The purpose of the program is twofold: (1) develop an in-house capability for interfacing small general purpose digital computers to medical research laboratory instrumentation, and (2) demonstrate applications in the research laboratory or clinic for very expensive computers that have been declared obsolete by DOD agencies.

Efforts have been directed toward adapting the M-18 FADAC (Field Artillery Digital Automatic Computer) system to a gas chromatograph system. The FADAC is a better documented system than the Minuteman computer. This documentation made possible a potentially greater usefulness for the amount of effort that could be applied.

During the past year, numerous subroutines have been written and tested on the FADAC. These routines are listed below:

Digital clock read and print
Display and trace
Resident and restore
Relocation
Memory dump and punch program tape

OCTAL to ASCII convert
Teletype output
Data coupler output
ACT output
ACT input
OPLE generator
Calculator program
Read/write on bulk storage
Bulk storage unit error interpreter
Bulk storage read/write interpreter
Floating point add
Floating point subtract
Floating point multiply
Floating point divide
Floating point absolute value
Floating point normalize
Floating point output routine

In addition to the above subroutines, a number of other software developments are in progress. An interface unit has been designed for coupling the gas chromatograph to the FADAC. Hardware fabrication and checkout is in progress. Data will be transmitted via intralaboratory telephone links from the instruments to the FADAC. This approach is believed to be a unique combination of the time-sharing and on-line techniques that are now being exploited successfully in commercial systems.

The D-17 computer from the Minuteman I ICBM guidance system has been powered and an interface design has been evaluated for coupling the computer to a Technicon AutoAnalyzer System. The actual hardware fabrication has been limited to procurement of bit parts at the present time. A considerable amount of time has been spent in defining the system requirements fo the D-17 due to the fact that documentation for this unit has been limited.

The FADAC is to be evaluated as a field tested medical laboratory data processor. The Minuteman computer is to be evaluated as a field instrument controller.

An automated gas chromatograph system utilizing a Hewlett-Packard 2116 mini-computer as the instrument controller has been leased for use in evaluating small laboratory computers in an analytical laboratory. This instrument will be utilized in support of the drug and antimalarial screening and research mission of the WRAIR.

Extensive utilization has been made of various time-shared computer systems for the processing and analysis of WRAIR generated data. One system is the GE-645 time shared computer system at Rome Air Development Center. Another is the local commercial GE system called DIALCOM.

A third system is the GE BASIC I computer program teaching system where individuals learn computer programming by a computer assisted teaching routine. This routine has been used for two WRAIR sponsored classes. A total of 90 physicians and laboratory officers have participated in the exercises. So far the acceptance of this technique has been favorable. Future use of this technique as an educational device will be encouraged.

SUMMARY.

Extensive efforts have been made in the acquisition and utilization of DOD surplus computer equipment in the analytical laboratory and the development of methods of using on-line or time-shared computers in the medical laboratory. These computer systems have been used in analysis of medical research data and the training of medical personnel in the programming of digital computers.

Walter Reed Research Reactor Dismantling Project.

Detailed plans and procedures have been completed for the dismantling of the Walter Reed Research Reactor. Meetings have been held with U.S. Atomic Energy Commission personnel, Department of the Army (ARCHS) personnel and Department of Transportation personnel for the purposes of reviewing and modifying the plans. Arrangements have been made for the return of the nuclear fuel to the U.S. Atomic Energy Commission for reprocessing. The expected project completion date is 1 October 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

70 07 01	D. Annual	U	
	61101A	3A061101A91C	

(U) Management of Primary Hypertension and Psychosomatic Dysfunction Using Operant Conditioning Techniques

Q134 01 Psychology

71 11	Cont.		
Not Applicable			
		71	59

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

Walter Reed Army Institute of Research
Division of Neuropsychiatry
Washington, D.C. 20012

Buescher, COL F.L.
202-576-3551

Sodetz, CPT F.J
202 576 2483

Moss, MAJ G.R.
Kotchen, MAJ T.

Foreign Intelligence Not Considered.

DA

(U) Blood Pressure; (U) Operant Conditioning; (U) Automnomic Dysfunction; (U) Psychosomatic Disease; (U) Primary Hypertension.

23. (U) Development of behavioral techniques for the outpatient management of primary hypertension through appropriate application of existing principles of operant and respondent conditioning and systematic exploration of the role of these principles in the pathogenesis of hypertension, autonomic dysfunction, and psychosomatic disease.

24. (U) Existing knowledge of operant principles is applied to both normal and hypertensive individuals to effect reductions in blood pressure of sufficient duration to warrant development and standardization of an optimal procedure for management of primary hypertension in outpatients. Concurrently, studies in non-human primates are conducted to update operant technology, to explore potentially productive methods for treatment of patients, and to facilitate development of required bioinstrumentation.

25. 70 07-71 06 During reporting period an apparatus for non-invasive continuous measurement of systolic and diastolic blood pressure in human subjects has been undergoing extensive testing. Inadequacies in design have been documented and plans are being developed for the needed modifications. The apparatus is critical to the work unit in that the conditioning of blood pressure responses requires that information regarding blood pressure be fed back to the subject on a contingent basis. Development of the apparatus is being given the highest priority. Several studies using animal subjects have been terminated to make space available for a non-human primate study in direct support of the work unit. The protocol for this study is now in preparation. For a brief report see Walter Reed Army Institute of Research Annual Progress Report, July 1 through June 71.

DD FORM 1498

[illegible] LABORATORY RESEARCH

[illegible], Laboratory, Independent research

Work Unit [illegible], Management of primary hypertension and autonomic dysfunction using operant conditioning techniques

[illegible]
Principal Officer: Frank J. Seidel, MSC

The technical objective of the work unit is the development of behavioral techniques for the outpatient management of primary hypertension through appropriate application of existing principles of operant and respondent conditioning and systematic exploration of the role of these principles in the pathogenesis of hypertension, autonomic dysfunction, and psychosomatic disease. Existing knowledge of operant principles is applied to both normal and hypertensive individuals to effect reductions in blood pressure of sufficient duration to warrant development and standardization of an optimal procedure for management of primary hypertension in outpatients. Concurrently, studies in non-human primates are conducted to update operant technology, to explore potentially productive methods for patient treatment, and to facilitate development of required biomedical instrumentation.

During the reporting period an apparatus for non-invasive continuous measurement of systolic and diastolic blood pressure in human subjects has been undergoing extensive testing. Inadequacies in design have been documented and plans are being developed for the needed modification. The apparatus is critical to the work unit in that the conditioning of blood pressure responses requires that information regarding blood pressure be fed back to the subject on a continuous basis. Development of the apparatus is being given the highest priority. Several studies using animal subjects have been terminated in an orderly manner to make space available for a non-human primate study in direct support of the technical objective of the work unit. The protocol for this study is now in preparation.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

NA	A. New								A. WORK UNIT

A. PRIMARY	62110A	3A762110A871	00	224

(U) Treatment of pain states arising from combat related peripheral nerve injuries by transcutaneous stimulation techniques (TENS). 04A

012900 Physiology 016200 Stress Physiology 016600 Psychology

70 07	CONT		A. In House	
NA				
			1.5	52
			1.5	25

Walter Reed Army Institute of Research	Walter Reed Army Institute of Research Division of Neuropsychiatry
Washington, D.C. 20012	Washington, D.C. 20012
[redacted]	[redacted]
Foreign Intelligence Not Considered	DA

(U) Pain; (U) Peripheral Nerve Injury; (U) Psychophysiology, (U) Psychophysics; (U) Analgesics; (U) Human Volunteers; (U) Combat

23. (U) Peripheral nerve injury, frequently resulting from a high velocity missile, may result in causalgia - a state of local hypersensitivity causing burning pain. Transcutaneous stimulation techniques may relieve this pain and other forms of currently intractable pain. The validity of this relief is tested 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 analysed. Methods are also used to study both clinical (nerve injury) and normal volunteers. Methods are designed to ensure that the volunteer experiences the minimum possible amount of pain and discomfort.

25. (U) 70 07 - 71 06. Preliminary results from the clinical testing of patients at Walter Reed and Camp Drake, Japan suggested that transcutaneous stimulation did reduce pain for substantial time periods. Results from normal volunteers, using induced rather than chronic pain, indicated an effect of stimulation on both pain judgements and peripheral autonomic activity. The effect was confined to intensely-painful as opposed to barely painful induced pain. These results are now being extended using a broader sample of volunteers and stringent experimental methods necessary to eliminate the influence of psychological suggestion upon pain judgements. For technical reports see Walter Reed Army Institute of Research Annual Progress Report, 1 JUL 70 - 30 JUN 71.

DD FORM 1498

PII Redacted

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 015, Treatment of pain states arising from peripheral nerve injuries by transcutaneous stimulation techniques (TST)

Investigators.
Principal: CPT J. Richard Jennings, MSC
Associate: Howard F. Fields, M.D.; Norman A. Krasnegor, Ph.D.; Harold F. Lawson, B.S.; CPT William C. Orr, MSC; David L. Winter, M.D.

Description.

Causalgia, a state of local hypersensitivity causing chronic pain, is associated frequently with peripheral nerve injuries inflicted by high velocity missiles. A number of clinical cases suggested that causalgia may be relieved by transcutaneous stimulation techniques (TST). The neurological, physiological and/or psychological bases for the analgesic effect of the low-level electrical TST remain unidentified. The examination of these mechanisms and the development of an optimum clinical treatment method are the primary goals of this project.

Progress.

1. Clinical testing

A field team of neurological specialists tested the method clinically with peripheral nerve injury patients at Walter Reed General Hospital and at Camp Drake, Japan. At both sites, low-level electrical stimulation of nerves innervating the hypersensitive area led to relief during and after stimulation. Relief after stimulation varied from minutes to two or three hours. Extensive clinical testing had been envisaged at Camp Drake; however, a recent drop in casualty rates and changes in evacuation policies forced a change in this plan.

2. Laboratory verification using normal volunteers

Pain is not only a physiological condition but also a highly subjective psychological experience. Many analgesic methods have been discarded after initial enthusiasm because their effect was found to be psychological rather than physiological. Laboratory testing of the analgesic effect of transcutaneous stimulation has three goals: 1) verify that the method has a physiological as opposed to psychological basis; 2) develop the optimum technique for clinical use; and 3) understand the mode of action of the transcutaneous stimulation.

These goals are being pursued by first bringing the pain state under experimental control using psychophysical techniques. Normal volunteers are exposed to a thermal pain stimulus (Hardy, Wolff & Goodell, 1967) and are trained to accurately describe different levels of pain. (Care has been taken to achieve a method which eliminates the possibility of tissue damage due to the thermal stimulus.) The volunteers are then stimulated using either the true transcutaneous stimulation technique or a placebo technique. Pain judgements are reassessed after both types of stimulation. This procedure allows an objective assessment of any analgesic effects of TST.

An initial attempt to understand the physiological effect of TST has been made by monitoring the galvanic skin response. This peripheral indicant of the sympathetic nervous system is implicated in clinical causalgia. Causalgia patients show a chronic galvanic skin response, or sweating, in the affected area. Clinical observations suggest that the sweating disappears upon application of TST.

Results of the first complete study of TST in normal volunteers have not yet been analyzed. Pilot work on three volunteers (investigators) indicated that normal persons do show analgesia in response to TST and do show an inhibition of the galvanic skin response. The pattern of these results and others in the literature suggested the viability of the physiological model of pain proposed by Melzack and Wall (1965). Such findings will provide an insight into the psychological and neurophysiological mechanisms associated with this most painful condition. In addition, this information and these techniques may provide a non-surgical or non-medicinal means of handling other forms of intractable pain.

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 015, Treatment of pain states arising from peripheral nerve injuries by transcutaneous stimulation techniques (TST)

Literature Cited.

References:

1. Hardy, J.D., Wolff, H.G., and Goodell, H. Pain sensations and reactions. New York, Hofner Publishing Co., 1967.

2. Melzack, R., and Wall, P.D. Pain mechanisms: A new theory. Science, 1965, 150, 971-979

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 08 6458	71 06 09	DD DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADE	8a. ORD'G INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
70 07 01	K. COMP	U	U	NA	NA	[X] YES [ ] NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	047
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Provide with Security Classification Code)

(U) Transport of Essential Metals in Simulated Biological Environments (21)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 09	Cont	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE 69 09 EXPIRATION

b. NUMBER DADA 17-70-C0014

c. TYPE S.CT d. AMOUNT 27,310

e. KIND OF AWARD f. CUM. AMT. 27,310

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PREVIOUS		
	70	1	23
	CURRENT		
	71	0.2	5

19. RESPONSIBLE DOD ORGANIZATION

NAME Walter Reed Army Institute of Research

ADDRESS Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME Buescher, COL E. L.

TELEPHONE 202-576-3551

20. PERFORMING ORGANIZATION

NAME University of Maryland

ADDRESS College Park, Md. 20740

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

NAME Rollinson, C. L.

TELEPHONE 301-927-3800

SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

NAME Angel, LTC C. R.

NAME

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Mineral Transport

(U) Sequential Dialysis; (U) Chromium; (U) Iron; (U) Insulin; (U) Chelating Agents;

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

23. (U) To perform kinetic studies on the interaction of trace metals with biological species, e.g., amino acids and hormones, with the aim of defining the role of trace metals in biochemical reactions.

24. (U) Through application of procedures devised by the investigator for the use of the method of sequential dialysis.

25. (U) 70 07 - 71 02 During the reporting period a number of potential ligands have been studied for Chromium (III) and Iron (III). Notably Iron (III) ligands are more rapid in reaction rate than chromium. Phosphate ion when used as a buffer was found to be reasonable ligand material at low concentration. The contract has been terminated and a final report has been received. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

* Applies to contractor name, address(es) approval.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 097, Transport of essential metals in simulated biological environments

Investigators.
Principal: C. L. Rollinson, Ph.D. (University of Maryland)
Associate: LTC C. R. Angel, MSC

DESCRIPTION.

The purpose of this contract is to perform kinetic studies on the interaction of trace metals with biological materials such as amino acids and hormones by sequential dialysis.

PROGRESS.

In previous studies under this contract, many biological substances were shown to be effective ligands for Cr (III); in the present investigation, histidine, gluconate, saccharate and threonine were found to have great coordinating tendency. The PO_4^{3-} ion itself is quite effective particularly at high PO_4^{3-}: Cr^{3+} ratios; it is tentatively concluded that the products formed in PO_4^{3-} buffered ligand solutions are mixed PO_4^{3-} ligand complexes. Sequential dialysis is as applicable to Fe (III) as to Cr (III), and probably to other essential metal ions; effective ligands for Fe (III) are ADP, pyrophosphate, triphosphate, citrate, isocitrate, saccarate, mesoxalate and oxalate. Some comparison of membranes was accomplished. Silver membranes are not useful because even the smallest pore diameter available (0.2 μ) is too large. Membrane filters are satisfactory but expensive and it is impossible to decontaminate them sufficiently to permit their re-use. Regenerated cellulose is by far the most satisfactory membrane but some lack of uniformity was observed.

SUMMARY.

A number of potential ligands have been studied for chromium (III) and iron (III). Notably Fe (III) ligands are more rapid in reaction than chromium. Phosphate ion when used as a buffer was found to be reasonable ligand material at low concentrations.

This contract was terminated on 31 August 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REPRODUCIBILITY	8A. [illegible]	8B. [illegible]	9. LEVEL OF SUM
70 07 01	D. Change	U	U	NA			A. WORK UNIT

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	022
b. CONTRIBUTING	62703D	ARPA AO 0794 9M10		
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code): (U) Further Studies on the Hazards of Microwave Irradiation as Indicated by CNS Neurotransmitters

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 012900 Physiology, 016200 Stress Physiology, 01400 Radio and Radiation Biol.

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 06	Cont.	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: 70 06 EXPIRATION: 71 06
b. NUMBER: DADA 17-69 C-9144
c. TYPE: S. CT d. AMOUNT: 27,810
e. KIND OF AWARD: Ext f. CUM. AMT: 57,175

18. RESOURCES ESTIMATE	PRECEDING FISCAL YEAR	CURRENT FISCAL YEAR	a. PROFESSIONAL MAN-YRS	c. FUNDS (In Thousands)
	71		0.5	25
	72		0.5	25

19. RESPONSIBLE DOD ORGANIZATION

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

RESPONSIBLE INDIVIDUAL
NAME: Buescher, COL E. L.
TELEPHONE: 202-576-3551

21. GENERAL USE

20. PERFORMING ORGANIZATION

NAME: The Johns Hopkins University School of Medicine
ADDRESS: 725 N. Wolfe Street Baltimore, Md. 21205

PRINCIPAL INVESTIGATOR (Name and Address if different from Performing Organization)
NAME: Snyder, Solomon H., M.D.
TELEPHONE: 301-955-3020
SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS
NAME: Sharp, Joseph C., Ph.D.
NAME: Meyerhoff, James L., MAJ

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Microwave Hazards; (U) Nonionizing Radiation Hazards; (U) Behavioral Effects; (U) Neurochemical Systems; (U) Military Medicine

23. TECHNICAL OBJECTIVE 24. APPROACH 25. PROGRESS (Precede text of each with Security Classification Code)

23. (U) To investigate the nature and extent of hazards to the central nervous system (CNS) and its function due to microwave and radio frequency emanations from military devices and equipment such as radar and communications gear.

24. (U) Chemical determination of changes in CNS levels and turnover rates of important neurotransmitters involved in thermoregulation as well as levels of consciousness and behavioral excitation. Those transmitters are serotonin (5-hydroxytryptamine) and norepinephrine.

25. (U) 70 06 - 71 06 Previous studies have shown that serotonin turnover is depressed by chronic microwave irradiation and by chronic heat stress. Extension of these studies suggests that chronic irradiation with low levels (10 milliwatts per square centimeter) may have a significant thermal component, and that drowsiness and related central effects reported in man may be related to effects on 5-HT metabolism. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

* Available to contractors upon originator's approval

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

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 099, Further Studies on the Hazards of Microwave Irradiation as Indicated by CNS Neurotransmitters

Investigators.
Principal: Solomon H. Snyder, M.D.
Associate: Joseph C. Sharp, Ph.D.;MAJ J.L. Meyerhoff, MC

The objective of this research is to investigate the nature and extent of hazards to the central nervous system (CNS) and its function due to insult by microwave and radio-frequency energy. The effects will be assayed by chemical determinations of changes in CNS levels and turnover rates of important neurotransmitters involved in thermoregulation as well as levels of consciousness and behavioral excitation, i.e., serotonin (5-hydroxytryptamine) and norepinepherine. Earlier studies have shown that serotonin turnover is depressed by chronic microwave irradiation and by chronic heat stress. Extension of these studies suggests that chronic irradiation with low levels (10 milliwatts per square centimeter) may have a significant thermal component and that drowsiness and related central effects reported in man may be related to effects of 5-HT metabolism. The final report on this work will be published during the last half of CY 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA [illegible]	72 [illegible]	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	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 01	D. Change	U	U	NA	NI	[X] YES [ ] NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A71	0	147
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Nuclear-Cytoplasmic Transplantation (24)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

006004 Biology; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 08	CONT	DA	B. Contract

17. CONTRACT GRANT

a. DATES/EFFECTIVE: 70 08 EXPIRATION: 71 07

b. NUMBER: DADA-17-69-C-9182

c. TYPE: C.CT d. AMOUNT: 0

e. KIND OF AWARD: CONT f. CUM. AMT: 23,974

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (Thousands)
FISCAL YEAR	PRECEDING 70	1	18
	CURRENT 71	0.1	5

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, DC 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: University of Minnesota

ADDRESS: Minneapolis, Minn. 55455

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

NAME: Estensen, R. D.

TELEPHONE

SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

NAME: Noyes, Howard E. Ph.D. 1

NAME

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Nuclear Transplantation; (U) Enucleated Cytoplasm; (U) Cytochalasins; (U)Sendai Virus

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

23. (U) To extend preliminary studies on nuclear transplantation of mammalian cells and to apply the technique to immunologic and other studies.

24. (U) Through a general methodology which in preliminary evaluations has been shown to be feasible; consists of separation of nucleus and cytoplasm by cytochalasins, followed by fusion of nucleus and cytoplasm with Sendai virus.

25. (U) 70 07 - 71 06 The feasibility of the nuclear transplant technique was successfully demonstrated. In addition, the investigator confirmed that the drug cytochalasin B (CB) inhibits cytokinesis while allowing karyokinesis to proceed. CB acts during metaphase or later and has little effect on growth of cells. These results are assumed to be the result of a functional failure of membrane fusion. CB's inhibition of cleavage in the Xenopus laevis egg has been defined temporally and histologically. Phagocytosis and pinocytosis were inhibited by CB. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

* 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 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 109, Nuclear-cytoplasmic transplantation

Investigators.
Principal: Richard D. Estensen, M.D.
Associate: Howard E. Noyes, Ph.D.

Preliminary work at WRAIR confirmed observations by Carter (1967) that the drug cytochalasin B (CB) had two dramatic effects. First, at doses of 1 ug/ml in cultures of mouse fibroblasts (L-cells) the drug stopped cytoplasmic division (cytokinesis) while allowing nuclear division (karyokinesis) to proceed. Second, at doses of 10 ug/ml a significant number of cells were enucleated, leaving nuclei surrounded by plasma membrane and intact cytoplasm in a culture. The latter observation served as a basis for the suggestion that nuclear transfer or exchange might be accomplished through the use of Sendai virus cell fusion (Okada, 1962). Investigations since the beginning of the contract in August 1969 have been directed toward determining the feasibility of this methodology. Three areas have been studied. 1) the mechanism of cytokinesis through the use of low doses of CB; 2) nuclear transfer through the use of CB in high doses; and 3) effect of CB on human polymorphonuclear leukocyte phagocytosis.

1. Mechanism of cytokinesis -- Two experimental systems have been used to study low dose (1 ug/ml) effects of CB: (1) N1S1-67 (Novikoff rat Hepatoma) cells; and (2) Xenopus laevis embryos. Experiments on N1S1-67 cells in culture have shown that the drug prevented cytoplasmic division (cytokinesis) while allowing nuclear division to proceed. N1S1-67 cells exposed to 2.1×10^{-6} M did not undergo cytoplasmic division, but cell volume and number of nuclei per cell increased at rates comparable to those of controls. Isotope studies suggested that the increases represented true nuclear and cytoplasmic growth. These results suggest that inhibition of macromolecular synthesis does not account for the action of CB and that inhibition of membrane fusion is a possible mode of action. Work on Xenopus laevis showed that fertilized eggs failed to complete cleavage although nuclear division and furrowing occurred normally. The effects of varied dose levels and changes in timing of exposure to CB indicated that a pool of substance(s) needed for subsequent cleavages becomes available for CB action just before first cleavage.

2. Nucleo-cytoplasmic transfer -- The concept of fusion of cytoplasm and free nuclei treated with CB needed several exploratory steps. First, a plasma membrane was necessary for fusion to take place. Ultrastructural studies demonstrated the presence of a plasma membrane surrounding the extruded nucleus. Further, cells treated with CB could be fused

with Sendai virus. It was possible to enucleate cells and to fuse enucleated cytoplasm to chicken red cells. The resultant hybrids showed incorporation of RNA precursors indicating that the cytoplasms were capable of supporting a transplanted nucleus. However, the final step remains to be accomplished, i. e., the transfer of enucleated nuclei from one cytoplasm to another. The technique offers exciting possibilities of study of nucleo-cytoplasmic interactions as well as possible therapeutic applications, e. g., restoring loss of enzyme or other activity in patients by transplanting competent nuclei into their own cytoplasms.

3. Inhibition of human polymorphonuclear leukocyte phagocytosis

Phagocytic mixtures containing CB at a concentration of 10 ug/ml showed little phagocytic activity. Subsequent experiments indicated that CB affected leukocyte phagocytosis by inhibiting bacterial uptake. CB had no effect on opsonization of bacteria. The effect of the drug was rapid and relatively complete and reversible. Cytochalasin B could inhibit phagocytosis by one of three mechanisms: (1) prevent attachment of opsonized bacteria to the cell surface, (2) prevent movement of the cell around the attached bacterium, or (3) prevent fusion of membrane surfaces after the membrane has moved around the adherent bacterium. Direct microscopic examination of phagocytizing PMN's and thin sections of similar cells suggested that bacteria did attach normally to the PMN surfaces. Data reported did not distinguish between the latter two possibilities.

Literature Cited.

References:

1. Carter, S. B.: Effects of cytochalasins on mammalian cells. Nature 213: 261-264, 1967.

2. Okada, Y.: Analysis of giant polynuclear cell formation caused by VSV virus from Earlich's ascites tumor cells. I. Microscopic observation of giant polynuclear cell formation. Exper. Cell Rsch. 26: 98-107, 1962.

Publications:

1. Kalin, R. L. and Estensen, R. D.: Introduction of a heterologous nucleus into enucleated cytoplasms of cultured mouse L-cells. Proc. Nat. Acad. Sci. 67: 1537, 1970.

2. Estensen, R. D.: Cytochalasin B. I. Effect on cytokinesis of Walker 256 carcinoma cells. Proc. Soc. Exp. Biol. Med. 136: 1256, 1971.

3. Hummer, M. L., Dalton, A. J., and Estensen, R. D.: Cytochalasin B. II. Selective inhibition of cytokinesis in Xenopus laevis eggs. Proc. Soc. Exp. Biol. Med. 136: 1260, 1971.

4. Davis, A. T., Estensen, R. D., and Quie, P. G.: Cytochalasin B. III. Inhibition of human polymorphonuclear leukocyte phagocytosis. Proc. Soc. Exp. Biol. Med. 137: 161, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6495	71 07 01	

3 DATE PREV SUM'RY	4 KIND OF SUMMARY	5 SUMMARY SCTY	6 RDNE SECURITY	7 REGRADING	8A THESIN INSTN	8B SPE [illegible]	9 TYP [illegible]
70 07 01	D. Change	U	U	NA	NE	YES NO	A. WORK UNIT

10 NO CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	115
b. CONTRIBUTING				
c. CONTRIBUTING				

11 TITLE (Procede with Security Classification Code)

(U) Effects of Physiological and Psychological Stress upon Infection and Disease (09)

12 SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 003500 Clinical Medicine 016200 Stress Physiology

13 START DATE	14 ESTIMATED COMPLETION DATE	15 FUNDING AGENCY	16 PERFORMING METH
64 10	Cont.	DA	C. In-House

17 CONTRACT GRANT Not Applicable

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. FUNDING (In Thousands)
PREFERENCE		
FISCAL YEAR 71	2	65
CURRENT FISCAL YEAR 72	2	65

19 RESPONSIBLE DOD ORGANIZATION

NAME Walter Reed Army Institute of Research

ADDRESS Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME Buescher, COL E. L.

TELEPHONE 202-576-3551

20 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 Mason, J. W., M.D.

TELEPHONE 202-576-3559

SOCIAL SECURITY ACCOUNT NUMBER [redacted]

PII Redacted

ASSOCIATE INVESTIGATORS

NAME Buescher, COL E. L. DA

NAME

21 GENERAL USE

Foreign Intelligence Not Considered

22 KEYWORDS (Provide EACH with Security Classification Code)

(U) Respiratory Infection; (U) Stress; (U) Endocrine Response; (U) Hormones; (U) Viruses; (U) Sociology; (U) Personality Type; (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) Definition and evaluation of various environmental and personal factors which contribute to physical and psychological stress experienced by military personnel, and determination of how these affect the overt clinical manifestations of naturally acquired infections. When factors are defined, efforts to modify clinical manifestations by modification of environment or human response to it are made.

24. (U) Endemic overt diseases in military populations are identified and studied for microbial etiology and variation in clinical manifestations. Environment in which they occur is defined. These findings are correlated with immunological susceptibility, physiological responses to environment and its stresses, and with personality types, and social backgrounds of patients. Factors suspected of influencing disease severity are evaluated in controlled experiments.

25. (U) 70 07 - 71 06 All hormonal and statistical analyses on this project are now complete. Because of several striking pre-illness hormonal differences in the sick and control groups, it is felt that the findings of this study merit publication and preparation of the manuscript is still in progress. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

* 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 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 113, Effect of physiological and psychological stress upon infection and disease

Investigators.
Principal: John W. Mason, M.D.; COL Edward L. Buescher, MC

Description.

This study was designed to explore the possibility that stress-related, pre-illness changes in hormonal levels may play a contributory role in the pathogenesis of acute respiratory infections. The feasibility of the study was suggested by the high incidence of acute adenovirus infections in Army recruits during basic training in the winter months at Ft. Dix, New Jersey. Furthermore, the great majority of such illnesses usually are clustered during the third and fourth week of basic training. It was, therefore, possible to study a population in which a very high incidence of respiratory illness could be predicted within a designated two-week period.

Progress.

All hormonal and statistical analyses on this project are now complete.

Summary and Conclusions.

Because of several striking pre-illness hormonal differences in the sick and control groups, it is felt that the findings of this study merit publication and preparation of the manuscript is now in progress. It is planned that work related to this project involving the study of host resistance will be resumed by Dr. Benjamin Natelson who will be arriving in July 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 0532	71 07 1	NA

3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY	6 WORK SECURITY	7 REGRADING	8 CHRON NBR	9 LEVEL	10 WORK UNIT
70 07 01	D. Change	U	U	NA	01	3	033

10 NO CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	033
b. CONTRIBUTING				
c. CONTRIBUTING				

11 TITLE (Provide with Security Classification Code)

(U) Biochemical Methodology and Laboratory Automation (09)

12 SCIENTIFIC AND TECHNOLOGICAL AREAS

008300 Inorganic Chemistry 003500 Clinical Medicine

13 START DATE	14 ESTIMATED COMPLETION DATE	15 FUNDING AGENCY	16 PERFORMING ORGANIZATION
69 10	Cont	DA	

17 CONTRACT/GRANT

a. DATE/EFFECTIVE NA EXPIRATION

b. NUMBER

c. TYPE d. AMOUNT

e. KIND OF AWARD f. CUM AMT

18 RESOURCES ESTIMATE

	PRECEDING	CURRENT	PROJECTED
FISCAL YEAR	71		
MAN-YEARS			

19 RESPONSIBLE (PERFORMING) ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D. C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Buescher, COL E. L.

TELEPHONE: 202-576-3551

20 PERFORMING ORGANIZATION

NAME: Walter Reed Army [illegible] Division of Biochemistry

ADDRESS: Washington, D. C. [illegible]

PRINCIPAL INVESTIGATOR (Provide last name, first name, middle initial)

NAME: Angel, LTC [illegible]

TELEPHONE: 20[illegible]

SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS

NAME: Beach, LTC D. L. DA

NAME:

PII Redacted

21 GENERAL USE

Foreign Intelligence Not Considered

22 KEYWORDS (Provide each with Security Classification Code)

(U) Automation; (U) ESR; (U) Atomic Absorption; (U) Toxicology; (U) Drugs of Abuse

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

23. (U) To develop and establish modern automated methods for qualitiative and quantitative analysis of important organic and inorganic materials.

24. (U) Automated, analytical instruments, data reduction equipment, electron spin resonance systems, and semi-automated manual techniques will be utilized to identify and quantitate various compounds for both developmental and research uses. Emphasis will be placed on fast analytical systems for identification of a variety of compounds, and on feasibility of systems for general laboratory use. Efforts will be concentrated on compounds of interest in red cell metabolism, on enzyme analysis, on the fast identification of drugs of abuse, and utilization of automated analytical systems for data collection.

25. (U) 70 07 - 71 06 Analytical chemistry methodology development and application continued in this work unit. Atomic absorption spectrophotometry was developed for the measurement of Zn, [illegible], Cd, Cu, As, Ca and Mg. Specific ion electrodes were utilized to determine ratios [illegible]ized calcium to total calcium. The development and utilization of ESR for dete[illegible]nd identification of drugs of abuse began and progressed to the point of comparison with gas liquid chromatography (morphine). Electrophoretic separation of lipoproteins and isoenzymes continued. Automated systems of measurement of 2,3-DPG, G.-6-PD, OH proline and sialic acid were assembled and comparative studies completed. There are now five cart mounted AutoAnalyzer systems. A detection method for tetrahydro cannabinol (THC) was established on the gas chromatograph-mass spectrometer (GLC-MS). Work continued on GLC-MS analysis and identification of drugs of abuse. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498-1 AND 1498-1 1 MAR 66 FOR ARMY USE ARE OBSOLETE

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 170, Biochemical methodology and laboratory automation

Investigators.
Principal: LTC C. R. Angel, MSC
Associate: LTC D. J. Beach, MSC; B. J. Boone, Ph.D.;
N. D. Brown, B.S.; E. S. Copeland, Ph.D.;
J. I. Davis, B.S.; B. P. Doctor, Ph.D.;
C. E. Emery, B.S.; L. Kazyak, B.S.;
J. A. Kintzios, B.S.; CPT P. A. Kramer, MSC;
CPT M. P. Kullberg, MSC; E. A. Levri, B.S.;
R. T. Lofberg, Ph.D.; E. J. Matusik, B.S.;
J. E. Matusik, B.S.; CPT W. L. Miller, MSC;
2LT P. E. Nino, MSC; N. M. Papadopoulos, Ph.D.;
R. C. Permisohn, B.S.; CPT G. A. Peyton, MSC;
J. T. Piechocki, B.S.; MAJ J. B. Powell, MC;
P. M. L. Siu, Ph.D.

DESCRIPTION.

The purpose of this project is to evaluate the existing techniques and methods for accurate, rapid and reproducible determinations of chemical compounds, enzymes and other body constituents which are of clinical and research significance. All available instruments, tests and chemical reactions are employed. Efforts are directed toward the conversion of these assays to automated systems. Use of computer programming for rapid and efficient dissemination of data thus obtained is included.

PROGRESS.

1. Micro-Analytical Measurements of Trace Minerals using Atomic Absorption Techniques.

The use of atomic absorption techniques for the trace mineral analyses in biological specimens is one of the integral parts of automated instrumental analysis of clinical chemistry. This in turn is intended to support other basic and applied military medical research and development projects. The following projects were carried out to this end.

a. In collaboration with LTC E. P. Quarantillo, Chief, USAMRT, WRAIR (Japan), sixty samples consisting of serum, red blood cells, and tissues were analyzed for zinc contents by atomic absorption techniques. The samples were prepared by two different methods of comparative purposes. The results obtained were compatible with the expected values.

b. The atomic absorption technique was applied to the measurement of the mercury contents of urine samples obtained from clinics.

This method was found to be sensitive and accurate at the 0-10 nanogram level and the results were reproducible at ±5% level.

c. Two samples of lyophilized serum containing iron, which will be employed for inter-laboratory testing by the World Health Organization, were compared with versatol A by the atomic absorption method. The results were in full agreement with the expected values, thus facilitating the standardization of these highly valuable samples.

d. The application of atomic absorption techniques to the determination of cadmium, copper and magnesium are being investigated at the present time.

e. A specially modified electrode (Orion Model 99-20 serum calcium flow through system) was designed to meet the requirements for accurate determination of total and ionized serum calcium. In order to apply this technique, the procedures for the preparation of samples were also modified to ascertain the highest level of accuracy, reliability and reproducibility.

2. Consolidation of Clinical Analyses Services Rendered for In-House Research Investigations.

The service support activities of clinical chemistry were consolidated from their respective locations within the WRAIR and Ward 30 into one large activity within the Division of Biochemistry. This consolidation of support and developmental activities has provided closer control of production, more efficient use of available instrumentation, and a better atmosphere for the AutoAnalyzer training program now in effect. The establishment of a policy of providing instrumentation, training, supervision and maintenance of equipment for any automated analysis requested by other divisions within WRAIR has been put in operation and is being further expanded. The requirement that all military personnel newly assigned to the division undergo a period of training in automated analysis has generated a pool of personnel capable of maintaining this policy.

Continuation of the use of modular analyzer units mounted on carts has permitted greater flexibility in the laboratory. The multichannel digitizer for peak height analysis continues to provide a great deal of time savings. Modified, high rise carts are in use and provide more efficient use of laboratory space compared to the larger horizontal carts.

An inventory of Division of Biochemistry AutoAnalyzer equipment was completed. Equipment requiring repair or replacement was identified and an equipment exchange system for supporting equipment loaned out (i.e., Ward 30) is now in effect. Close contact has been maintained with the WRGH Clinical Chemistry Unit. It is anticipated that further close cooperation with both of these areas will result in more cooperative investigations.

3. Evaluation of Methods for the Determination of Glucose.

A complete evaluation of the methods available for the determination of glucose was carried out in order to incorporate them into the general scheme of automated instrumental analysis. This evaluation included studies of interference of such compounds as uric acid, creatinine, lactic acid, fructose, urea, etc., in glucose determinations. Results show that the ortho-toluidine and glucose oxidase methods most accurately measure true glucose levels. In uremics, the glucose oxidase method is the only valid method. Less desirable methods are ferricyanide and neocuproine methods.

4. Studies on Screening Tests for Sickle Cell Hemoglobin.

The existing test for Sickle cell hemoglobin, i.e., the "Solubility Test" developed by Dr. H. Itano of NIH, was modified in order to make it available as an inexpensive screening test. This modified method was compared with Sickledex™ and electrophoretic analysis and found to be in excellent agreement. In addition, a microtiter plate test method was developed and found to be highly accurate. These methods were evaluated and modified in order to make them applicable for large numbers of tests in the military medical program. Proper and accurate evaluation of incidences of this disease can now be performed.

5. Evaluation of Blood 2,3-Diphosphoglyceric Acid Determination Methods.

Several available methods and the one developed in this division are presently under extensive evaluation for incorporation in overall programs. Preliminary results are very encouraging and it is anticipated that a method for the determination of this compound will be integrated in the very near future.

6. Studies on the Determination of Sialic Acid Contents of Vaccine Samples.

Meningococcal vaccine, developed and studied extensively by the Department of Bacteriology, DCD&I, WRAIR, contains sialic acid polymer as a presumed active component. An accurate and rapid determination of this compound thus becomes imperative in order to follow the various stages of purification of this antigenic component. Extensive evaluation of the available methods was carried out. The selected modified method was further altered and is now successfully adapted for analysis by the AutoAnalyzer. It is hoped that this effort will increase the pace of research on meningococcal vaccine preparation.

7. Studies on Genetic Diseases.

"Maple Syrup" disease is a genetic abnormality observed in newborn infants. The genetic defect is the lack of enzyme(s) for the

decarboxylation of branched chain amino acids (valine, isoleucine and leucine). These amino acids are accumulated in the blood stream to a toxic level and eventually results in death. Prompt detection and careful dietary planning can prevent this complication.

In collaboration with Dr. Giacoia, DeWitt Army Hospital, Fort Belvoir, a complete screening system has been set up to identify these amino acids. Quantitative analyses further characterized the approaching toxic levels. One infant under the care of Dr. Giacoia was assisted by this system. In addition, several other Army installations have been using this test system on a routine basis.

8. Multiple Use of Commercial and Non-Commercial T4 Resin Columns.

This project compares the practicality in cost and convenience of reusing different pre-packed and laboratory-packed thyroxine determination resin columns. Implicit in the development is also an efficiency rating of the kits offered under normal use.

Due to the undesirable serum constituents left on the column after elution of T_4 and T_3, the project utilizes an in-column regeneration and a stronger follow up method to enable cleansing of the anion-exchange resin.

Completed development will demonstrate to any thyroxine testing laboratory how to decrease the cost of each determination from approximately $.91 to approximately $.08 each and will also allow the center to "customize" their system to avoid commercial error for a more precise T_4 value.

9. Modifications and Improvements in Automated Clinical Chemistry.

Improvements and modifications have been made in inulin, para-amino hippuric acid, micro blood urea nitrogen and creatinine, simultaneous glucose and lactic acid determinations in order to improve the methods and increase their efficiency.

The following table shows an example of the workload carried out under this program.

In addition, approximately 110 complete amino acid analyses were carried out as per equivalents for various on-going research projects.

It should be emphasized that these and other analyses were performed primarily for methodological improvements, research and clinical studies and not for routine clinical purposes. The improved methods are continually made available for routine clinical tests to various laboratories and clinics in the Army Medical Department.

CLINICAL ANALYSIS WORKLOAD

Test	Number Performed
Inulin	4777
Para-amino hippuric acid (PAH)	3944
Blood urea nitrogen (BUN)	931
Creatinine	1076
Hydroxyproline	5168
Glucose	2197
Lactic acid	2120
Sodium (Na)	327
Potassium (K)	327
Ammonia (NH_3)	667
Triglycerides	180
Dapsone	153
Sialic acid	1600
Chloride (Cl)	217
Methemoglobin reductase	2786
Hydroxyproline (by amino acid analyzer)	1560
	28,030

10. Studies on Marihuana.

In conjunction with behavioral studies conducted by the Division of Neuropsychiatry (Dr. Elsmore) on monkeys given Δ9 tetrahydrocannabinol (THC), urine collected from these animals has been analyzed for unchanged drug as well as the hydroxylated metabolite of THC. Ethyl ether extracts of the urines have been chromatographed by thin layer, paper, and gas chromatography. After numerous attempts to isolate the metabolite proved unsuccessful, a group of urine specimens from one of the animals produced consistent indications of minute amounts of unchanged tetrahydrocannabinol which is presumed to be the Δ9 isomer. Detection of the THC was accomplished by mass spectrometry in the combined gas chromatograph-mass spectrometer. Before any definite conclusions can be advanced, efforts are being directed toward confirmation of these findings in the urine specimens of other monkeys that received similar doses of the drug. If labeled Δ9 tetrahydrocannabinol can be obtained, quantification of this compound in the monkey urines will be attempted in order to determine if any correlation exists with the behavioral data.

11. Laboratory automation.

With the acquisition of automated gas chromatography equipment, the development of an automated toxicology laboratory will proceed with

particular emphasis on rapid determination of narcotics, amphetamines, and barbiturates in urine specimens.

Progress to date has been mainly in the area of sample preparation where a reduction in the volume of solvent used for extractions has reduced the analysis time without decreasing the recoveries of the drugs or quantitative accuracy of the procedure. The smaller solvent volume is a significant improvement for those extracts that must be concentrated for analysis by gas chromatography which is the nucleus of the automated system.

With the reduced solvent volume principle, amphetamines can be re-extracted without an evaporation of the solvent, and the determination can be completed within about 5 minutes. If no other compounds are required to be analyzed, amphetamine analyses could be performed at a rate of 80 to 100 specimens a day. With proper coordination of the system, the combined amphetamine-narcotic determination will be made at a rate of 40 to 50 specimens in an 8-hour day. As a definitive analytical scheme for reference purposes, the system ought to be able to serve as a back up for a screening operation capable of processing several hundred specimens per day.

12. Computer Programs for Toxicological Information Retrieval.

A registry of human toxicoloty is being maintained at the WRAIR Computer Center (Division of Biometrics) for the purpose of providing information on drug levels in various organs and body fluids of deceased individuals suspected of drug overdose. Various laboratories including the Army Area Medical Laboratories are contributing to this data bank, and the information is available to these laboratories as well as to pathologists and clinicians who must evaluate cases of drug intoxication. This program and the existing file is a result of efforts by this laboratory to promote an information exchange and data compilation service to rapidly update files by computerized systems, and to provide toxicological information rapidly when requests are received. These efforts are primarily directed toward new drugs or drugs and compounds for which very little information now exists. With the introduction of new drugs every year by the pharmaceutical industry and the use of certain non-proprietary drugs, centralized data banks are the most effective means of collecting and dispersing this vital information to those chemists and physicians who need it.

13. Studies on Electrophoretic Analysis of Serum Lipoproteins.

The electrophoretic method for the fractionation of serum lipoproteins was further developed in order to determine a larger number of samples for screening purposes and to provide more details of lipoproteins and better clarity of patterns for practical applications. With the present method 8 samples can be determined simultaneously; in addition to the beta and alpha lipoproteins, one, two and three pre-beta

lipoproteins can be demonstrated. In collaboration with the Cardiology Service, WRGH, the method has been applied in studies of correlation of serum lipoproteins with clinical (cardiovascular) conditions.

Lipid abnormalities have been associated with cardiovascular disease (CVD) and myocardial infarction (MI). Therapeutic measures using diets and drugs have been established by other workers and are utilized to treat and prevent CVD.

Attempts have been made to identify the lipid abnormality and study the mechanism of the abnormal process in order to provide early diagnosis and proper treatment to victims of the disorder.

Serum lipoprotein fractionation has accurately classified individuals into specific lipoprotein types (according to the Fredrickson's classification). Some of these types are high risk candidates for MI; a screening program could identify them and therapeutic measures could be obtained.

Some information has been obtained by studies of lipoprotein patterns in the serum of 50 patients with MI. Two pre-beta lipoproteins have been found in these patients. This finding raised the question whether they are the result of MI or an indicative risk factor of MI.

Pre-beta lipoproteins are formed in the liver. The clear demonstration of several pre-beta bands by this technique allowed the study of serum lipoprotein patterns in the serum of patients with liver disease. One hundred samples were analyzed and the most significant findings were as follows: absence of pre-beta lipoprotein in cirrhosis, disappearance of pre-beta lipoproteins during the acute phase of hepatitis and reappearance during the recovering phase; increased amounts of the beta-lipoprotein in primary biliary cirrhosis. These findings could be used for developing simple methods for differential diagnosis of liver disease and hepatitis in particular.

14. Assay System for Detection and Identification of Narcotic Metabolites in Biological Fluids.

Antibodies are made to an antigenic complex of the drug of interest. The drug is spin-labeled with an appropriate nitroxide spin label. ESR observation of the spin labeled drug - antibody complex reveals strong immobilization of the label. When this complex is exposed to body fluids containing the drug of interest, some of the labeled drug is released from the complex. The increase in signal observed is proportional to the body fluid concentration of the drug of interest.

The Free Radical Assay Technique (FRAT) was developed by SYVA Corporation, Palo Alto, California. Our evaluation is continuing and current studies indicate a considerable variation in the amount of spin-labeled morphine released from the morphine-antibody complex upon

exposure to normal, morphine-free urine. Other drugs such as Tigan and Thorazine may influence this background level.

To date, only commercial spin labeled morphine and antimorphine antibody have been tested. We are developing techniques for spin-labeling morphine free base and in cooperation with Dr. Donald Catlin of the Division of Medicine, WRAIR, are preparing antimorphine antibody.

After the technique has been developed for morphine assay, we will extend it to other drug classes. Evaluation of the commercially available system will continue.

15. Role of Inorganic Ions in the Physiology and Biochemistry of Man.

During this year, the electrochemical technique of the measurement of serum ionized calcium as reported last year was further developed and a reproducible method established. The means of sample collection, the effect of storage, electrode standardization and the influence of various electrode parameters were evaluated. The range of normal values was determined using sera from 397 healthy adult volunteers. A value of 1.22 $\pm$ 0.09 mM (mean + 2 S.D.) was obtained. The normal value for total calcium, as measured by atomic absorption spectrophotometry, was determined concurrently as 2.29 $\pm$ 0.24 mM (mean $\pm$ 2 S.D.).

Using this method, studies of the variations of serum ionized calcium in various disease states and therapeutic situations were performed in collaboration with the Division of Medicine. It was found that (1) serum ionized calcium represented a more accurate means to diagnose primary hyperparathyroidism. In 15 patients admitted to the Endocrine-Metabolic Ward, WRGH, for evaluation of this disease, serum ionized calcium values ranged from 1.40 - 1.98 mM, well outside the 3 S.D. of the mean value for normals. In contrast, 5 out of 15 patients had values well within the normal range. On operation, parathyroid adenomata or adenomatous hyperplasia were identified in all 15 patients. Thus it is apparent that the high diagnostic accuracy of the method provides an important clinical diagnostic tool for parathyroid disorders, particularly in instances of normocalcemic hyperparathyroidism.

(2) Immobilization of patients, e.g., in treatment of fractures, results in a rise of serum ionized calcium. Ten patients have been studied by sequential measurements of ionized and total serum calcium values. It was found that 24 of 38 measurements of ionized calcium were abnormally high (outside of the 2 S.D. of the normal mean) while only 3 of 38 total calcium values were abnormal. To better define the chronology and physiology of the serum ionized calcium rise, 8 normal male volunteers have been studied in detail under controlled conditions of intake and output. Immobilization produced a rise in serum calcium by the fourth day of bedrest. In marked contrast, total serum calcium was

normal although urinary calcium excretion was greatly increased. Thus serum ionized calcium more clearly reflects the alteration in calcium metabolism associated with immobilization, and should be useful in further defining the mechanism of the calcium loss of immobilization. As a corollary, caution must be used when ionized calcium values are used diagnostically in patients at bedrest.

(3) Transfusions can dramatically lower serum ionized calcium, but in spite of this, no clinical evidence of tetany occurred in infants undergoing exchange transfusion. The effects of using heparinized versus citrated (ACD) blood upon serum ionized and total calcium values were compared as well as the effect of administering calcium gluconate to counter the fall in ionized calcium. While heparinized blood produced negligible changes, ionized calcium fell dramatically with ACD blood. Administration of 0.1 g calcium gluconate per 100 ml ACD blood exchanged was unable to maintain a normal ionized calcium concentration, but total calcium rose to hypercalcemic levels. Since the ionized calcium values did not correlate with the clinical state, the established use of calcium gluconate during exchange transfusions certainly will need re-evaluation.

16. Evaluation of a New Fluorometric Method for Determination of Cortisol in Serum.

Although fluorometric methods for the determination of serum 11-hydroxycorticosteroids have been available for many years, only those which involve lengthy chromatographic separation and purification methods have been shown to possess specificity for cortisol or corticosterone. In 1969, Clark and Rubin (Anal. Biochem. 29: 31, 1969) described a new method which is simple and rapid, yet purported to retain the specificity and accuracy of lengthier procedures. The method utilizes the initial conversion of cortisol and corticosterone to the 17-β-carboxylic acid derivation with metaperiodate, extraction of contaminants and fluorometric analysis. If this method can be documented to be specific, it would be of great practical value for serum cortisol determinations. Hence an evaluation was conducted in collaboration with the Division of Medicine, comparing this method against others, including the Porter-Silber method, competitive protein binding method, and the double isotope derivative method which chromatographically separates cortisol from other derivatives. Sera from patients known to have low, normal and high cortisol values were employed. It was found that of all the methods examined, the Clark and Rubin method correlated most closely with true cortisol values as measured by the double isotope derivative method. Thus this fluorometric method can now be the one routinely used with confidence in clinical laboratories for the diagnosis of adrenocortical abnormalities. It is simple and rapid. Forty samples may be analyzed in 8 hours with minimal glassware and equipment.

SUMMARY.

Analytical chemistry methodology development and application to clinical use and research is being pursued. Atomic absorption spectrophotometry was developed for the measurement of Zn, Hg, Fe, Cd, Cu, As, Ca and Mg. Specific ion electrodes were utilized to determine ratios of ionized calcium to total calcium. The development and utilization of ESR for detection and identification of drugs of abuse began and progressed to the point of comparison with gas liquid chromatography (morphine). Electrophoretic separation of lipoproteins and isoenzymes continued. Automated systems of measurement of 2,3-DPG, G.-6-PD, OH proline and sialic acid were assembled and comparative studies completed. There are now five cart mounted AutoAnalyzer systems. A detection method for tetrahydrocannabinol (THC) was established on the gas chromatograph-mass spectrometer (GLC-MS). Work continued on GLC-MS analysis and identification of drugs of abuse.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 170, Biochemical methodology and laboratory automation

Literature Cited.

Publications:

1. Battistone, G. C., Levri, E. A., and Lofberg, R. T.: A simplified ultramicro-determination of copper in biological specimens by neutron activation analysis. Clin. Chim. Acta 30: 429, 1970.

2. Lofberg, R. T.: Gas chromatographic analysis of aminothiol radioprotective compounds. Anal. Letters 4: 77, 1971.

3. Li, T.-K., and Piechocki, J. T.: Determination of serum ionic calcium with the ion-selective electrode: Evaluation of methodology and normal values. Clin. Chem. 17: 411, 1971.

4. Low, J. C., Schaaf, M., Earll, J. M., Piechocki, J. T., and Li, T.-K.: The value of serum ionized calcium in the diagnosis of primary hyperparathyroidism. Clin. Research 19: 375, 1971.

5. Heath, H., Earll, J. M., Schaaf, M., Piechocki, J. T., Li, T.-K., and Baker, G. I.: Abnormal serum ionized calcium with immobilization and bedrest. Clin. Research 19: 477, 1971.

6. Maisels, M. J., Li, T.-K., Piechocki, J. T., and Werthman, M. W.: Effect of exchange transfusion on serum ionized calcium. Ped. Research, May 1971.

7. Powell, J. B., and Djuh, Y.: A comparison of automated methods for glucose analysis in patients with uremia before and after dialysis. Am. J. Clin. Path. 56: 8, 1971.

8. Kazyak, L., and Permisohn, R. C.: Retention indices for compound identification by gas chromatography. J. Forensic Sci. 15: 3, 1970.

9. Papadopoulos, N. M., and Kintzios, J. A.: Human serum lipoprotein pattern varieties by agarose gel electrophoresis. Clin. Chem. 17: 427, 1971.

10. Papadopoulos, N. M., and Charles, A.: Serum lipoprotein patterns in liver disease. Proc. Soc. Exp. Biol. Med. 134: 797, 1970.

11. Papadopoulos, N. M., and Bedynek, J.: Serum lipoprotein electrophoretic patterns in patients with myocardial infarction. Lab. Med. 2: 38, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
[illegible]		[illegible]

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. DOD SECURITY	7. REGRADING	8. DISCR INSTR	8a. TYPE SPEC DATA (CONTRACTOR ACCESS) YES / NO	9. LEVEL OF SUM
70 07 01	D. Change	U	U	NA	[illegible]	[illegible]	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A0[illegible]01A	[illegible]	[illegible]
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Migratory Animal Pathological Survey - JA

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

[illegible] 003 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMING ORGANIZATION
[illegible] 07	CONT	DA	[illegible]

17. CONTRACT GRANT

a. DATES/EFFECTIVE NA EXPIRATION

b. NUMBER

c. TYPE a. AMOUNT

d. KIND OF AWARD f. CUM AMT

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN-YRS	b. FUNDS (Thousands)
PREVIOUS FISCAL YEAR	[illegible]	[illegible]
CURRENT FISCAL YEAR	[illegible]	[illegible]

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Puescher, COL W. W.

TELEPHONE: AC 202-576-3211

20. PERFORMING ORGANIZATION

NAME: [illegible]

ADDRESS: [illegible]

PRINCIPAL INVESTIGATOR (Provide name if this is a collateral investigation)

NAME: [illegible], M. F.

TELEPHONE

SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

NAME

NAME

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Ornithology; (U) Migration; (U) Vectors

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

23. (U) Particular interest is in the role of migrating animals in the transmission of disease.

24. (U) Major effort will be on bird banding and recovery in various areas of SEA. Ectoparasites will be collected, blood and tissues will be examined. Area surveillance will be from Bangkok.

25. (U) 70 07 - 71 06 Current report of activities is not yet available from FEBA.

(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 66 AND 1498 1 1 MAR 66 FOR ARMY USE ARE OBSOLETE

RESEARCH AND TECHNOLOG WORK UNIT SUMMARY

70 07 01					
a. PRIMARY	61101A	3A062101A91C	00	175	
b. CONTRIBUTING					
c. CONTRIBUTING					

(U) X-Ray Diffraction Studies of Organic Compounds (27)

01 70 Intramural

		2A		3. Contract
		TRAVEL	1.5	31
			1.5	6

1

Walter Reed Army Institute of Research

Washington, D.C. 20307

Foreign [illegible]

1

University of Maryland

College Park, Md. 20740

[illegible], M.

[illegible]-6100-2636

Angel, Dr. R.

1

(U) Chemistry; (U) Analytical Chemistry; (U) Biochemistry; (U) X-Ray; (U) Pharmacology

24. (U) To study chemical structure and analogues of chemical compounds and the products of the interaction of these compounds in biochem. ally important systems, to learn more regarding the specificity of chemical structure in treatment of disease.

24. (U) To relate crystal structure analysis to the configurations of the chemical molecules which exhibit pharmacologic activity in the chemical and to establish sufficient reference data to evaluate structure-activity relationships.

25. (C) 70 07 - 71 02 The contract was extended without additional obligation of funds until 71 02 28. All the structures defined during previous reporting periods have been processed and the findings are being assembled in a final report. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 66 AND 498-1 1 MAR 66 FOR ARMY USE ARE OBSOLETE

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 175, X-ray diffraction studies of biological interest

Investigators.
Principal: J. M. Stewart, Ph.D (University of Maryland)
Associate: LTC C. R. Angel, MSC

DESCRIPTION.

The single crystal X-ray diffraction studies on the antimalarials, their analogues and their products of interaction in biological systems were carried out under this contract. These studies are intended to relate the specific configuration of these chemical compounds to their protective effects as antimalarials in biological systems.

PROGRESS.

The crystal structures of the antimalarials diaminodiphenylsulfone, chloroquine diphosphate, methylene blue, and 1-(3'4'-dichlorobenzyloxy)-2,2-dimethyl-4,6-diamine-1,2-dihydrotriazine hydrochloride have been determined, providing much information about molecular geometry, hydrogen bonding, and molecular packing as well as, in some cases, anion-cation interactions in the solid state.

The structure of a complex formed between 8-aminoquinoline, an antimalarial precursor and zinc (II) chloride has been determined, with some difficulty, to provide information about coordination geometry of the 8-aminoquinoline class of antimalarials. Also determined is the crystal structure of another antimalarial precursor, 2-(2,4-dimethylphenyl)-3-methyl-6-chloro-7,8-benzoquinoline-4-carboxylic acid.

The structural studies efforts are summarized in the accompanying tables. Table I is a list of solved antimalarial structures. Table II is a list of other solved structures. Table III is a list of structures under investigation.

Table I

SOLVED ANTIMALARIAL STRUCTURES

1.	4,4'-sulfonyldianiline	Dickenson	Intramolecular H-bonding; molecular geometry
2.	Chloroquine Phosphate	H. Preston	Side chain configuration; phosphate binding and H-bonding network

3.	Methylene Blue (hydrated)	H. Marr	Location of charge on cation; H-bonding interactions
4.	2-(2,4-diamethyl phenyl-3-methyl-6-chloro-7,8 benzo-quinoline-4-carbox-cyclic acid methyl ester	E. Boonstra	Antimalarial precursor
5.	Ethyl-5-phenyl-2-imino-4-oxo-1-imidazolidine car-boximidate	L. Plastas	Antimalarial and Zwitterionic effects
6.	1-(3'4'-dichloro-benzyloxy)-2,2-dimethyl-4,6-di-amino-1,2-dihydro-triazine hydrochloride	L. Plastas	Molecular geometry
7.	Transaquobis (8-amino-quinoline) Zinc (II) Tetrachlorozincate III	C. Kerr	Chelation geometry of 8-aminoquinoline

Table II

OTHER SOLVED STRUCTURES

1.	Picryl Chloride	J. Willis	Densely packed explo-sive substance
2.	Sodium Chloride	E. Boonstra	Diffractometer align-ment check
3.	Pentacarbonytri-phenylphosphite-chromium (0)	H. Plastas	Backbonding theories
4.	Pentacarbonxyltri-phenylphosphine-chromium (0)	H. Plastas	Backbonding theories
5.	1,1-dimethyl-3-phenylpyrazolium-5-oxide	W. DeCamp	Antipyrine structural isomer

6.	Exo-1-chloro[2.1.1] bicyclohexane-5-carboxylic acid	P. Watts	Parent (2.1.1) bicyclic compound
7.	5-Phenylpseudohydantoin	L. Plastas	Zwitterionic effects
8.	2-Methyl-5-bromo-7-trifluoroacetyl-3-azo-2H-3-pyridine	P. Watts H. Ammon	Determination of positions of substitution
9.	Thiepin-1,1 dioxide	H. Ammon P. Watts	Aromatic character determination
10.	3-methyl-6-isopropyl 5'-parabromobenzoyl 8H-azuleno[1,8-bc] thiophene	H. Ammon P. Watts	Identification of molecules
11.	5-phenyl-2-imino-4-oxo-1-imidazolidine	R. Chastain	Blocking agent in protein degradation
12.	Tetracarbonylbistriphenylphosphie chromium (0)	H. Preston H. Plastas	Coordination back-bonding theories
13.	3-methyl-pyrazolin-5-one	W. DeCamp	Parent of many steroids
14.	1,3-dihydroxy-2-CH_3 2-nitropropane	H. Marr	Geometry in di-alcohols
15.	bis (triphenyl-phosphine) diphenyl-acetylene platinum (II)	H. Plastas	Coordination geometry

Table III

STRUCTURES UNDER INVESTIGATION

1-hydroxy-2-methyl-2-nitropropane	H. Marr	Geometry in mono alcohols
Trans-iridium (I) Chlorocarbonyl Bis[tri(o-tolyl)] phosphine	W. DeCamp	

Diphenyl-dimethyl phosphine chloride	H. Plastas	Coordination geometry
2-methyl-4-amino quinoline	P. Watts C. Kerr	Accurate bond lengths to give geometry and bond order of the 4-aminoquinoline system
Quinine Sulfate	P. Watts C. Kerr	Absolute configuration and conformation; interaction between quinine and the anion
Primaquine Phosphate	C. Kerr	Side chain configuration; phosphate binding and H-bonding network
11-Bromoundecanyl anhydride	W. Pangborn	Structures of membranes
Ethylene didodecanoate	P. Watts W. Kopecky	Structures of membranes
4-hydroxypyrimidone	W. Kopecky	Structures of membranes

Intensity data have been collected for quinine sulfate and 2-methyl-4-aminoquinoline and attempts are now in progress to solve these two structures; both are acentric structures and are offering some resistance to solution. Only preliminary cell data have been collected for primaquine diphosphate, which crystallizes in extremely thin plates unsuitable for an intensity study.

Complexes of chloroquine with cobalt (II), copper (II), and zinc (II) salts have been prepared and preliminary conductivity and spectral measurements have been made. The preparation of complexes of quinine, chloroquine, and primaquine with organic and organophosphoric acids is in progress; subsequent structure determination should yield further valuable information as to the binding capabilities of these drugs.

The specific mission of solving the structures of antimalarial compounds has required a parallel effort in the field of crystallographic computing. A major problem arises in the structure determination of optically active (therefore acentric) light atom drugs. The method of solution being developed for these acentric compounds is similar to that presently used for compounds crystallizing in centrosymmetric space groups, but is more complex and has required a major computational effort. A new program has been developed for acentric phase determination and has been used recently for the solution of two previously

unsolved compounds. Five noncentrosymmetric light atom structures have now been solved.

Review of quality of the programs in the X-ray crystallographic computing system continues and several new programs have been added. This system is distributed for use in other laboratories throughout the world and has, in fact, been referenced in approximately 10% of the articles appearing in recent issues of Acta Crystallographica.

SUMMARY.

This contract was terminated on 71 02 28 and this report constitutes the final summary of the accomplishments under this project. Studies of correlation of structure and function of antimalarials will in the future yield worthwhile knowledge in understanding of their mode of action. X-ray diffraction studies will be a fruitful tool for structural determination of antimalarials.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6439	71 06 30	DD DR&E(AR)636

3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. WORK UNIT NUMBER	8B. SOR OR OTHER DATA CONTRACT OR GRANT ACCESS YES / NO	9. LEVEL OF SUM
70 07 01	A			NA			A. WORK UNIT

10. NO	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B74C	00	140
b. CONTR/GRANT				
c. CONTRIB				

11. TITLE (Procede with Security Classification Code)

(U) The Importance of Chromium in Disorders of Human Glucose Tolerance

12. SCIENTIFIC AND TECHNOLOGY AREAS

002300 Biochemistry 002500 Clinical Chemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 05	Cont	DA	B. Contract

17. CONTRACT/GRANT

a. DATES/EFF 69 08 EXPIRATION 72 07

b. NUMBER DADA 17-68-C-8119

c. TYPE S/T AMT 29,524

d. KIND GRANT f. CUM AMT 54,870

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN-YRS	b. FUNDS (In thousands)
FISCAL YR 71 (CURRENT)	1	27
72	0.1	2

19. RESPONSIBLE DOD ORGANIZATION

NAME Walter Reed Army Institute of Research

ADDRESS Washington, D.C. 20012

20. PERFORMING ORGANIZATION

NAME State University of New York

ADDRESS Stony Brook, New York 11790

PRINC INVESTIGATOR (Provide last name, first name, middle initial)

NAME Doisy, R

TELEPHONE 516 [illegible]

SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

NAME Angel, [illegible]

NAME

1

RESPONSIBLE INDIVIDUAL

NAME [illegible]

TELEPHONE 202-[illegible]

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEY WORDS (Provide each with Security Classification Code) (U) Chromium

(U) Diabetes Mellitus; (U) Glucose Tolerance; (U) Insulin Antibodies; (U) Absorption;

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

23. (U) To study chromium metabolism in normal, diabetic and elderly human subjects and to biochemically define defects in absorption, handling and excretion of chromium associated with impaired glucose metabolism. To identify the nature of the circulating chromium complex which appears in the plasma in response to glucose ingestion.

24. (U) Oral Chromium-51 will be administered and plasma and urinar, Chromium-51 concentrations will be determined at intervals for three days. The same measurements will be made following intravenous injection of Chromium-51. Comparison of experimental data will allow assessment of intestinal absorption, plasma half-life and excretion in all three types of subjects. Studies of the nature of circulating chromium will initially use animals. Electrophoretic patterns of chromium containing plasma fractions, before and after a glucose load, will be compared and the eluted fractions tested for biological activity in the epididymal fat pad assay.

25. (U) 70 07 - 71 02 The study has been carried out in two parts. The organ and intracellular distributions of chromium in the rat under varying dietary and hormonal chromium in normal and insulin requiring diabetics. Absorption of 2-7 times the quantity of chromium in diabetics in contrast to normal absorption is reported. A concomitant increase in urinary excretion has been documented. A final report has been prepared and disseminated. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

*Available [illegible] upon request of approved

DD FORM 1498 [illegible]

3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 180, The importance of chromium in disorders of carbohydrate metabolism

Investigators.
Principal: R. J. Doisey, Ph.D. (State University of New York)
Associate: LTC C. R. Angel, MSC

DESCRIPTION.

Previous investigations under this contract suggested that chromium deficiency may exist in the elderly population. Impaired glucose tolerances in some elderly subjects were normal after supplementation of their diet with trivalent chromium. Tests on oral ^{51}Cr absorption suggested no evidence of malabsorption of chromium in elderly subjects. However, it was observed that insulin-requiring diabetics appeared to absorb more chromium than did maturity-onset diabetics or normal subjects.

PROGRESS.

a. Clinical Studies.

Oral administration of a tracer dose of $^{51}CrCl_3$ has shown that insulin-requiring diabetics absorb two and one-half to seven times the amount of chromium than normal subjects absorb. It is not known whether the increased absorption reflects possible tissue deficiency or not. The increased urinary excretion observed may be due to the increased absorption, or due to impaired metabolism of the ingested chromium.

Intravenous administration of ^{51}Cr into normal subjects and juvenile diabetics demonstrated that the diabetics again lose more chromium in the urine than do normal subjects or maturity-onset diabetics. This finding clearly suggests that the insulin-requiring diabetic is unable to metabolize chromium in a normal manner. Whether this is a cause or result of the disease is not known.

b. Animal Studies.

The organ and intracellular distribution of ^{51}Cr in normal and diabetic rats were determined. Attempts to alter the distribution of ^{51}Cr by dietary and normal influences are described.

SUMMARY.

This contract was terminated on 31 July 1970.

PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1 AGENCY ACCESSION: DA OB 6440

2 DATE OF SUMMARY: 71 07

REPORT CONTROL SYMBOL: CSRD AR 0610

3 DATE PREV SUM: 70 07

4 SUMMARY LEVEL: T

5 WORK SECURITY: C

7 REGRADING: NA

8A ORGN INSTR: 20

8B SPECIFIC DATA: N

8C LEVEL OF SUM: A

8D WORK UNIT: 181

9 TYPE PROGRAM: 6.1 Meningococcal Immunologic Agent (09)

10 PRIMARY: 31 02 00 EW

11 START: 70 07

12 ESTIMATED COMPLETION DATE: CONT

13 FUNDING AGENCY: DA

14 PERFORMANCE METHOD: In House

15 RESOURCES ESTIMATE: FISCAL YEAR 71 — PROFESSIONAL MAN/YRS: 3 — FUNDING (In Thousands): 05

16 RESPONSIBLE INDIVIDUAL: NAME: Walter Reed Army Inst of Research — ADDRESS: Washington, DC 20012

17 PERFORMING ORGANIZATION: NAME: Walter Reed Army Inst of Research — Div of Med — ADDRESS: Washington, DC 20012

NAME: Artenstein, M. S., M.D. — TELEPHONE: (AC 202) 576-3705 — [redacted]

NAME: DA

18 GENERAL USE: Foreign Intelligence Not Considered

19 KEYWORDS: (U) N. meningitidis; (U) Meningitis; (U) Polysaccharides; (U) Vaccines; (U) Human Volunteers

20 TECHNICAL OBJECTIVE & 21 APPROACH & 22 PROGRESS (Furnish individual paragraphs identified by number. Proceed from left to right with Security Classification Code.)

23 (U) - To isolate, purify and characterize antigens from meningococci. To determine the protective capacity of immunogenic fractions.

24 (U) - To survey military and selected civilian populations to determine the prevalence of those serogroups of N. meningitidis causing disease. Polysaccharides will be purified and characterized by chemical and physicochemical methods. The response of animals and human volunteers will be measured by hemagglutination and bactericidal antibody tests.

25 (P) - 70 07 - 71 06 Group C polysaccharide vaccines have been administered to recruits in six basic training centers with apparent success in preventing meningococcal disease. Several group B polysaccharide vaccines have been tested in volunteers without toxicity but also without significant immunogenicity. Group B vaccines with various adjuvants have been tested in rabbits and have produced only minimal antibody responses. One of four human volunteers who received group B vaccine mixed with influenza virus vaccine developed a significant group B antibody rise. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

DD FORM 1498 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE FOR ARMY USE ARE OBSOLETE

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 181, Development of a meningococcal immunizing agent

Investigators.

Principal: Malcolm S. Artenstein, M.D.

Associate: Frederic A. Wyle, MAJ, MC; Brenda L. Brandt, M.S.; Edmund C. Tramont, MAJ, MC; Dennis L. Kasper, MAJ, MC; Wendell D. Zollinger, CPT, MSC; Charles Harkins; Richard L. Cohen, CPT, MSC; Benjamin E. Hoover, SP5; Joseph R. Pinson, Jr., PFC; Eugene E. Limberg, PFC; and Adam D. Druzd, SFC.

Description.

Meningococcal polysaccharide vaccines have been developed against organisms of serogroups A and C. The group C vaccine has been proven to be safe and effective in preventing disease and has been used for prophylaxis this past winter in six basic training posts. The group A vaccine is safe and immunogenic but has not as yet been field tested for efficacy since group A disease has been nonexistent in CONUS for many years.

Isolation and purification of a group B polysaccharide has been accomplished this past year. The results of animal and human volunteer immunizations have been rather disappointing, as will be shown, and suggest the need for other approaches to the problem.

Progress.

1. Purified group B polysaccharide vaccines.

Lot B-1 vaccine was tested initially in six laboratory volunteers (intradermally, 50 micrograms) with the following results (Table 1).

The initial testing with the FAB assay indicated that all six volunteers showed antibody response within two weeks of injection. One volunteer (MP) also showed HA-B rise. The initial high titers of HA antibody, however, make this method rather difficult to interpret. Sera of three subjects (MSA, BLB and MP) were tested by FAB using serogroup A and C antigens with no titer increases demonstrable. Thus the group B rise was specific. Testing with the B-Farr technique at a much later time showed no antibody increases in any of the volunteers. This assay is the most sensitive yet devised to detect antipolysaccharide antibody and, therefore, is preferable for vaccine studies. However, despite the rather clear cut negative response one is faced with a difficulty in interpretation of the data

Table 1. Antibody response to group B vaccine (Lot B-1), November 1969.

Name	Test*	Serum date 0	1 wk	2 wk	3 wk	5 wk	6 mo.
MSA	FAB-B	1:2	1:4	1:32			
	HA-B	1:64	1:64	1:64			
	B-Farr	4.4	3.3	5.0	[illegible]	[illegible]	[illegible]
H.D.	FAB-B	1:4	1:64	1:64		[illegible]	
	HA-B	1:32	1:64	1:64			
	B-Farr	5.5	5.6	6.8		[illegible]	[illegible]
H.S.	FAB-B	1:2	1:4	1:32			
	HA-B	1:32	1:32	1:32			
	B-Farr	1:5	1:2	0.5	0.0		0.0
BLB	FAB-B	1:2	1:8	-	1:8	1:256	
	HA-B	1:64	1:64	1:64			
	B-Farr	6.4	7.6	4.4	5.3	4.4	4.6
J.Z.	FAB-B	1:4	-	1:16	1:32		
	HA-B	1:128	-	1:128			
	B-Farr	11.1	-	12.5	10.7	11.6	9.8
M.P.	FAB-B	1:2	1:8	1:32	1:16	1:8	
	HA-B	1:64	1:64	1:256			
	B-Farr	3.8	3.3	2.7	3.1	2.4	2.1

*FAB-B = fluorescent antibody, B organism

HA-B = hemagglutination, B antigen

B-Farr = radioactive gp B binding assay, % binding

since the B-Farr test was performed over a year after the sera were collected and the specimens had been thawed and refrozen many times. This type of treatment, however, has not noticeably reduced anti-C antibody in several individuals previously vaccinated with group C vaccine.

Subsequently, two laboratory volunteers were administered 250 micrograms of Lot B-1 polysaccharide by the subcutaneous route. Twenty-four hrs. later erythema measuring 2 to 3.5 cm was visible but tenderness was very minimal; the local reaction disappeared within the . 20 hrs. Antibody response was as follows (Table 2).

Table 2. Antibody response to 250 mcg group B vaccine (Lot B-1), January 1970.

Name		Titer at indicated week						
		0	1	2	4	8	13	16
MD	FAB-B	64	128	128	128	128	64	-
	B-Farr	8.1	10.5	13.1	14.5	10.8	-	-
JS	FAB-B	16	16	16	64	64	-	32
	B-Farr		0.9	0.9	0.0	1.6	1.5	-

JS showed a 4-fold FAB-B titer rise at 4 weeks; MD had no titer increase but his initial titer was high. Subject MD showed a suggestion of increased binding of the radioactive B antigen.

After these preliminary studies a titration of Lot B-1 was approved and carried out at Fort Bragg, N. C. beginning 25 Feb 1970. Two hundred and seventy-five recruits in reception week underwent venipuncture and nasopharyngeal cultures. Of these men, 183 volunteered to receive the group B vaccine and were divided into four groups, each of which received a different dose administered subcutaneously. Following this injection each man received the mandatory influenza and tetanus-diphtheria inoculations. Local reactions observed at 24 hrs were minimal (1 to 3 cm erythema) and were correlated (Table 3) with dose administered.

Table 3. Local erythema following group B vaccine (Lot B-1) administered subcutaneously.

Dose	No. men reacting/No. men vaccinated
10 mcg	0/48
50 mcg	3/49
100 mcg	8/54
250 mcg	8/32

Carrier cultures and blood speciens were obtained at two week intervals from the entire group.

Meningococcal carrier surveys showed very little group B transmission but serogroups C, Bo and nongroupable organisms were eventually carried by over 60 percent of the men.

The antibody response to the vaccine was interpreted with the knowledge that the FAB test system detects antibody crossreactive with other serogroups. Therefore, the data on vaccine response was also tabulated after deleting all men who were carriers of any meningococcal serogroup.

The results of FAB and Latex-B tests on these individuals are shown in Table 4.

Table 4. Antibody responses to graded doses of group B polysaccharide vaccine (Lot B-1, Ft. Bragg, Feb. 1970).

	Total	Non-carriers	
Dose	No. Pos.*/No. Tested	No. Pos./No. Tested	Date of non-carrier rise
10γ	15/34	2/8	Week 4; 7
50γ	9/24	4/9	2 at wk 4; 2 at wk 7
100γ	9/38	1/18	Week 2
250γ	8/30	1/16	Week 2 Lx and FAB pos.
No vaccine	2/14	0/8	

*4-fold or greater increase in titer by FAB-B and/or Latex-B tests.

Although many men showed significant titer rises in the FAB test relatively few individuals who never acquired a throat culture positive for meningococci developed antibody increases. Only two individuals in this latter category developed a titer rise within two weeks and only one of these two rises was confirmed by latex test. A total of 16 FAB positive subjects were checked by the Lx test and only the above mentioned individual showed a rise (2 tubes). Even if all antibody rises in noncarriers are tabulated (8/41 = 20%) the response to B-1 vaccine must be considered poor. In addition the response seemed unrelated to the dose administered.

Two more experiments were carried out in lab volunteers when preliminary results on the recruit sera suggested a very poor response. These were done in order to determine whether the group B vaccine had deteriorated during storage. In April 1970, seven subjects received a triple vaccine consisting of 50 mcg doses of each polysaccharide, A, B and C, given by subcutaneous route. Results of antibody tests are shown in Table 5.

Of this group, five showed antibody rise to A polysaccharide, six to C polysaccharide, but only one responded to the B component (R. Sco.) by FAB. This same individual, however, showed no antibody response in the Latex-B and B-Farr tests. Subject Macl., who showed no response in the FAB-B test, showed a low level, but consistent titer increases in the Lx-B and B-Farr assays, both within the first week following injection.

One more group of lab volunteers was injected with monovalent Lot B-1 vaccine, 50 mcg subcutaneously, in May 1970. Antibody responses of these seven subjects are shown in Table 6. None showed antibody rise by the FAB-B technique.

Five subjects failed to show antibody change by B-Farr test; Br. showed a falling titer and one individual, Ry., showed a low level but consistent antibody increase by this technique.

One further group of laboratory personnel were immunized with triple vaccine (Lot A-7, B-1 and C-8, 50 micrograms of each mixed together and injected subcutaneously) on 30 July 1970. Local reactions were negligible; antibody responses to A and C components were demonstrated in each of the six volunteers.

Four subjects showed no antibody response in the B-Farr test over a two month period; three of these were also tested by Lx-B and were negative. Two volunteers became serogroup B carriers four months after vaccination and their antibody studies are shown in Table 7. On the same date, two months after immunization, both subjects showed significant group B antibody increases by the Farr test, one also showed a Lx-B increase. Both men had negative throat

Table 5. Antibody responses to triple vaccine.

Name	Week after vaccination	HA-C	FAB A	FAB C	FAB B	FAB B repeated	Lx-B	B-Farr %
Adk.	0	8	4	16	16	32	<2	8.3
	1	128	16	128	8	-	<2	6.0
	2	256	128	128	16	-	<2	5.7
	4					32	<2	5.8
	8					16	<2	6.3
Chad.	0	<2	16	2	32	32	8	12.6
	1	16	16	16	32	32	8	13.4
	2	32	32	32	8	64	8	13.0
	4					32	8	13.2
	8					64	8	10.8
D.Sco.	0	<2	32	8	64	32	<2	1.1
	1	8	32	8	32	-	<2	1.4
	2	16	128	8	32	-	<2	2.0
	4					32	<2	1.9
	8					64	<2	3.8
MacL.	0	16	8	16	64	32	<2	8.3
	1	32	32	32	32	-	8	13.3
	2	32	32	32	32	-	8	13.0
	4					64	8	10.4
	8*					32	8	9.2
R.Sco.	0	<2	8	2	4	4	<2	0.0
	1	32	32	32	2	-	<2	0.1
	2	32	32	16	16	-	<2	0.0
	4					16	<2	0.0
	8					8	<2	0.0
R.Coh.	0	2	4	4	<2	4	<2	1.7
	1	64	32	32	<2	-	<2	0.3
	2	64	32	64	<2	-	<2	0.4
	4					4	<2	0.4
	8					4	<2	0.0
Roa.	0	<2	8	8	4	8	<2	1.1
	1	8	8	64	4	-	<2	0.0
	2	32	8	64	2	-	<2	3.2
	4					8	<2	0.7
	8					8	<2	0.8

*Nasopharyngeal culture revealed four colonies of group Y meningococcus. All the other cultures were negative.

Table 6. FAB titers following vaccination with Lot B-1 vaccine.

Volunteer	Antibody test	Serum titer (reciprocal) at indicated week			
		0	1	2	3
Tr.	FAB-B	16	32	16	16
Ae.	FAB-B	32	16	16	16
Je.	FAB-B	16	16	-	16
Pa.	FAB-B	32	8	8	32
Fo.	FAB-B	32	-	16	32
Br.	FAB-B	16	32	16	-
	B-Farr (%)	24.3	21.7	18.5	10.1
Ry.*	FAB-B	64	32	32	64
	B-Farr (%)	12.3	14.0	17.8	18.2

*Carrier of serogroup X.

Table 7. Antibody response of two subjects who received triple vaccine and subsequently became nasopharyngeal carriers.

Subject	Week after vaccination	Carrier state	Serum titer	
			B-Farr test (%)	Lx-B
J.W.	0	-	1.8	<1:2
	1	-	2.2	<1:2
	2	-	2.7	<1:2
	4	-	2.6	<1:2
	8	-	7.7	<1:2
	16	B	6.8	<1:2
	37	B	6.6	<1:2
A.D.	0	-	2.1	<1:2
	1	-	6.4	1:2
	2	-	4.3	1:2
	4	-	3.9	1:2
	8	-	25.7	1:8
	16	B	6.7	1:2
	37	B	8.0	1:2

cultures at this time. When next tested two and seven months later both were found to be group B carriers (it should be mentioned that both had laboratory exposure to group B organisms). Antibody titers of A.D. fell markedly to prevaccination levels despite the fact that he remained a carrier. J.S.'s antibody titers did not change very much despite prolonged meningococcal group B carriage.

Attempts to produce a larger molecular weight B polysaccharide based upon harvesting cultures of different ages culminated in the preparation of five different group B antigens labelled B-3, 6 hr., 8 hr., 10 hr., 12 hr. and 14 hr. (Fig. 1). From this figure it is apparent that the peak of sialic acid shifted to the right (smaller MW) in the polysaccharides prepared from older cultures.

Prior to studies in recruits, six laboratory volunteers were inoculated with B-3, 8 hr. vaccine by the intradermal route. Results of antibody tests are shown in Table 8.

Although two of the six volunteers (Rom. and Hoov.) showed suggestive FAB-B rises within one week following vaccination these were not confirmed by the other two assays.

All five B-3 vaccine preparations were tested in Fort Dix recruit volunteers (50 micrograms subcutaneously) with throat cultures and serum specimens being collected at two week intervals. No adverse reactions were noted. Antibody studies are summarized in Table 9.

Table 9. Antibody response to group B vaccines of different molecular size (Lot B-3, Ft. Dix).

Vaccine	No. tested	No. pos. by FAB-B
6 hr.	17	2
8 hr.	11	1
10 hr.	10	0
12 hr.	7	0
14 hr.	5	0
Total	50	3

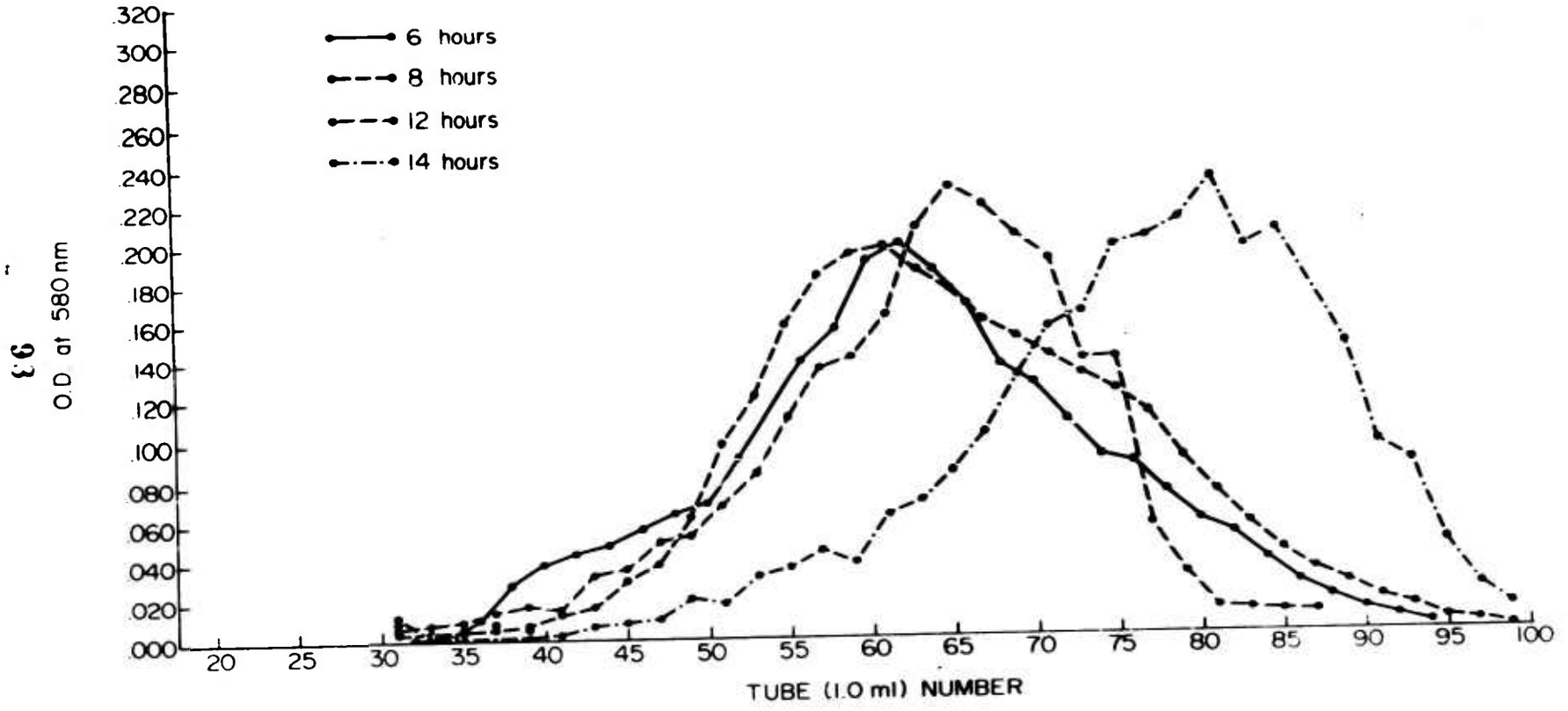

Fig. 1. Sepharose fractionation of four group B polysaccharide vaccines (Lot B-3). Sialic acid determinations on polysaccharides prepared from cultures of different ages.

Table 8. Antibody response to group B vaccine (Lot B-3, 8 hr., 50 micrograms, intradermal).

Subject	Week after vaccination	Antibody titers vs. indicated antigen		
		FAB-B	Lx-B	B-Farr (%)
O.Br.	0	8	<2	2.7
	1	16	<2	5.0
	2	16	<2	5.5
	4		<2	3.6
VanD.	0	32	<2	1.3
	1	32	<2	1.2
	2	32	<2	0.6
	4	32	<2	2.9
Rom.	0	8	<2	0.8
	1	32	<2	0.9
	2	32	<2	1.5
	4		<2	0.1
Hoov.	0	8	<2	11.3
	1	32	<2	13.3
	2	32	<2	9.2
	4		<2	14.3
Dal.	0	16	4	13.8
	1	16	4	16.1
	2	16	4	14.2
	4		4	12.2
Sang.	0	16	4	13.8
	1	32	4	14.7
	2	16	4	17.1
	4		4	15.9

Fifty subjects whose throat cultures failed to yield meningococci at two and, in many cases, four weeks after vaccination were tested for antibody response. Three individuals showed a rise by FAB-B test and these results were reproducible. However, none of the volunteers were positive by Latex-B test. Forty subjects who received the 6 hr. vaccine were tested by the B-HA assay, only one person showing a four fold increase in titer. This subject and eight others failed to show any antibody increase when sera were tested with the B-Farr antigen.

The results of the above studies suggest that group B polysaccharide vaccines can be produced with varying molecular size; but even those with the largest sized molecules are extremely poor immunogens in man.

Further studies were carried out with a fourth lot of group B vaccine made with a more highly buffered medium (B-4, 3XCA) containing three times the standard concentration of casamino acids. Eight laboratory volunteers received 50 microgram doses of vaccine (four intradermally, four subcutaneously). Sera obtained at weekly intervals were tested for B-Farr and Lx-B antibody; none showed significantly increased titers over a four week period. Four months later three of these volunteers were injected again with the same vaccine; none showed B-Farr antibody titer change.

2. The use of adjuvants to enhance the immunogenicity of group B meningococcal polysaccharide antigens.

As evidence mounted that the meningococcal group B purified polysaccharide vaccines were not immunogenic in man a series of experiments were carried out to test the combination of polysaccharide with various adjuvants.

a. Studies in rabbits.

The following adjuvants were prepared:

Name	Preparation	
Alum	Al $Cl_3.6H_2O$ 12 gms/L add 0.1M NaOH 1.8 vol to 1 vol Al Cl_3 for pH 7	5 ml B4 vaccine 1 ml Al Cl_3 1.8 ml NaOH 2.2 ml H_2O
Methylated albumin	Methylated bovine albumin 50 mcg/ml water	Equal vol mixed with B4 vaccine
Influenza virus	A2/Jap/305/57 E-10 Allantoic fluid	Mix equal vol with B4 vaccine
B4 vaccine	Lot B4 - 3XCA	Dilute 1:2 with water to equal 50γ/0.4 ml

In addition, various crude preparations of group B polysaccharide were also tested (Table 10).

Table 10. Chemical characterization of group B polysaccharides tested in rabbits.

Sample	% protein	% nucleic acids	% sialic acid*
B-36 crude	47.3	6.6	52
B-36 sevag	14.5	1.6	91.0
B-36 FP	0.84	1.07	94.0
B-36 FP-1	0.57	0.72	99

*% of a standard preparation.

Two kilogram male rabbits were injected into the rear footpads at weekly intervals for three weeks, rested two weeks, injected again, rested two weeks and injected a final time. Vaccines were refrigerated the first week and subsequently frozen between injection dates.

Results shown in Table 11 indicate that the three adjuvants were approximately equal in their effects, namely very low anti-B antibodies were induced at four weeks which, in the few animals tested, could not be boosted by a sixth injection. Purified polysaccharide alone, B-4 or B-36, were essentially nonimmunogenic but cruder antigens induced very low levels of HA-B antibodies. One injection of live group B organisms intravenously gave serum titers of 1:8 after one week. Subsequently, injection of viable organisms, three times in a week, produced HA titers from 1:64 to 1:1024 in all rabbits regardless of previous group B antigen injections. After a one month rest period, these titers declined significantly but were rapidly boosted by 2 I.V. injections of live organisms. The low levels of antibody in rabbits who had not received live organisms fell even lower or became negligible during the one month rest period and were returned to the 1:5 level by two injections of sensitized latex particles.

In a subsequent experiment, B polysaccharide vaccines were prepared with an improved alum (Merck formula), latex or mixed with typhoid vaccine and injected into rabbits three times at weekly intervals. Only the rabbit given latex-B developed a trace (1:4) of anti-B antibody.

Thus, extracted and purified (crude or with adjuvants) group B polysaccharides were poorly immunogenic in rabbits. Titers could not be boosted with purified polysaccharides even when attached to latex particles. However, live group B organisms injected

Table 11. Antibody response (B-HA) of rabbits to B polysaccharide in adjuvant or to crude B antigen.

Vaccine and weekly dose	Rabbit No.	Reciproval of B-HA titer at indicated week														
		↓ 0	↓ 1	↓ 2	↓ 3	4	↓ 5	6	↓ 7	8	9	10	11	12	13	14
M-BSA-B4	032	0		0		8		8	+		8				<5 ++	5
100γ	038	0		0		8		8								
Alum-B4	025	0		0		8		8	+		8				<5 ++	5
100γ	031	0		0		4		4	+		8				5 ++	5
Flu-B4	024	0		0		8		8								
100γ																
B-4 alone	035	0		0		4		4								
100γ	042	0		0		4		4	***		64				<5 **	320
B-36 crude	034	0		0		8		4	***		512				80 **	320
400γ	032	0		0		8		8	***		512				160 **	640
B-36 sevag	037	4		0		<4		<4								
400γ	027	0		0		8		8	+		8				<5 ++	5
B-36 FP	041	0		0		0		0	***		256				20 **	640
400γ	048	0		0		0		0								
B-36-FP-1	040	4		<4		<4	*	8	***		256				20 **	640
400γ	030	4		<4		<4	*	8	***		1024				320 **	640
Control	026						*	8	***		1024				320 **	640

* One injection of viable group B organisms (0.5 ml I.V.).
- 100γ I.V. of purified B4 polysaccharide.
↓ Injection
++I.V. B-4 sensitized latex, two injections.

intravenously produced prompt, high titers of group B antibody. These data suggest that the group B polysaccharide in its native state is significantly different from the purified products tested.

An alternative explanation of the results, however, would be that immunological tolerance, or paralysis, to the polysaccharides has been induced and that this can be broken by the antigen when presented as a large particle (whole organism).

b. Studies in human volunteers.

Based upon the rabbit data which showed a slight response to group B polysaccharide mixed with influenza virus, four laboratory volunteers were inoculated with a mixture of influenza vaccine and group B meningococcal vaccine to determine antibody responses.

Influenza vaccine was Lederle Bivalent containing A_2 and B antigens. Lot B_4-3XCA meningococcal group B vaccine (0.2 ml containing 50 micrograms) was mixed with 0.5 ml flu vaccine and injected together by the subcutaneous route. Throat cultures and serum specimens were collected prior to vaccination and at weekly intervals.

Three volunteers, E.D., R.W. and J.P., developed local erythema which measured from 1 cm. to 6.5 cm. in diameter after 24 hrs. R.W. also complained of dizziness and feeling weak shortly after injection but this was transient. Three of the volunteers received booster injections of freshly mixed vaccine three weeks later.

Antibody studies are shown in Table 12. Only one subject, R.W., showed an antibody response to the group B polysaccharide. Booster injections had no effect in elevating antibody titers.

Conclusions:

Studies of group B polysaccharide vaccines in human volunteers have been disappointing in that antibody rises were infrequent (Table 13). Initially it was believed that the vaccine could be improved in terms of molecular size and concentration of antigenically active material. This was achieved by changes in culture conditions, but even the best preparations were not satisfactory immunogens. When improved serological assays were developed these results were confirmed and antibody responses were even more uncommon.

Attempts to improve the immunogenicity of the purified polysaccharides by use of adjuvants in rabbits yielded very low grade, although definitely positive responses. The final study presented; ie, influenza and meningococcal vaccines combined, provided only one definite antibody response in a total of four subjects, an inconclusive result.

Table 12. Group B meningococcal antibody response to influenza-group B polysaccharide vaccine combination.

Volunteer	Serum date (week)	Throat culture	Antibody test: Latex-B	Antibody test: B-Earl % binding
1. G.L.	0	C	1:2	5.5
	1	C	1:2	4.8
	2	-	<1:2	5.3
	3*	+		4.9
	6	+		4.6
2. E.D.	0	-	<1:2	0.5
	1	-	<1:2	2.0
	2	-		0.7
	3*	-		0.0
	4	-		0.2
	6			0.0
3. R.W.	0	-	<1:2	12.6
	1	-	<1:2	13.8
	2	-	<1:2	30.7
	3	-	1:4	29.4
	4	-	1:4	36.8
	5	-	1:4	37.5
4. J.P.	0	B	1:8	11.9
	1	B	1:8	13.4
	2	Ro	1:8	12.9
	3*			15.8
	4	NT		16.1
	5	+		14.3

*Booster injection

Table 13. Summary of group B vaccines in man.

Vaccine	Subjects	Pos. antibody rises/Total tested	Remarks
B1 50γ	Lab vol.	? 6/6	FAB+, Farr-
250γ	Lab vol.	? 2/2	1 FAB+, ?1 Farr+
B1 10-250γ	Ft. Bragg recruits	? 8/41	FAB+, only one confirmed by Lx+
B1 triple vacc.	Lab vol.	? 2/7	1 FAB+, 1 Lx+
B1	Lab vol.	? 1/7	FAB+, Farr and Lx-
B1 triple vacc.	Lab vol.	2/6	Both became B carriers
B3 8 hr.	Lab vol.	? 2/6	FAB+, Farr and Lx-
B3 6 and 8 hr.	Ft. Dix recruits	? 4/59	3 FAB+, Lx-, 1 HA+. Farr-
10,12,14 hr.		0/22	FAB-
B4 3XCA	Lab vol.	0/8	Farr-
B4 + influenza	Lab vol.	1/4	Farr+, Lx+

There is some evidence that the immune response in man to group B infection is different from that with group C organisms, at least in terms of antipolysaccharide antibody. Only about 10 percent of group B carriers develop antipolysaccharide antibodies. Blood stream inoculation, however, is an effective stimulus both in man (cases) and rabbits.

These data suggest either a different host immune mechanism for the group B organism or perhaps an important alteration in the polysaccharide molecule when extracted from the intact organism. Ongoing and planned studies will consider these problems.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

AGENCY ACCESSION	DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0[illegible]644	71 07 01	DD DR&E/AR 636

		90	182

Dominance, Performance, Aggression, Stress, Combat
[illegible] Testicular and Adrenal Secretions. (U)

			3. Contrast
		0.1	15
	7.	0.3	8.5

Emory University
Atlanta, Ga. 30303

[illegible], M.D.
404-963-6201

Rose, R. M., M.D.

Foreign [illegible] considered

1

b. Performance under Stress, (U) Psychiatry;
[illegible] Behavior, 16. Aggressive behavior

20. [illegible] assess the relevant social behaviors that operate to influence future performance and endocrine response to stressful [illegible] simulating military training and combat.

24. [illegible] relatively complete control and observation of a group of unrestricted monkeys is feasible, animals with known behavioral histories will be subjected to varying stressors and their behavioral and endocrine responses recorded to clarify social influences on success of task performance and aggressive behavior. The relationships between hormone secretions and performance success, breakdown, and interactions between groups will be determined.

[illegible] established that the effect of dominance rank and [illegible] testosterone secretion, work continued to investigate other relevant social variables. Access to receptive females was established as a potent stimulus for testosterone secretion. Conversely, exposure of individual males to [illegible] led to a marked suppression of testosterone secretion, accompanied by behavioral withdrawal and a depression like picture. Males in an established group were observed to gain dominance over a larger group of more adult [illegible] together. Experiments commenced studying the [illegible] during conditioned avoidance. For [illegible] see Army Institute of Research Annual Progress Report,

10

DD [illegible] 1498

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 182, Correlation of performance aggression, stress, combat and group position with testicular and adrenal secretion

Investigators.
Principal: Irwin S. Bernstein, Ph.D.
Associate: Robert M. Rose, M.D.

DESCRIPTION

Dr. Rose, Dr. Bernstein, and Mr. Gordon at Yerkes Regional Primate Center continued a systematic evaluation of the relationship between plasma testosterone levels and dominance rank and aggressive behavior. They demonstrated that exposure of adult male rhesus monkeys to receptive females resulted in a two to three-fold increase in endogenous testosterore secretion. This is the first documentation of stimuli resulting in increased testosterone secretion in primates. Preliminary evidence also was obtained, suggesting that exposure of an adult male to a strange group of other males resulted in dramatic suppression of testosterone which lasted for many weeks. This suppression was associated by withdrawal and a decrease in motor activity in the male who is a stranger to the group. Further studies were done on endocrine responses of animals subsequent to exposure and confrontation by two intact but separate groups of rhesus monkeys; this data awaits analysis.

In collaboration with CPT Frank Sodetz, Department of Experimental Psychology, animals of both high and low dominance rank were studied during Sidman shock avoidance. This will provide information on the relationship between aggressive behavior, dominance rank, performance, and endocrine response under stress.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 182, Correlation of performance aggression, stress, combat and group position with testicular adrenal secretion

Literature Cited.

Publications:

1. Rose, R.M., Holaday, J.W., and Bernstein, I.S.: Plasma testosterone, dominance rank and aggressive behavior in male rhesus monkeys. Nature. 231: 366, 1971

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY CODE	7. REPRADING	8A. CITIZEN-ALIEN	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUMMARY
70 07 01	P. Change	U	U	NA	N	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061102B97	00	183
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Diseases of Military Animals in [illegible]

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 005900 Environmental bio

13. START DATE	14. ESTIMATED COMPLETION DATE	15. PERFORMANCE METHOD
68 07	Cont	C. In-House

16. CONTRACT/GRANT		17. PROFESSIONAL MAN YRS	18. FUNDS (In Thousands)
a. DATE/EFFECTIVE	NA	EXPIRATION		
b. NUMBER				120
c. TYPE				
d. KIND OF AWARD				120

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research

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22. GENERAL USE

Foreign intelligence not considered.

23. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military Dogs; (U) Tropical Canine Diseases; (U) Tropical Canine Pancytopenia; (U) Ehrlichia canis; (U) Babesia gibsoni

24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Continue on separate paper. Precede entries with Security Classification Code.)

23. (U) To define, study, diagnose and control known and potential infectious diseases of military dogs in Southeast Asia and other tropical and semi-tropical areas of potential military significance. The major effort is directed toward the cause, pathogenesis, treatment and control of tropical canine pancytopenia, which has jeopardized the operational efficacy of military dogs in Asia. Studies are also being conducted on the epidemiology, treatment and control of [illegible] parasitic infections which are medical problems in military dogs.

24. (U) Conventional methods are employed for epidemiological, pathological and microbiological examinations, and new procedures are developed as needed.

25. (U) 70 07 - 71 06 Tropical canine pancytopenia (TCP), a highly fatal hemorrhagic disease caused by Ehrlichia canis, has been responsible for the death of many US military dogs in Southeast Asia. Studies on the pathogenicity of Ehrlichia canis are being continued in both intact and splenectomized dogs. Tetracycline was shown to be highly effective in the treatment and prevention of TCP. Ultrastructure examination of lung tissue revealed elementary bodies with the same general features of rickettsiae and large particles of the chlamydia group. Blood monocyte cultures of TCP infected dogs developed typical morulae of Ehrlichia canis. Material from such cultures produced TCP in experimental dogs and infection was transferred to normal monocyte cultures. Studies to concentrate and purify this agent and its antigen are in progress. In comparative studies Neorickettsia helminthoeca has been propagated by the same procedure. The pathogenesis of Babesia gibsoni has been defined in laboratory Beagles and studies are in progress to determine the efficacy of suitable drugs in treating B. gibsoni infections. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

27. Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE AND 1498 1 MAR 65 FOR ARMY USE UNTIL EXHAUSTED.

PII Redacted

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 183, Diseases of military animals in Southeast Asia

Investigators:
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LTC D. B. Tuthill, MC; I. E. Hemelt, AB;
A. R. Warner, Jr.; E. C. Lazar, BA;
COL E. W. Grogan, VC

Description.

To define, study, diagnose and control known and potential infectious diseases of military dogs in Southeast Asia and other tropical and semi-tropical areas of potential military significance. The major effort is directed toward the cause, pathogenesis, treatment and control of tropical canine pancytopenia, which has jeopardized the operational efficacy of military dogs in Southeast Asia. Studies are also being conducted on the epidemiology, treatment and control of Babesia and other parasitic infections which are medical problems in military dogs.

Progress.

I. Tropical Canine Pancytopenia (TCP)

The history of tropical canine pancytopenia, the clinical and pathological descriptions of the natural disease, transmission studies and etiology have been described in detail in previous Annual Reports.[1,2] During the past year the investigation of TCP has included studies on the ultrastructure of the causative agent, pathogenesis of the disease in both intact and splenectomized dogs, chemotherapy and chemoprophylaxis, tick transmission, and in vitro cultivation of the causative agent, Ehrlichia canis.

1. Pathogenicity of Ehrlichia canis, the Causative Agent of Tropical Canine Pancytopenia, in German Shepherd Dogs.

A. Introduction

Tropical canine pancytopenia (TCP) was observed as early as 1963 in Southeast Asia in British military dogs in Singapore.[3] Between 1963 and 1968 this disease was responsible for the death of numerous military and privately-owned dogs in Singapore and Malaysia.[3,4,5] In the Republic of Vietnam TCP was first recognized in 1967 in several Labrador Retrievers which had previously been trained as tracker dogs in Malaysia.[6] During the following year an epizootic of the disease occurred in Vietnam among German Shepherd dogs which had originated in the United States. To date over 200 U.S. military dogs have died of the disease in Southeast Asia. TCP has also been reported in military and privately-owned dogs in the Caribbean.[7,8]

Specimens from affected dogs from geographically isolated areas in Southeast Asia as well as Puerto Rico, the Virgin Islands, Florida, and Panama were used to transmit the disease to laboratory dogs. Ehrlichia canis, a member of the family Rickettsiaceae, has been identified as the etiologic agent of TCP.[8] Transmission studies provided preliminary evidence that German Shepherd dogs experimentally infected with the agent developed signs of disease identical to those of the natural disease; whereas the disease in the experimentally infected Beagle was milder and clinical signs of hemorrhage were not observed.[8]

The current study was initiated to determine the pathogenicity of Ehrlichia canis in mature and young German Shepherd dogs.

B. Materials and Methods

Two groups of German Shepherd dogs were used in the study. The first group consisted of 11 mature dogs 2-3 years of age, and the second group was made up of 6 immature dogs 12-14 weeks of age. The dogs were inoculated intravenously with ml of whole blood collected in sterile sodium citrate from a common donor dog infected with Ehrlichia canis. In the first group 8 dogs were infected and 3 were retained as uninfected controls. In the second group 4 were infected and 2 were used as controls.

Two weeks of baseline data were collected on each dog prior to inoculation. Each dog was examined and its temperature recorded daily. Blood was collected twice weekly for WBC, RBC, PCV, hemoglobin, erythrocyte sedimentation rate, differential, SGPT, BUN, thrombocyte, bilirubin and reticulocyte determinations. In addition, blood was taken weekly

for coagulation studies. Serum was collected once a week for serum protein studies and serologic examinations. Each dog was weighed twice weekly. Bone marrow specimens were taken periodically. When possible urine was collected for routine urinalysis and urobilinogen studies. Standard procedures were used in all tests. A complete necropsy was performed on all dogs that died and dogs that were destroyed at the termination of the study.

C. Results

Analysis of results has not been completed. All infected dogs developed a conjunctivitis accompanied by an ocular and nasal discharge. Other consistent clinical signs were pyrexia, anorexia and weight loss (Table 1). Anemia, leukopenia and thrombocytopenia characterized the infected dogs (Fig. 1-5). A high erythrocyte sedimentation rate was also characteristic of the disease. Most dogs showed an elevation of SGPT during the course of the disease; however, no alteration was noted in BUN. During the course of the disease an increase in reticulocytes was noted in all dogs.

The variation in response of individual dogs is evident in the extent of fluctuation in red, white and thrombocyte count, relapses and length of time between inoculation and death. Some dogs showed no evidence of recovery from the initial phase of the disease and died as early as 60 days post inoculation (Fig. 1). Other dogs, as illustrated in Fig. 2, showed an early drop in red, white and thrombocyte counts, followed by a return to near normal values and then relapsed with a severe recurrence of earlier signs. A few dogs as illustrated in Fig. 4 showed early signs but tended to recover, and when the study was terminated 147 days post inoculation, blood values were near normal.

D. Discussion

The thrombocyte count appeared to be a sensitive indicator of the status or prognosis of the disease. In all instances there appeared to be a sharp drop in thrombocyte count early in the disease. The slope of the curve suggests a complete cessation in production or release of thrombocytes in the bone marrow. However, the fact that all dogs showed an increase in the reticulocytes provides evidence that the erythroid elements of the bone marrow are functional. In addition, increases in white cell counts observed in many of the dogs suggest the myeloid elements are also functional.

The mechanism whereby the hemopoietic system is altered by the infection is still under study.

Table 1. Summary of Clinical Signs in German Shepherd Dogs Experimentally Infected with *Ehrlichia canis*

Signs	Mature German Shepherd	Young German Shepherd
Ocular & Nasal Discharge	8/8	4/4
Weight Loss	8/8	4/4
Death	5/8	4/4
Epistaxis	5/8	1/4
Melena	7/8	0/4
Septicemia	2/8	1/4
Vomiting & Diarrhea	1/8	1/4
Skin Lesions	2/8	0/4
Hematuria	1/8	0/4
Hyphema	1/8	0/4
Corneal Opacity	1/8	0/4
Edema*	1/8	0/4

* Hind limb & scrotum

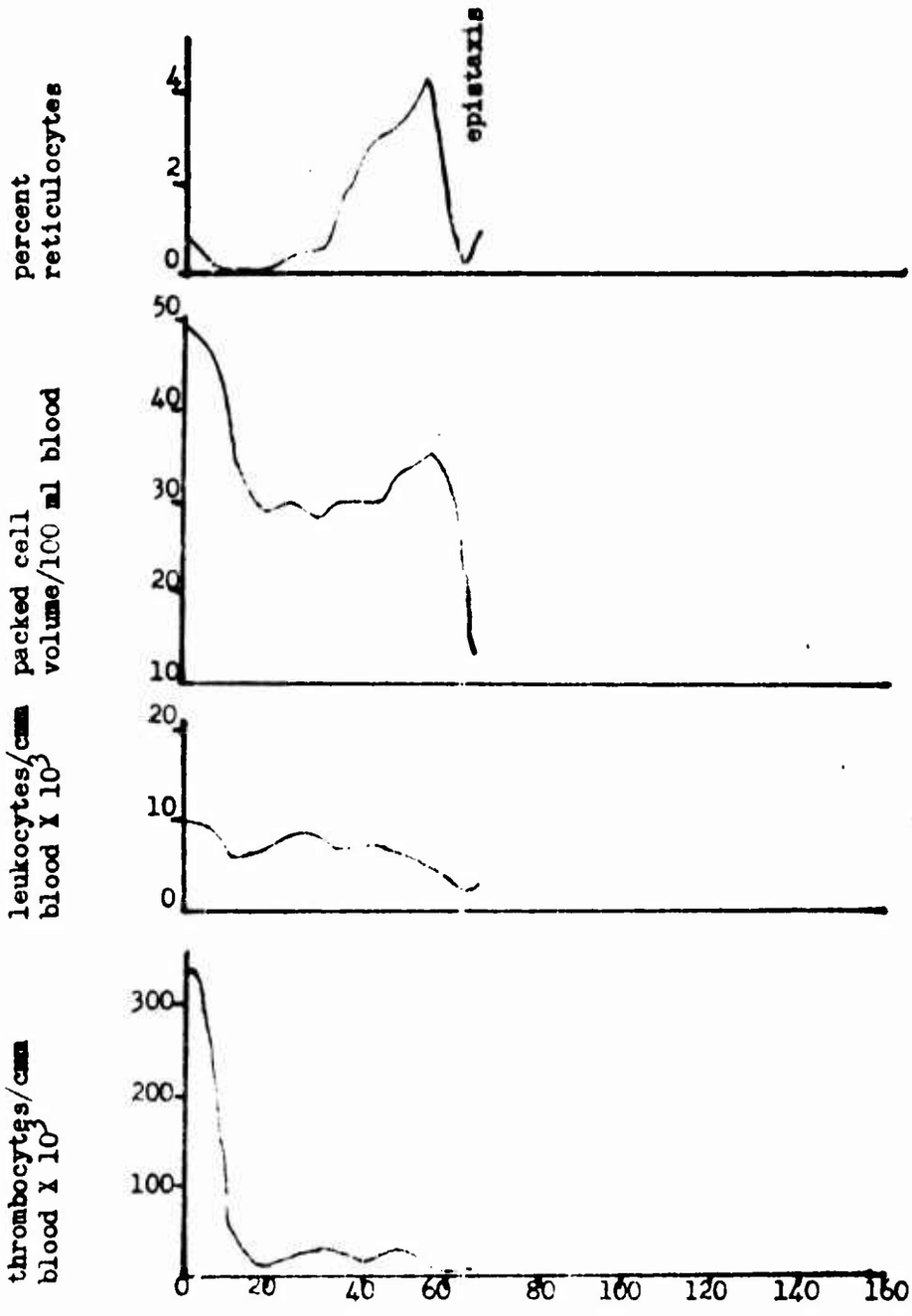


Fig. 1 Thrombocyte count, leukocyte count, packed cell volume, and percent reticulocytes of a mature German Shepherd following inoculation with Ehrlichia canis.

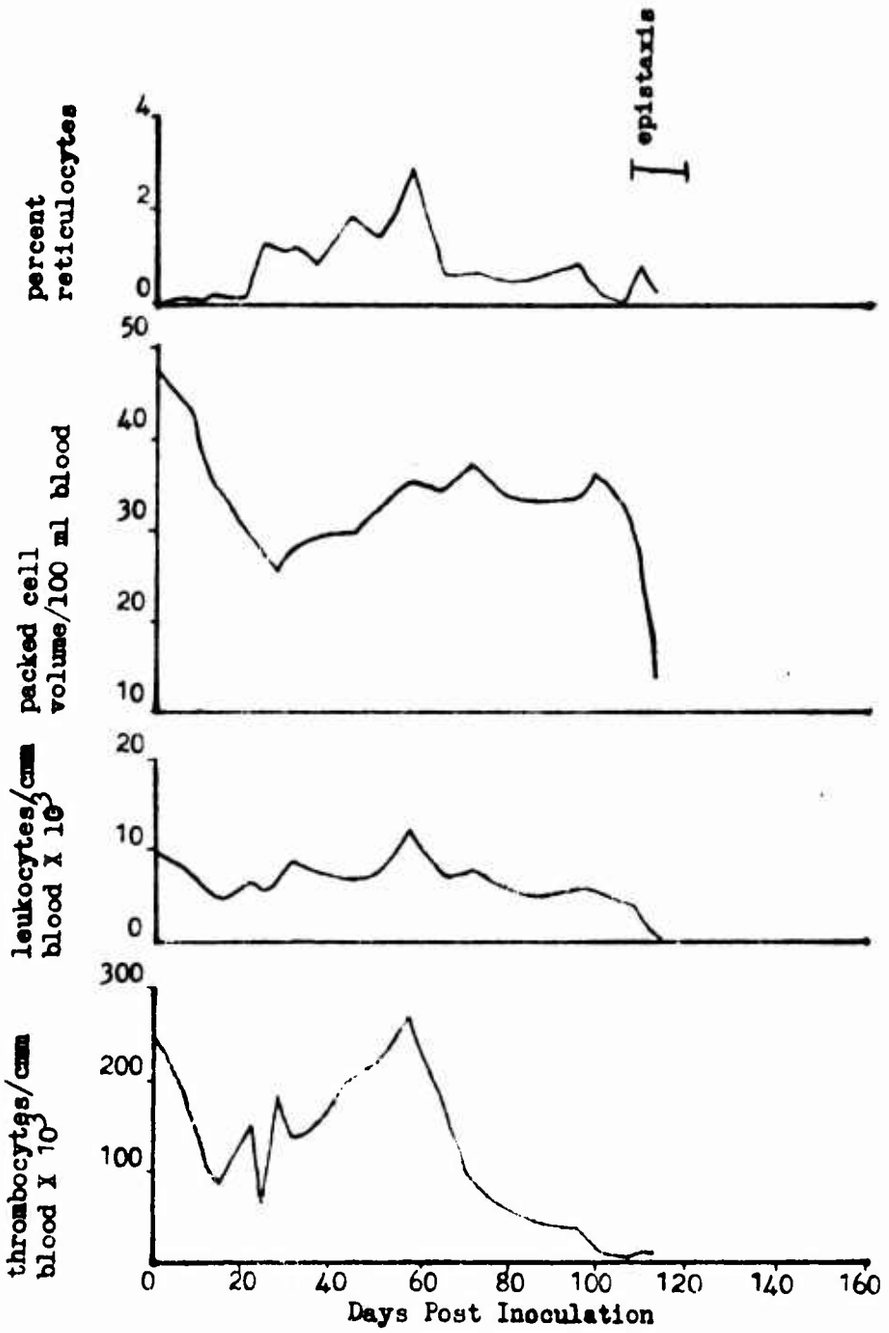


Fig. 2 Thrombocyte count, leukocyte count, packed cell volume, and percent reticulocytes of a mature German Shepherd following inoculation with Ehrlichia canis.

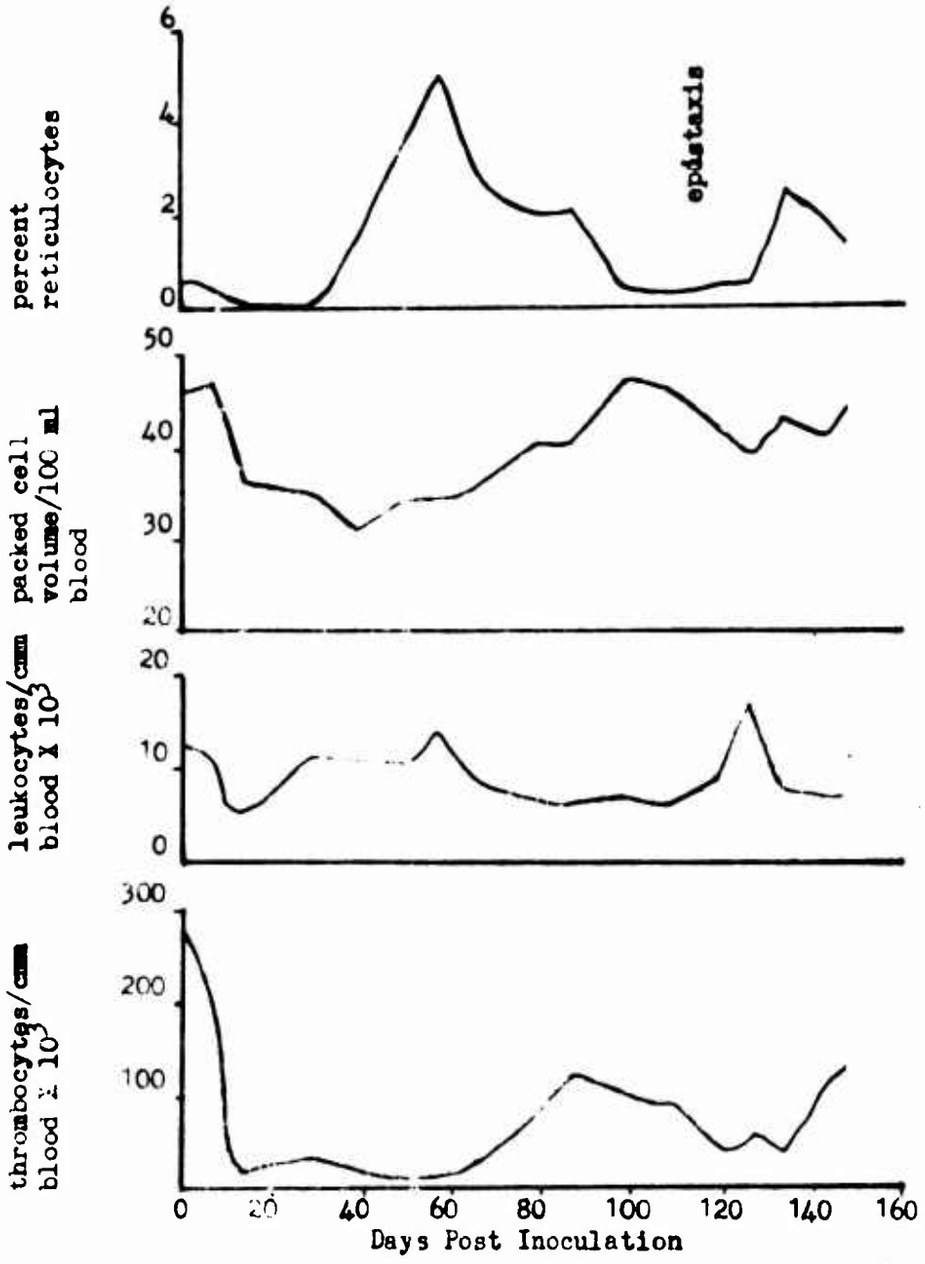


Fig. 3 Thrombocyte count, leukocyte count, packed cell volume, and percent reticulocytes of a mature German Shepherd following inoculation with Ehrlichia canis.

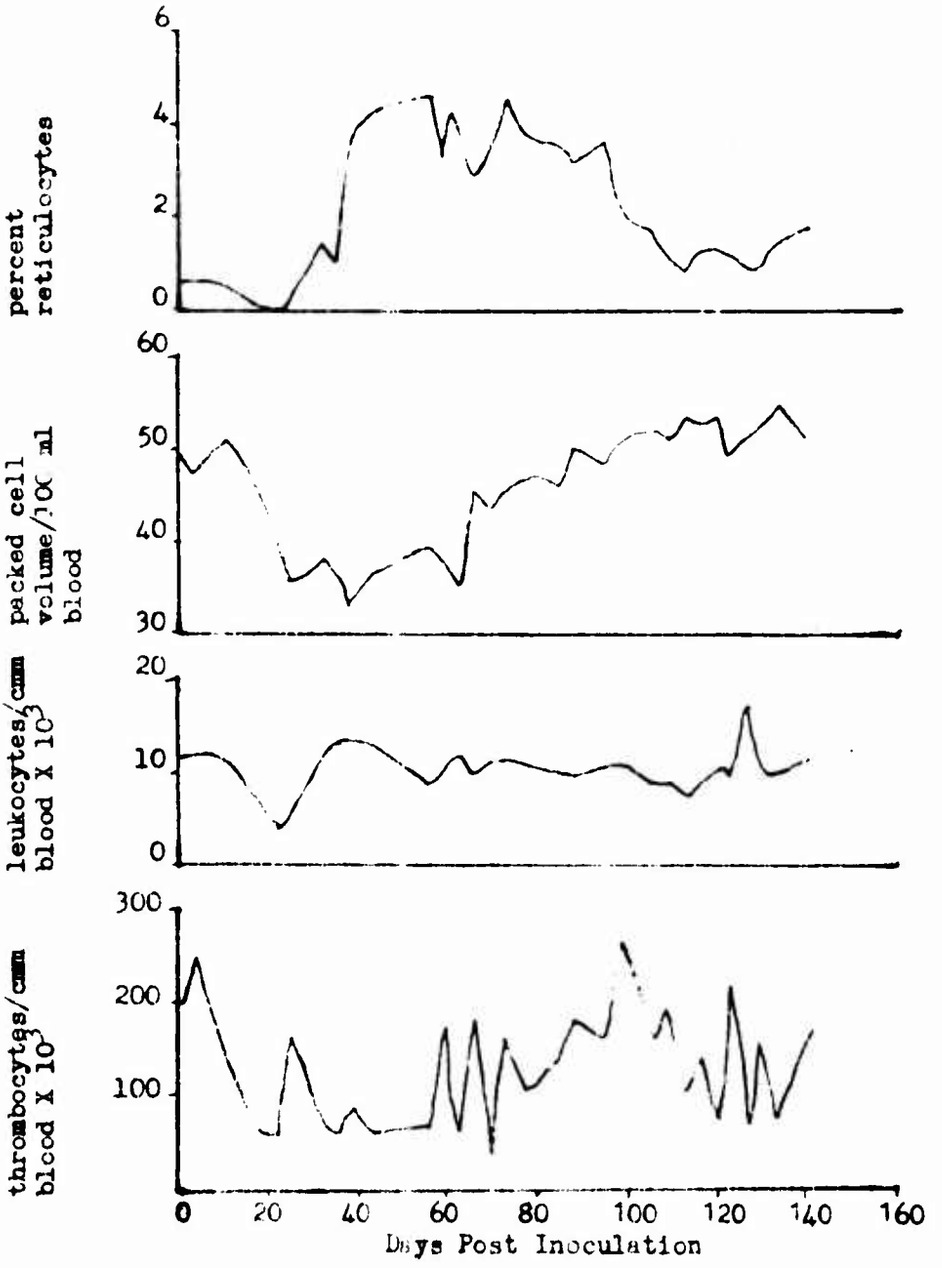


Fig. 4 Thrombocyte count, leukocyte count, packed cell volume, and percent reticulocytes of a mature German Shepherd following inoculation with Ehrlichia canis.

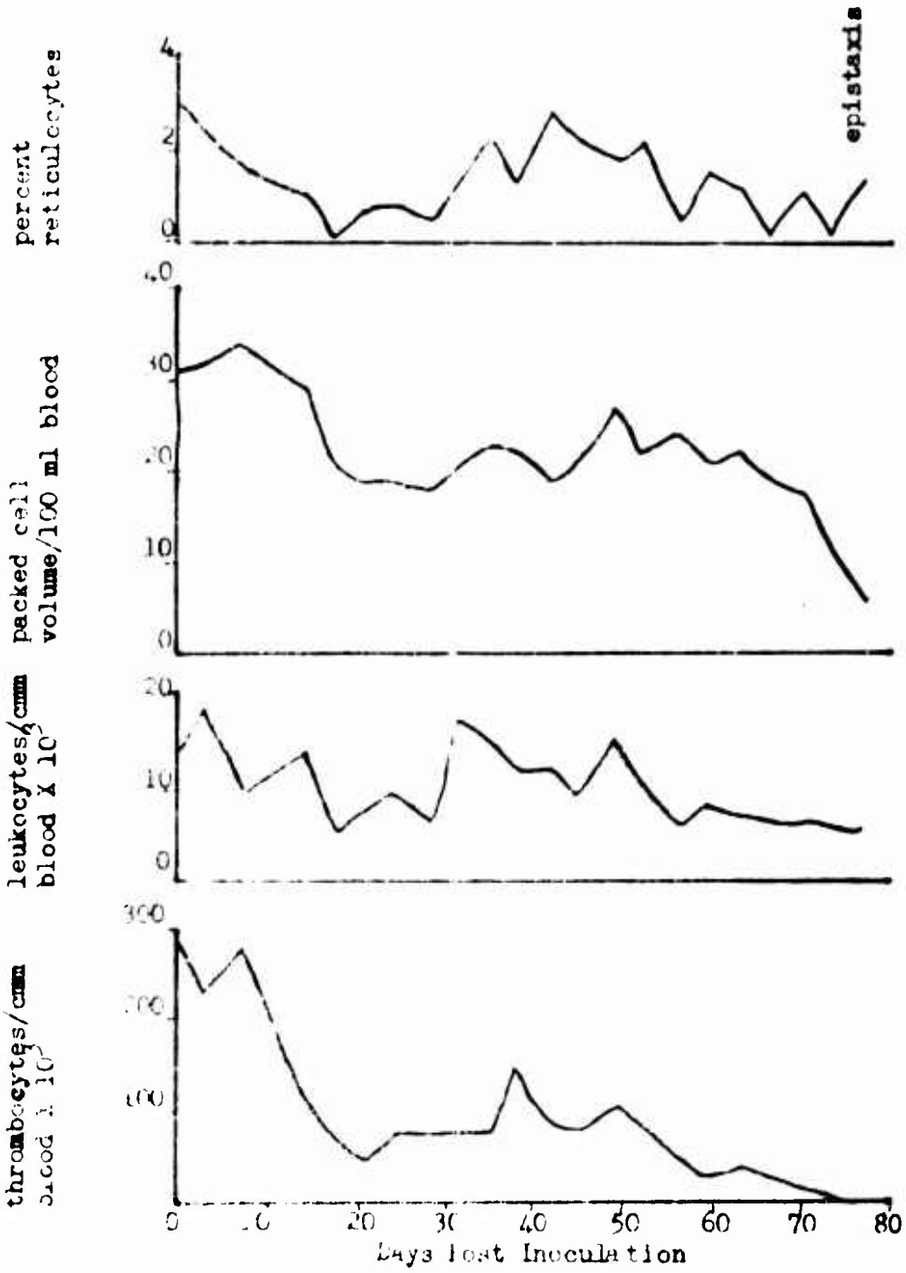


Fig. 5 Thrombocyte count, leukocyte count, packed cell volume, and percent reticulocytes of a fourteen week old German Shepherd following inoculation with Ehrlichia canis.

2. Pathogenicity of Ehrlichia canis in Newborn Beagle Pups

A. Introduction

In endemic areas TCP is most often observed in aged, purebred dogs.[9] Information is lacking on the disease in the very young puppy. It has been speculated that the disease in the young puppy may be less severe and often not recognized. Such findings would have epidemiologic significance. The study was initiated to determine the pathogenicity of E. canis infection in young, nursing puppies.

B. Materials and Methods

A litter of 5 Beagle puppies, 7 days of age, was used in the study. Three puppies were inoculated intravenously with blood collected in EDTA from an acutely affected dog. The remaining puppies were retained as controls. Baseline data were collected prior to inoculation. Each dog was examined, weighed, and rectal temperature recorded daily. Blood was collected twice weekly for WBC, RBC, PCV, hemoglobin, and erythrocyte sedimentation rate. Peripheral blood smears were prepared twice weekly.

C. Results

All inoculated pups had a severe nasal and ocular discharge 11 days post inoculation. Clinical signs of anemia were evident at 14 days. This correlates with the low packed cell volumes of the infected dogs 14-21 dogs post inoculation (Fig. 6). The infected dogs also showed a severe leukopenia. During the course of disease the infected puppies gained little as compared with the uninfected controls which showed a normal weight gain (Fig. 7). During the acute phase of the disease morulae of E. canis could readily be demonstrated in peripheral blood smears.

D. Discussion

The pattern of disease in the newborn Beagle puppy is similar to that observed in experimentally infected mature Beagle dogs. The signs of anemia and the nasal and ocular discharges could easily go unrecognized in natural infection. The difference in weight gain is more evident. Many natural infections of E. canis in adult Beagle dogs are not recognized. Although the disease is more severe in the young Beagle puppy, it mimics other puppy diseases and a specific diagnosis of E. canis may not be made.

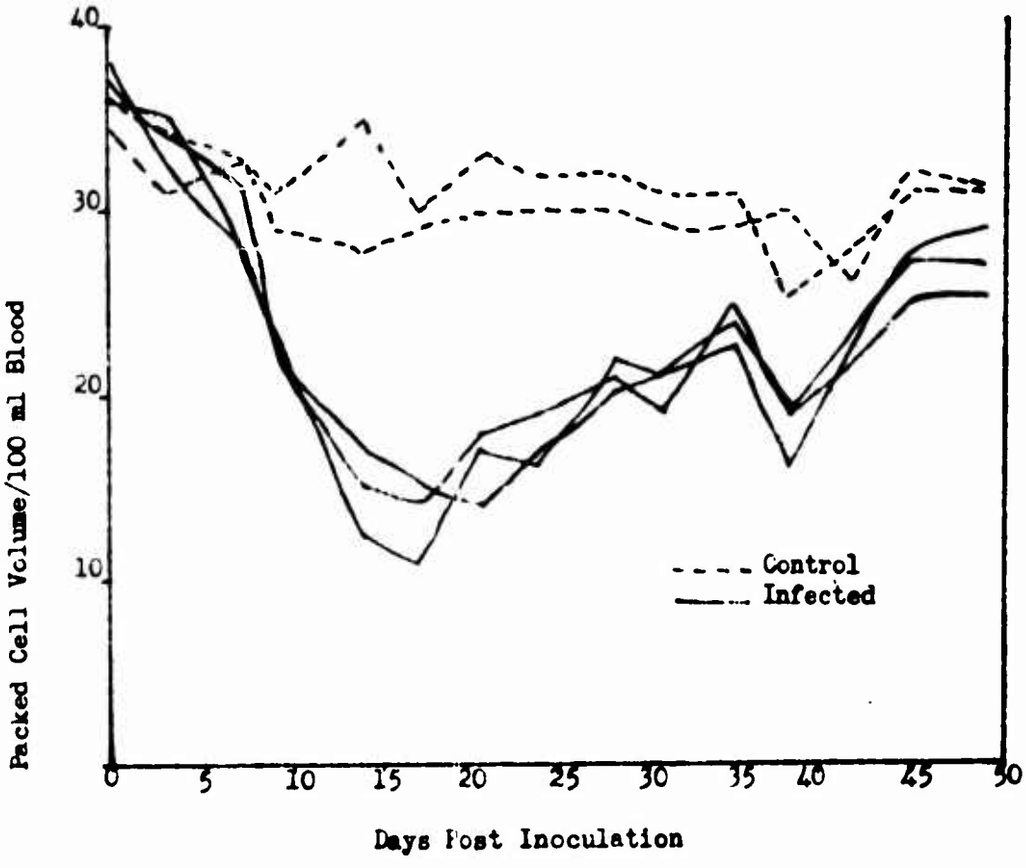


Fig. 6 Packed cell volume of newborn beagle pups following inoculation with Ehrlichia canis.

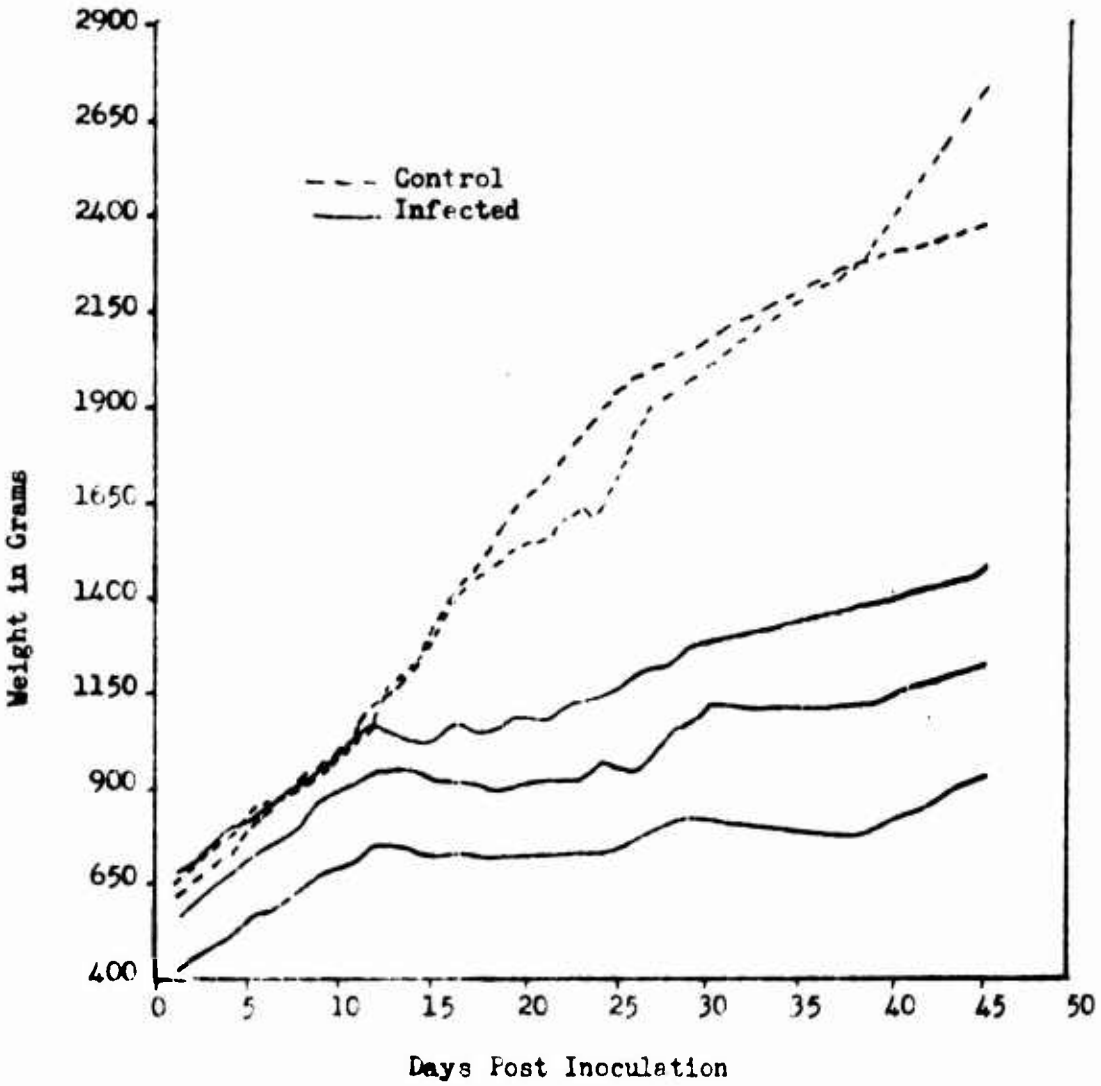


Fig. 7 Body weight of newborn beagle pups following inoculation with Ehrlichia canis.

3. Pathogenicity of Ehrlichia canis in Splenectomized Dogs.

A. Introduction

The hemorrhagic signs of TCP are associated with severe thrombocytopenia. In dogs with epistaxis the platelet counts are often below 5000/cu mm of blood and in many instances no platelets are observed. In dogs with thrombocytopenia purpura removal of the spleen will cause the platelet counts to return to normal. Splenectomy has been used in treating hemorrhage in dogs with TCP.[9] However controlled studies in splenectomized dogs have not been done.

A study was initiated to determine not only the pathogenicity of E. canis infection in dogs, but also the effect of splenectomy on the severe hematological changes which occur in the course of this disease.

B. Materials and Methods

Four German Shepherds and four Beagles were used. Two of the German Shepherds and two of the Beagles were splenectomized while the other two in each group were left intact. The baseline data were collected for 39 days at which time the normal WBC, RBC, PCV, ESR, thrombocyte count, hemoglobin, SGPT levels, BUN levels, and temperatures were determined for each dog (Table 2).

All eight dogs were inoculated with 5 cc of whole blood from a Beagle which was in the acute phase of TCP. Temperatures were taken daily for the first 9 days, twice daily for the next 10 days, and daily thereafter. The dogs were bled three times weekly and the following were determined: WBC, RBC, PCV, ESR, thrombocyte count, hemoglobin, SGPT and BUN levels.

Six days after splenectomy, the thrombocyte counts increased nearly 3-4 fold in the Beagles and 2 fold in the German Shepherds.

C. Results

Following inoculation the two intact German Shepherds and one of the splenectomized Beagles showed secondary infections. One intact Shepherd (#42) 14 days post inoculation developed an anal gland abscess that healed completely by the 37th day with no treatment. The other intact Shepherd (#43) developed a severe case of spirochetal trench mouth 25 days post inoculation and was treated with furacin and 2% iodine solutions. The mouth still showed some mild inflammation 115 days post inoculation. All dogs developed nasal and ocular discharge at 17 days which cleared up in all except Beagle 270, by 30 days post inoculation. Splenectomized Beagle 270 showed signs of an upper

Table 2. Average Baseline Values

DOG	TEMP (°F)	WT (lbs)	PCV	WBC ($X10^3$)	RBC ($X10^6$)	MCV	ESR (mm/hr)	HGB (gm/100ml)	THROMBOCYTES ($X10^3$)
40	102.4	44.0	38.0	16.3	5.8	65.9	4.4	12.7	563.3
41	102.3	51.1	41.3	19.3	6.0	69.7	6.5	13.6	393.8
42	102.3	55.4	39.3	16.2	5.8	68.4	6.7	13.1	308.1
43	102.2	54.3	46.7	14.9	6.7	68.7	.3	15.4	346.3
268	102.3	15.6	44.3	13.2	5.8	67.2	2.6	12.9	371.1
269	101.7	15.2	37.1	16.0	5.5	67.7	2.8	12.3	435.3
270	101.9	15.4	34.7	17.5	5.3	65.1	10.4	11.3	1044.3
271	102.1	15.5	35.5	12.6	5.3	66.2	5.1	11.8	725.0

PCV -- Packed cell volume
MCV -- Mean corpuscular volume (cubic microns)
ESR -- Erythrocyte sedimentation rate

respiratory infection at 15 days which subsided without treatment by 59 days post inoculation.

On the 11th day, all dogs were considered infected when the morula stage of Ehrlichia canis was found in monocytes from a capillary smear. The morulae were observed in monocytes up to 66 days post inoculation, but were not seen in capillary smears after day 66.

Two to 4 days post inoculation there was a sharp decrease in the platelet counts of the intact dogs, whereas the splenectomized dogs showed no decrease until the seventh day (Fig. 8-11). The low point in the splenectomized dogs during the acute phase occurred at the 11th day post inoculation with a sharp rise on 14-18 days. The intact group reached a low point on the 14-18th days post inoculation but have not demonstrated a sharp rise except for German Shepherd #43. German Shepherd 43 demonstrated a sharp rise at the 56th day and has maintained counts within the normal range.

All dogs showed a decrease in the WBC, RBC, and PCV but the degree of decrease was less in the splenectomized dogs. All showed nearly the same amount of increase in ESR although the rise was delayed in the splenectomized dogs by 12-14 days.

BUN levels remained normal in all dogs, whereas the SGPT levels showed slight elevations in all dogs beginning approximately at 11 days.

Two of the German Shepherds died. One was splenectomized and one was intact. The intact dog died 72 days post inoculation with epistaxis. Internal hemorrhages were marked in the intestine, mesenteric lymph nodes, kidney, spleen, liver, and lungs. The splenectomized German Shepherd died 102 days post inoculation showing epistaxis and internal hemorrhages. The intact dog lived 12 days with a platelet count of zero, the WBC dropped to 410 cells, and the PCV was 9%. The splenectomized dog had a platelet count of 5000 which had been under 10,000 for 14 days, the WBC was 3400 and the PCV was 30%. No deaths have occurred in the Beagles.

Further studies are in progress.

D. Discussion

At onset of infection, the splenectomized dogs all had higher platelet counts than the intact dogs. However, all dogs showed a sharp decrease in platelets. This decrease may be due entirely to an alteration in the production or release of platelets.

Soon after the initial sharp decline, the splenectomized dogs all demonstrated a sharp rise. The intact dogs remained low with one exception. In this case, the intact dog demonstrated a sharp rise on

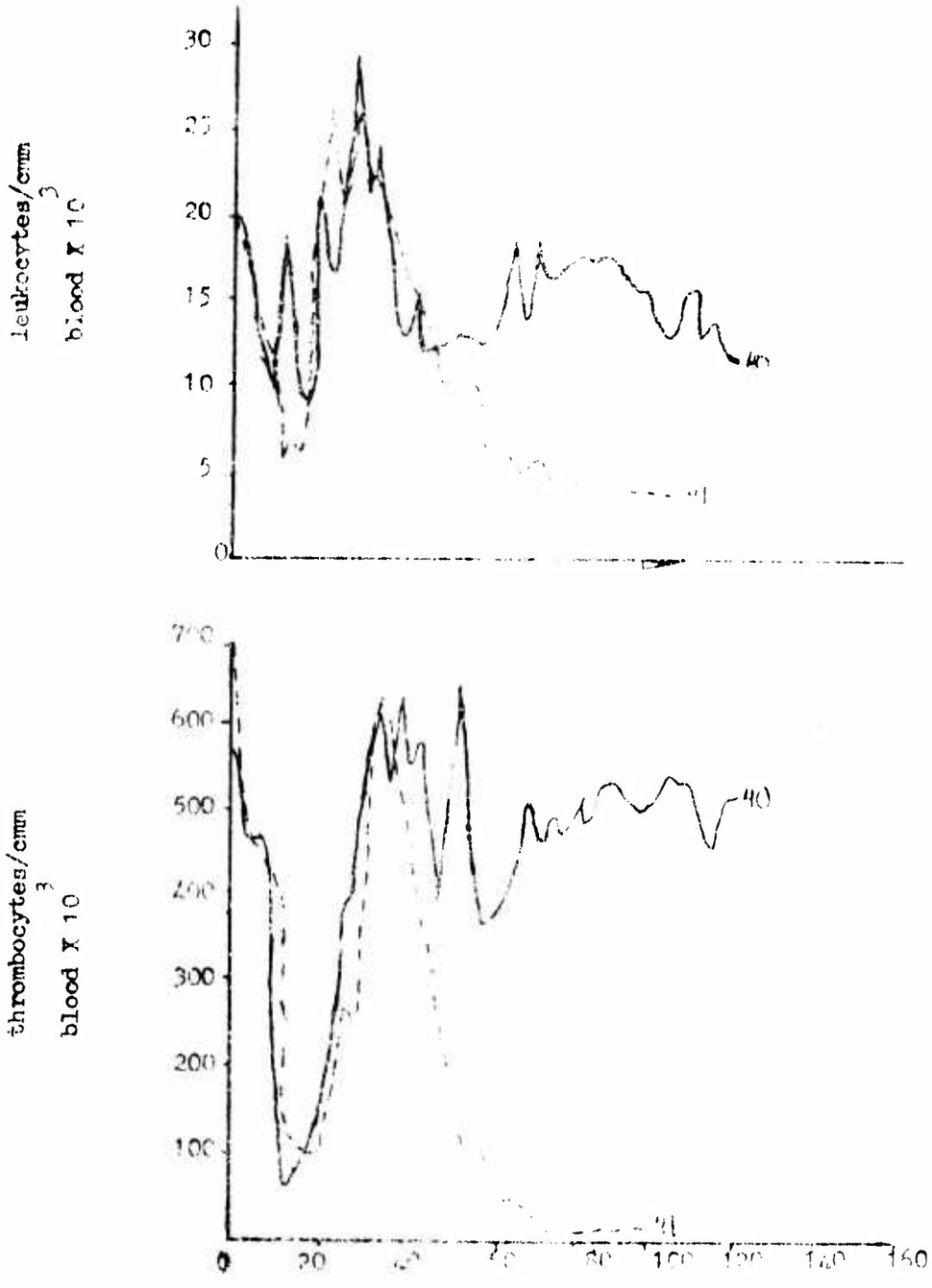


Fig. 8. Thrombocyte and leukocyte counts in splenec on red ocrnas Sheplerds inocula ed with Ehrlichia canis

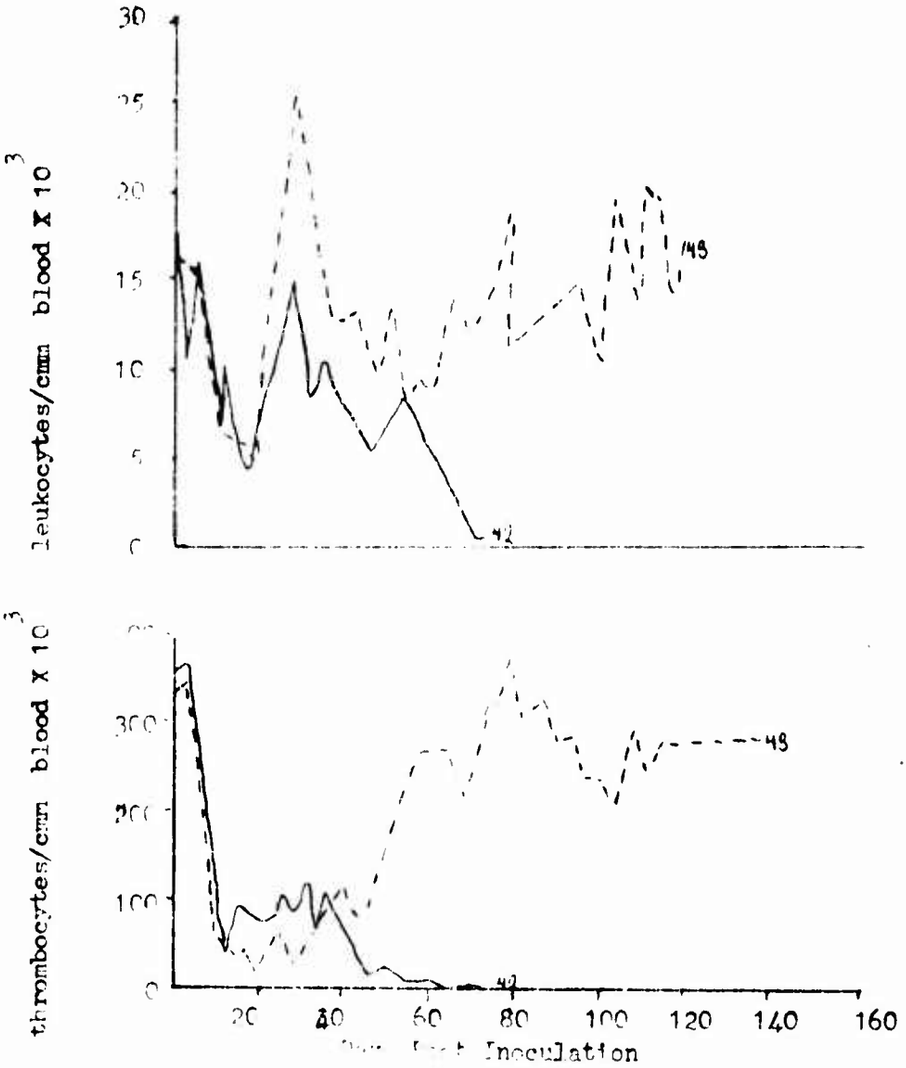


Fig. 9. Thrombocyte and leukocyte counts in intact German Shepherds inoculated with Ehrlichia canis.

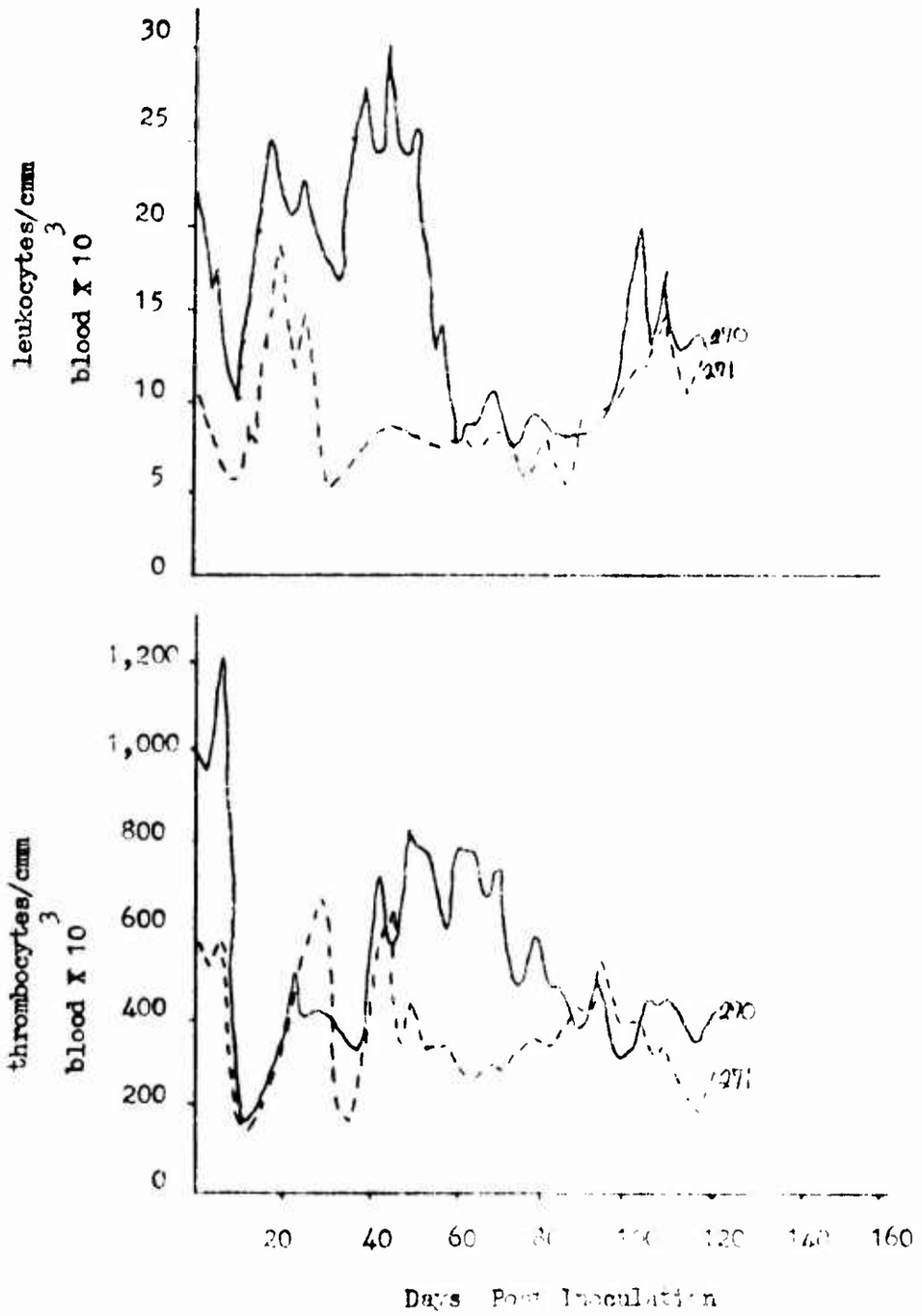


Fig. 10. Thrombocyte and leukocyte counts in splenectomized bovines inoculated with

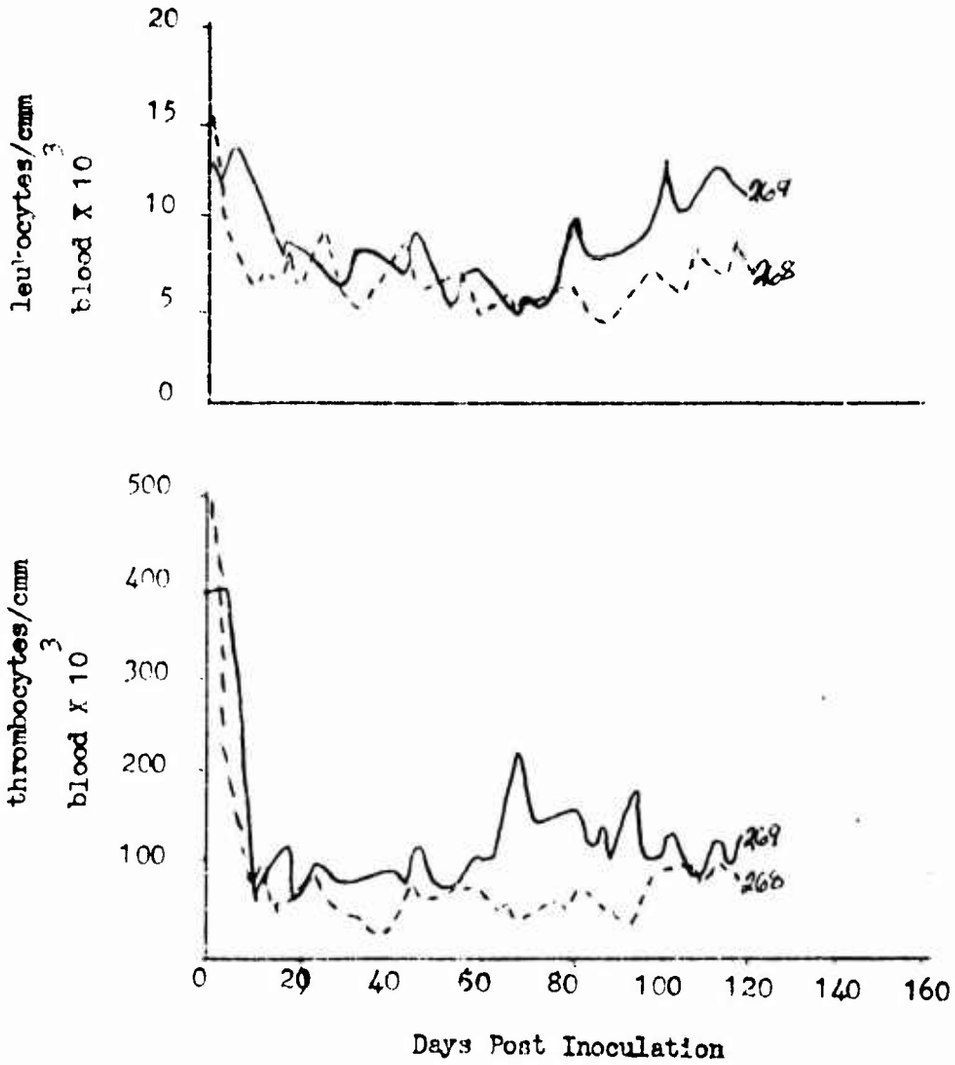


Fig. 11. Thrombocyte and leukocyte counts in intact beagles inoculated with Ehrlichia canis.

day 56 as compared to day 14 for the splenectomized dogs.

Due to the low WBC, anemia and inappetence, secondary infections are characteristic in TCP. The greatly elevated WBC demonstrate that the dogs still have the ability to respond to secondary invaders.

4. Electron Microscopic Examination of Ehrlichia canis, the Causative Agent of TCP

A. Introduction

Ehrlichia canis, the causative organism of TCP, is characterized by the intracytoplasmic inclusions which it produces in circulating monocytes, lymphocytes and rarely neutrophils. Blood films prepared from acutely ill dogs and stained with any of the Romanowsky stains usually contain mononuclear cells with single or multiple morula-like colonies which apparently consist of aggregates of smaller elementary bodies. Although these morulae can be demonstrated more readily in impression smears prepared from lung tissue, the true origin of these infected mononuclear cells and the site of multiplication have not been well established.

Ehrlichia canis has been included in the family Rickettsiaceae on the basis of its morphologic characteristics and arthropod transmission. The organism has not been adequately compared to other microorganisms within the group. All observations have been made by means of light microscopy which does not suffice in disclosing the true structure of the agent. Electron microscopy has been hampered by the fact that the organism has not been grown in any host system other than the dog and attempts to concentrate the organism from dog tissues have been unsuccessful. Early in the course of the disease small blood vessels in the lungs often contained numerous infected mononuclear cells. In the lumina of these same vessels organisms were found frequently in what appeared to be sloughing endothelial cells. It became apparent that the number of infected cells in these areas might permit electron microscopic studies.

B. Materials and Methods

A young adult Beagle dog was inoculated with 10 ml of whole blood collected in EDTA from an acutely ill dog. At 10 days post inoculation when early signs of infection were evident the dog was killed. The lungs were removed and perfused with glutaraldehyde. Multiple sections of the lungs were made and stained with hematoxylin and eosin to determine areas of high concentration of infected mononuclear cells. Opposing surfaces of fixed lung containing numerous infected cells were selected for electron microscopic examination. The tissue was processed by standard methods and after staining with 2% lead acetate was examined in a Siemens Elmiskop-1A electron microscope at 80 Kv.

C. Results

Elementary bodies of Ehrlichia canis were detected in endothelial cells. The round to ovoid particles ranged in size from 0.5 u to 1.5 u and were enclosed in a membrane-lined vacuole adjacent to the nucleus of the host cell. In some instances the vacuole membrane was not discernible. The number of particles in each vacuole ranged from 2 to 40; however, this is dependent on the manner in which the vacuole is sectioned. Each particle or elementary body was bound by two distinct membranes, each of which was tri-layered. The outer membrane, the cell wall, tended to be rippled while the underlying plasma membrane appeared to be fused to the underlying constituents of the particle. The inner structure of each particle consisted of dense and pale areas intermingled with each other. The more dense areas contained ribosome-like granules.

D. Discussion

This is the first reported electron microscopic observation of E. canis. The ultrastructure of the organism is similar to that which has been described for the rickettsiae and large particles of the Chlamydia group.[10]

It is apparent that the inclusions or morulae observed by light microscopy consist of a group of individual elementary bodies within a cytoplasmic vacuole. A cycle of development similar to that which has been described for the Chlamydia was not evident in this study; however, only a limited number of observations were made and all specimens were taken from a single dog, 10 days post inoculation. Determining the true cycle of development may await electron microscopic studies of infected cells grown in cell culture systems.

5. Prophylactic and Therapeutic Values of Tetracycline on TCP.

A. Introduction

Ehrlichia canis, the causative agent of TCP, has been identified in dogs in diverse geographical areas. In Southeast Asia as well as in other areas of potential military significance the utilization of military dogs is dependent on establishing means of testing and controlling this disease.

Antibiotics as well as sulfonamides have been used in attempts to treat Ehrlichia canis infection. Carmichael and Fiennes[11] reported that sulfapyridine was effective in treatment of the disease. Malherbe[12] found penicillin to be ineffective although he reported good results with sulfamethazine. Later Bool and Sutmoller[13] reported sulfapyridine and sulfamethazine to be effective during the early stages of the disease. Buckner and Ewing[14] found chloramphenicol and oxytetracycline

to be efficacious in treating the disease, and Ferrell[15] has suggested that drugs effective in treating the salmon poisoning complex are effective in treatment of canine ehrlichiosis. Most investigators, however, agree that relapses occur and infections persist following treatment.

Tetracycline was reported to be effective in treating early stages of TCP in military dogs in Southeast Asia.[16] In addition, preliminary laboratory studies showed that tetracycline, when administered prophylactically, will prevent infection with Ehrlichia canis. Based on these observations the efficacy of tetracycline as a chemotherapeutic and chemoprophylactic drug was examined in controlled laboratory studies.

B. Materials and Methods

The isolate of Ehrlichia canis used in this study was recovered from a German Shepherd dog with typical signs of TCP in Southeast Asia. The organism has been maintained by blood passage in laboratory Beagles. The inoculum for each dog consisted of 5 ml. of whole blood collected in EDTA from a laboratory Beagle acutely ill with the disease. The inoculum was determined to be free of Babesia and Hemobartonella by passage in splenectomized dogs.

Purebred Beagle and German Shepherd dogs of both sexes were used. Each dog received distemper, hepatitis, leptospirosis and rabies vaccinations according to the standard recommendations.

All experimental dogs were examined and rectal temperatures recorded daily. Three times per week 10 ml. of blood were collected in sealed vacuum tubes containing ethylene diamine tetra-acetic acid (EDTA) for clinical laboratory examination. All laboratory tests were conducted within 3 hours after specimens were collected. White and red blood cell counts were determined with an electronic cell counter. Thrombocyte counts were made with a phase contrast microscope. A standard micro-hematocrit centrifuge was used for all PCV determinations. Hemoglobin determinations were made by the cyanmethemoglobin method. The Wintrobe tube was used for determination of erythrocyte sedimentation rates. Urograph was employed for determination of BUN, and SGPT determinations were made by the modified Reitman-Frankel method. Capillary blood smears were stained using Giemsa and May-Grunwald Giemsa methods.

Tetracycline HCl in tablet form was used in all studies.

Experimental Design of Therapeutic Study

Thirteen adult laboratory Beagles and six six-month old German Shepherds were divided into 2 groups: a treated group consisting of 10 Beagle and 5 German Shepherd dogs; and an untreated group of 3 Beagle and 3 German Shepherd dogs. All dogs were inoculated intravenously with 5 ml.

of whole blood collected in EDTA from an acutely ill dog. Treatment was initiated after all dogs had a rectal temperature of 104°F or greater, and were showing altered hemograms. This was 14 days post inoculation in the Beagles and 13 days post inoculation in the German Shepherd. Each treated dog received 30 mg. per pound of body weight of tetracycline HCl daily for 14 days. The tetracycline was administered orally in a divided dose, half in the morning and half in the evening. Thirty days after the last day of treatment, 20 ml. of blood was collected in EDTA from each dog with the exception of 5 treated and 2 untreated Beagles. The blood was inoculated intravenously into susceptible laboratory Beagles to determine the infectivity of each dog. Ninety days after the last day of treatment blood was collected from the remaining dogs and subinoculated as above. All treated dogs cleared of the infection were reinoculated with the homologous strain of Ehrlichia canis to determine susceptibility to reinfection.

Experimental Design of Prophylactic Study

Ten young adult Beagle dogs were used in the study. Eight were administered tetracycline prophylactically and 2 dogs were held as untreated controls. The tetracycline was administered daily in a single oral dose at the rate of 3 mg. per pound of body weight. Treatment was initiated 1 week prior to challenge and was continued 1 month after challenge. All dogs were inoculated with 5 ml. of whole blood collected in EDTA from an acutely ill dog. Each dog was monitored carefully for 30 days after tetracycline was discontinued. Five ml. of blood were then collected in EDTA from each dog and were inoculated intravenously into susceptible laboratory Beagles. All protected dogs were reinoculated with the homologous strain of Ehrlichia canis.

C. Results

Therapeutic Study

All dogs developed signs of disease within 2 weeks following inoculation. The signs included pyrexia, anorexia, conjunctivitis, elevation of the erythrocyte sedimentation rate and a lowering of the red cell, white cell and thrombocyte counts (Fig. 12-15). Morulae of Ehrlichia canis were demonstrated in monocytes in capillary blood smears. Twenty-four hours after initiation of tetracycline therapy the rectal temperature of the treated dogs had returned to normal (Fig. 14 & 15). During the following two weeks the hemograms of the treated dogs returned to normal while the untreated dogs continued to show severe hematological signs (Fig. 12-15).

Following the acute stage of the disease the red cell and white cell counts of one untreated German Shepherd (Fig. 12) returned to normal. Approximately 75 days post inoculation a relapse, characterized by a reappearance of earlier signs, was noted in this dog. The dog died

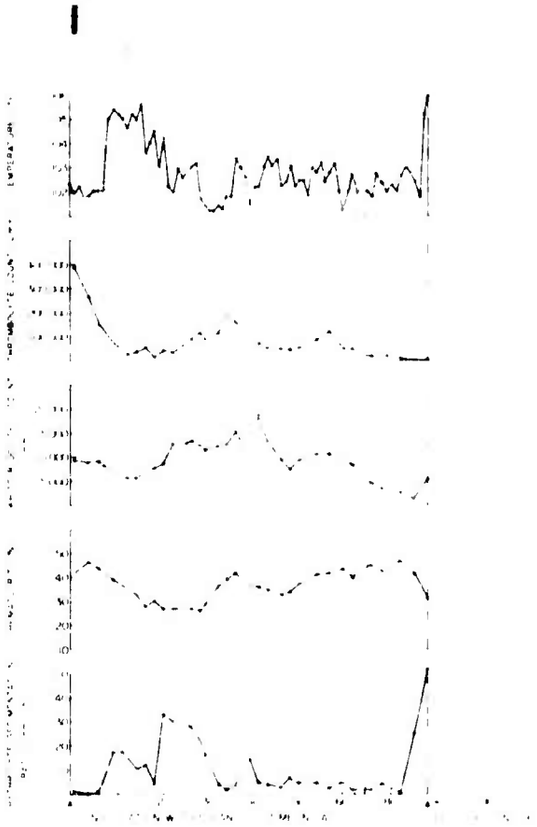


Fig. 12 Rectal temperature, thrombocyte count, leukocyte count, hematocrit, and erythrocyte sedimentation rate of an untreated 6-month old German Shepherd dog following inoculation with Ehrlichia canis. Dog died with epistaxis 88 days post inoculation.

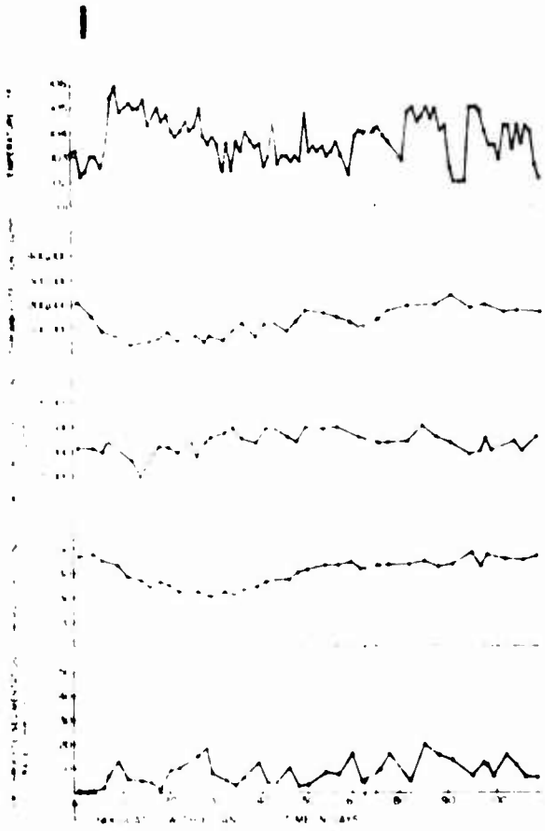


Fig. 13 Rectal temperature, thrombocyte count, leukocyte count, hematocrit, and erythrocyte sedimentation rate of an untreated 6-month old German Shepherd dog following intravenous inoculation with *Ehrlichie canis*.

Fig. 14 Rectal temperature, thrombocyte count, leukocyte count, hematocrit, and erythrocyte sedimentation rate of a treated 6-month old German Shepherd dog following intravenous inoculation with Ehrlichia canis. Administration of tetracycline was initiated 13 days post inoculation and continued for 14 days. Dog was reinoculated with Ehrlichia canis on day 86.

Fig. 15 Rectal temperature, thrombocyte count, leukocyte count, hematocrit, and erythrocyte sedimentation rate of a treated 6-month old German Shepherd dog following intravenous inoculation with Ehrlichia canis. Administration of tetracycline was initiated 13 days post inoculation and continued for 14 days. Dog was reinoculated with Ehrlichia canis on day 86.

with signs of hemorrhage 88 days post inoculation. Throughout the infection the thrombocyte count remained extremely low and at the time of death thrombocytes could not be detected in the blood. The red cell and white cell counts of a second untreated German Shepherd (Fig. 13) gradually returned to near normal over a 60 day period; however, during this time febrile episodes were common and the thrombocyte counts remained depressed.

With the exception of 2 Beagles, the treated dogs showed no evidence of a relapse following treatment (Fig. 14 & 15). The 2 Beagles relapsed approximately one month after treatment, and the relapses, as in the case of the untreated German Shepherd, were characterized by a reappearance of earlier signs. Ehrlichia canis was recovered from the blood of these 2 Beagles by inoculation of susceptible dogs. The organism was not recovered from the blood of the 13 treated dogs which showed no evidence of relapse. No differences were noted between the group of Beagles subinoculated at 30 days and the group subinoculated at 90 days. Each group contained 1 dog which remained infected.

All dogs cleared of the infection responded to reinoculation with the homologous strain of Ehrlichia canis (Fig. 14 & 15). The second infection was equally as severe as the first, and intracytoplasmic inclusions of Ehrlichia canis were easily demonstrated in capillary blood smears of all reinfected dogs.

Prophylactic Study

Tetracycline administered daily at the rate of 3 mg. per pound of body weight protected all dogs from infection with Ehrlichia canis, whereas untreated dogs developed typical signs of the disease. The organism could not be recovered from the blood of treated dogs 30 days after the tetracycline was discontinued, and the dogs remained fully susceptible to infection with the homologous strain of Ehrlichia canis.

D. Discussion

The results of these studies indicate that tetracycline HCl is an effective therapeutic agent for canine ehrlichiosis and that most of the treated dogs were cleared of the infection. The use of tetracycline HCl in treating Ehrlichia canis, which is classified in the order Rickettsiales, had not been reported until recently when Walker and co-workers[6] found tetracycline to be effective in treating early stages of TCP. However, these investigators made their observations on field cases, and followup studies were not done to determine if the dogs had been cleared of the infection.

The tetracycline antibiotics have been widely used in treating rickettsioses of man and have been recommended for canine rickettsiosis.[15,16] Buckner and Ewing[14,17] reported that dogs infected with Ehrlichia canis improve clinically when treated with chloramphenicol and oxytetracycline

but are not cleared of the infection. In the current studies 13 of 15 dogs were cleared of the infection when treated with tetracycline HCl for 14 days. The tetracycline antibiotics are rickettiostatic and not rickettsiocidal[18]. Therefore, the length of treatment may be very important.

Canine ehrlichiosis is frequently complicated by concurrent infections with babesia, Hemobartonella or Hepatozoon. The effect of these concurrent infections on the efficacy of various antibiotics in treatment of Ehrlichia canis is not known. Only uncomplicated infections were examined in this study.

Low levels of tetracycline HCl can be used effectively as a prophylactic for canine ehrlichiosis. When administered daily at the rate of 3 mg. per pound of body weight, tetracycline rendered all dogs refractory to infection. Since vaccines for ehrlichiosis are not available, the prophylactic use of an antibiotic may represent the only practical means of control in endemic areas. This may have application in the control of the disease in military working dogs where a highly susceptible dog population may be deployed in Ehrlichia endemic areas. This may also be a means of controlling the disease in pets in highly endemic areas. Neitz and Thomas[19] have reported that in areas where ehrlichiosis is endemic it is practically impossible to maintain dogs for any length of time. The continued daily administration of antibiotics is not unprecedented. Chlortetracycline has been given continuously to both domestic farm animals and human beings for long periods of time with no deleterious side effects noted.[20]

Dogs cleared of infection with Ehrlichia canis are fully susceptible to reinfection with the homologous strain. This is in contrast to the other well known rickettsial disease of dogs, salmon poisoning, in which treated and recovered dogs are resistant to reinfection.[15] Since infection with Ehrlichia canis confers no immunity to reinfection, there appears to be little hope for development of a killed vaccine. Veterinarians responsible for the care and treatment of dogs in Ehrlichia endemic areas should be aware that apparent relapses in treated dogs may be due to reinfections.

The Pathogenicity of Ehrlichia canis in Gray and Red Foxes

During the last few years, the widespread distribution of Ehrlichia canis infections has become evident. Due to recent isolations of Ehrlichia from clinical cases of F P in Florida and Texas, veterinarians in the region have become aware that Ehrlichia infections can be a serious problem in the United States, as well as other countries.

While basic pathogenicity and chemotherapy studies have been done, the epidemiology of this disease remains for the most part speculative. Host ranges and natural cycles of transmission have not been established.

Neitz and Thomas have suggested that wild dogs (Lycaon pictus) served as a reservoir for Ehrlichia in the Kruger National Park in Africa, and have reported that the jackal can be infected. The only evidence of reservoir hosts in the United States was supplied by Ewing and co-workers when they experimentally infected a coyote, Canis latrans.

The red fox, Vulpes fulves, and the gray fox, Urocyon cinereoargenteus are excellent candidates as potential reservoirs of Ehrlichia. Their range is spread throughout the United States, and population densities are high in areas where Ehrlichia have been identified. In addition, they are commonly parasitized by Rhipicephalus sanguineus, a tick which has recently been shown to transmit Ehrlichia canis. Studies have been initiated to determine the susceptibility of these two species to experimental infection with Ehrlichia canis.

In our laboratory, three red foxes and one gray fox have been experimentally inoculated with an isolate of Ehrlichia canis. Two foxes (one of each species) are being used as uninfected controls. Results of this study are not yet available.

7. Tick Transmission of Ehrlichia canis

A. Introduction

Donatien and Lestoquard in 1937 incriminated the common dog tick, Rhipicephalus sanguineus, as the vector of E. canis;[21] however, others have not been able to substantiate this.[22] Both tick transmission studies were done in enzootic areas of E. canis, and prior infection with or immunity to E. canis in the experimental dogs could not be excluded.

In this laboratory over 150 laboratory Beagles have been infected with E. canis, and all have been susceptible. Furthermore none of the Beagles used as controls in Ehrlichia studies have become infected. Using the laboratory's supply of susceptible, uninfected Beagles, studies to define the role of R. sanguineus as a vector of E. canis are being done.

We have previously reported the transtadial transmission of E. canis by R. sanguineus.[2] One strain of ticks, VN 6, was fed on an infected dog as nymphs and subsequently transmitted E. canis as adults. Another strain, VN 48, transmitted E. canis as nymphs and adults after feeding on an infected dog as larvae.

B. Materials and Methods

The establishment of a R. sanguineus colony, colony husbandry, and feeding methods were detailed in the previous annual report.

Two strains of R. sanguineus, VN 6 and VN 48, were maintained during the year, and one strain, VN 52, was eliminated from the colony. A fourth strain, RM 1, was added to the colony. This strain was obtained from the Rocky Mountain Laboratory, Hamilton, Montana, courtesy of Dr. W. Burgdorfer. The RM 1 strain was fed only on rabbits for several generations prior to acquisition by this laboratory.

Additional transstadial transmission studies with strains VN 6, VN 48, and RM 1 were done using methods outlined in last year's report.

Four attempts to prove transovarial transmission were done. From each of 4 different groups of adult ticks which had transmitted E. canis to normal dogs, 50 engorged females each were selected and allowed to oviposit. Larval offspring from the four ova pools were then fed separately on 4 normal dogs to determine if transovarial transmission occurred.

Using methods reported by Burgdorfer[23], hemolymph smears were made from adult VN 6 and RM 1 ticks fed on infected and uninfected dogs as larvae and nymphs. Smears were stained with Giemsa and examined for E. canis organisms.

C. Results

Tick strains VN 6 and VN 48 have been maintained in the laboratory through four complete life cycles (larva-nymph-adult-ova) and are currently in the fifth generation. The RM 1 strain has completed one life cycle.

Transstadial transmission of E. canis occurred in 4 groups of ticks. One group of VN 6 ticks fed as larvae on an infected dog transmitted E. canis as nymphs and adults, and a second group of VN 6 ticks fed as larvae and nymphs on infected dogs transmitted E. canis as adults. One group of VN 48 adults transmitted E. canis after feeding on an infected dog as nymphs. Adults of the RM 1 strain transmitted E. canis after the larval and nymphal stages had fed on infected dogs.

Transovarial transmission of E. canis did not occur with any of the 4 larval pools.

Comparative examination of a limited number of hemolymph smears taken from ticks selected from infected and uninfected tick pools did not reveal any cell inclusions or differences that would indicate E. canis infection in the cells.

D. Discussion

It is now clearly evident that R. sanguineus is capable of transstadial transmission of E. canis. Both strains of ticks acquired from dogs in

Vietnam, VN 6 and VN 43, have transmitted the disease from stage-to-stage after feeding on infected dogs as both larvae or nymphs. More significantly the RM 1 strain was also capable of transtadial transmission even though these ticks had been maintained on rabbits for several generations.

The ease with which transtadial transmission occurs leads one to wonder what other species of ticks can transmit E. canis. During the next year transmission experiments using the Brown Dog Tick, Dermacentor variabilis are planned.

The inability to achieve transovarial transmission of E. canis with the larval offspring from 4 groups of 30 engorged female ticks does not eliminate the possibility. The 30 female ticks selected represented only a small fraction of the total population of adult ticks from which they were derived. Because the percentage of infected ticks is unknown, only a small number of the many hundreds of ticks placed on a dog may transmit the disease. In this case it would, therefore, be easy to select only "clean" females for study and cull the small number of infected females from the colony.

During the next year we plan to feed small numbers of adult females (whose preadult stages have fed on infected dogs) on normal dogs. If transmission occurs, then all female ticks will be saved and their offspring fed on normal dogs.

8. In Vitro Study of Ehrlichia canis

A. Propagation of Infected Monocytes

(1) Procedure

Propagation of TCP agent in vitro has been achieved using the following monocyte culture technique first described by Nyindo et al.[24] An acutely affected dog with a high erythrocyte sedimentation rate was bled using sterile heparinized syringes for collection. The blood-filled syringes were positioned vertically in an incubator at 37 C for 30 to 45 minutes to permit sedimentation of erythrocytes. The overlying supernatant consisting of plasma, leukocytes, and platelets was transferred to culture flasks and incubated at 37 C. In 4 to 6 hours the supernatant was discarded and the developing monolayer was rinsed with Hanks balanced salt solution. The cell culture was maintained with Eagle's minimum essential medium with Earle's balanced salt solution containing 20% canine serum plus L-glutamine.

Microscopic examination of cover-slip cultures stained by the May-Grunwald Giemsa method has been used for visualization of the agent in cell culture. Elementary bodies and morulae characteristic of E. canis may be identified in an occasional cell by 24 to 48 hours. The percent

of infected cell gradually increases and by 8 to 12 days E. canis may be found in various developmental stages in cells throughout the monolayer.

(2) Comparison of Selected Media for Growth of E. canis

Selected media were tested for ability to support growth of monocytes and development of E. canis. The reference medium was Eagle's Minimum Essential Medium (EMEM) with Earle's Balanced Salt Solution supplemented with 20% canine serum and 1% L-glutamine. The media tested and the results are summarized in Table 3. None of the test media was superior to the reference medium for maintaining monocyte cultures or development of E. canis.

(3) Effect of Selected Antibiotics on Development of E. canis

Studies of the growth of E. canis in monocyte cultures were often compromised by bacterial contamination. To overcome this difficulty selected antibiotics, either alone or in combination, were incorporated in the media and their effect on the development of E. canis determined (Table 4).

At the concentration of antibiotic tested, only aureomycin completely suppressed the development of E. canis in monocyte cultures. Although morulae of E. canis were evident in cultures containing the other antibiotics there was some reduction in percentage of infected cells. Further experiments are in progress to quantitate the effect of antibiotics on this organism. The use of monocyte culture appears to be a practical, inexpensive means of screening potential anti-ehrlichia agents. It should be noted that aureomycin, an agent highly effective against rickettsia-psittacosis organisms was also effective in this system. Furthermore, studies of anti-ehrlichia agents may provide clues on the taxonomic status of the organism.

B. Propagation of Normal Monocytes

Monocyte cultures were prepared from normal dogs by two modifications of the previously described technique. First, two hours prior to collection of blood the level of circulating monocytes was increased by the oral administration of diethylcarbamazine (Caricide^{R}) at the rate of 30 mg per pound. Second, to produce rapid sedimentation of erythrocytes the blood was mixed with a solution of 3% dextran in 0.85% sodium chloride in a ratio of 2:1, respectively. The technique is completed as previously described and a confluent monolayer of monocytes are formed in 5 to 7 days.

Table 3. Comparison of Selected Media for Growth of E. canis

Media*	Morulae of E. canis
Eagles MEM	+
Eagles MEM + Glucose	+
McCoy's 5A	-
RPMI (1640)	+
Medium 199	+

* All media supplemented with 20% canine serum and 1% glutamine as required.

Table 4. The Effect of Selected Antibiotics on the Development of E. canis

Antibiotic (dose)	Morulae of E. canis
Aureomycin (12.5 ug/ml)	0
Streptomycin (25 ug/ml)	+
Vancomycin (50 ug/ml)	+
Fungizone (0.5 ug/ml)	+
Penicillin (25 ug/ml), Streptomycin (25 ug/ml) Fungizone (0.6 ug/ml)	+
Vancomycin (50 ug/ml), Fungizone (0.5 ug/ml) Streptomycin (25 ug/ml)	+

C. Infectivity of E. canis Propagated in Monocyte Cultures

Non-infected monocyte cultures were infected by an inoculum of infected cell culture suspension containing a high concentration of infectious agent. Elementary bodies and morulae could be demonstrated in these cultures in 10 to 12 days. The percentage of cells infected is variable as evidenced by cytopathic effect (CPE) and formation of morulae within the cytoplasm. A satisfactory method of titrating E. canis has not been developed, but apparently CPE is not directly related to E. canis concentration in the inoculum. After 3 passages in monocyte cultures over a 3-month period the agent produced typical signs when inoculated intravenously into dogs. Reisolation of monocyte culture was successful.

D. Discussion

Cultivation of E. canis in an in vitro system has provided the means whereby its basic properties can be investigated. Perhaps of more practical importance this system may provide a concentrated antigen for subsequent development of a diagnostic serological test. Taxonomic classification of TCP has previously been based upon limited factual information. A more detailed study of its physical and biochemical characteristics will provide the basis for a more precise classification.

9. Comparative Study of Ehrlichia canis and Neorickettsia helminthoeca

A. Introduction

Presently most texts list Ehrlichia canis in the Order Rickettsiales, Family Rickettsiaceae. The only other rickettsiae commonly recognized as a pathogen for the dog are the agents classified under the heading salmon (poisoning) disease complex. The infectious agent usually incriminated as the cause of salmon disease has been given the generic name Neorickettsia helminthoeca. Because of its apparent similarities to TCP the salmon poisoning agent (SPA) was acquired for comparative purposes.

B. Agent and Disease

Six small trout were obtained from the Washington State Fish and Wildlife Hatchery, Cathlamet, Washington. These trout were triturated and fed in equal amounts to each of two Beagles. A fever developed in both dogs in 5 days and was followed by typical signs described for salmon disease. Both dogs died 13 days following infection. Prior to death blood was collected from these 2 dogs and inoculated intravenously into laboratory Beagles. When these dogs became acutely ill, they were killed and the spleen and mesenteric lymph nodes removed. Twenty percent tissue suspensions of spleen and lymph nodes were made in Snyder's

solution. The suspensions were rapidly frozen in a dry ice-alcohol bath and stored at -90C. The infectivity of this suspension has been retained as confirmed by its ability to produce typical disease when inoculated into laboratory Beagles.

C. Propagation of Neorickettsia helminthoeca in Monocyte Cultures

From an acutely ill dog an infectious agent with morphological characteristics of a rickettsia was isolated using the same technique described above for E. canis. In some cells the organism appeared to be confined to vacuoles within the cytoplasm, whereas in other cells the organism was dispersed throughout the cytoplasm. In contrast to monocyte cultures of E. canis in which little CPE can be noted, the SPA produces characteristic cellular changes which can be readily observed with light microscopy. Infected cell suspensions when inoculated intravenously into laboratory dogs produced typical signs of salmon disease.

D. Discussion

This study has demonstrated the monocyte culture technique can be used for the isolation and propagation of 2 canine rickettsial agents. It is possible that other rickettsial agents can be cultured in a similar in vitro system.

II. Babesiosis

1. Experimental Babesia gibsoni Infections in Laboratory Beagles

A. Introduction

Babesia gibsoni is a small babesia capable of infecting a number of species in the family Canidae. Naturally occurring infections in the domestic dog have been reported from India, Ceylon, Malaysia, and Korea.

Studies to define B. gibsoni infection in dogs have, with one exception, been done in enzootic areas of the parasite either in clinical situations or with locally procured experimental dogs. Concurrent disease processes and prior exposure to babesia parasites could not be ruled out. Also detailed blood and urine studies were not done.

The purpose of the following report is to define the signs, clinical pathology, and pathology of experimental b. gibsoni infections in laboratory Beagles.

B. Materials and Methods

The strain of *P. vivax* used in the study has been maintained in laboratory beagles at the Walter Reed Army Institute of Research since August 1965. The strain was originally obtained from a Gurl carrier that contracted [illegible] in Malaysia.

Thirteen, one-year-old, [illegible] beagles were used in the study. All dogs [illegible] were inoculated intravenously with 1 ml of blood containing 152 parasites/1000 red blood cells (RBC's). A 5th dog was used as infected with blood from an inapparent [illegible] carrier. The parasite served as the donor. The remaining 2 dogs [illegible] were used as normal controls.

The dogs were examined and temperatures recorded daily. Total and differential white blood cell counts, RBC count, reticulocyte counts, hematocrit [illegible], hemoglobin determinations, and parasites/1000 RBC's were made twice weekly except during periods when they were done daily. Serum glutamic pyruvic transaminase (SGPT), blood urea nitrogen (BUN), [illegible] total protein, and urinalyses determinations were made twice weekly. Blood values and serum chemistries were done twice a week for 2 weeks, prior to infecting the dogs.

Two infected dogs were euthanized at the peak of disease, 20 days post infection (PI) and examined for pathological lesions. The remaining 7 dogs were allowed to [illegible] their normal state before being euthanized and examined for pathological lesions on day 90 PI.

Blood samples were collected in sealed vacuum tubes containing sodium ethylenediamine tetraacetic acid (EDTA) and examined within 4 hours. An electronic cell counter was used to compute RBC and total WBC counts, mean corpuscular volumes (MCV), and packed cell volumes (PCV). Reticulocyte counts were made from new methylene blue, supravitally stained, thin blood films. Parasites/1000 RBC's and differential WBC counts were determined from Giemsa-stained thin blood films.

Total bilirubin, SGPT, and hemoglobin concentrations were done spectrophotometrically. Paper chromatography of serum was used to measure BUN. Urine was collected by catheterization and analysis made with dip stick reagent strips.

C. Results

Control dogs: No abnormal values were noted during the 90-day study.

Infected dogs: Parasitemia. *P. vivax* parasites were present in the RBC's of the 7 infected dogs within 5 days PI. The parasitemias rapidly increased and attained their highest counts of 57-274.

parasites [illegible]. The parasitemias of individual dogs occasionally decreased slightly for 1 or 2 days during the acute phase (day 7-26 PI) only to increase again. A rapid [illegible] occurred between the 21st and 24th day [illegible]. By day 30 PI all dogs had detectable [illegible] (films range 1-4/1000 RBC's).

[illegible] By day 21 PI all infected dogs demonstrated [illegible] which were sustained over the [illegible]. The lowest PCV's recorded for the 9 [illegible] were [illegible]. Recovery was a very slow, steady, [illegible]. Changes in RBC counts paralleled those of the PCV's. The lowest recorded counts ranged from [illegible] million/cu. mm.

[illegible] cubic microns with [illegible] (range 200 m). Limits of test [illegible] day 27-90 PI) stages of [illegible] poikilocytosis, anisocytosis, macrocytosis, [illegible]. The reticulocyte count increased [illegible] peak of 30% was ended [illegible]. During acute stages counts ranged from 3-28 reticulocytes/100 RBC's with an average of 7/100 RBC's. Numerous nucleated RBC's were [illegible].

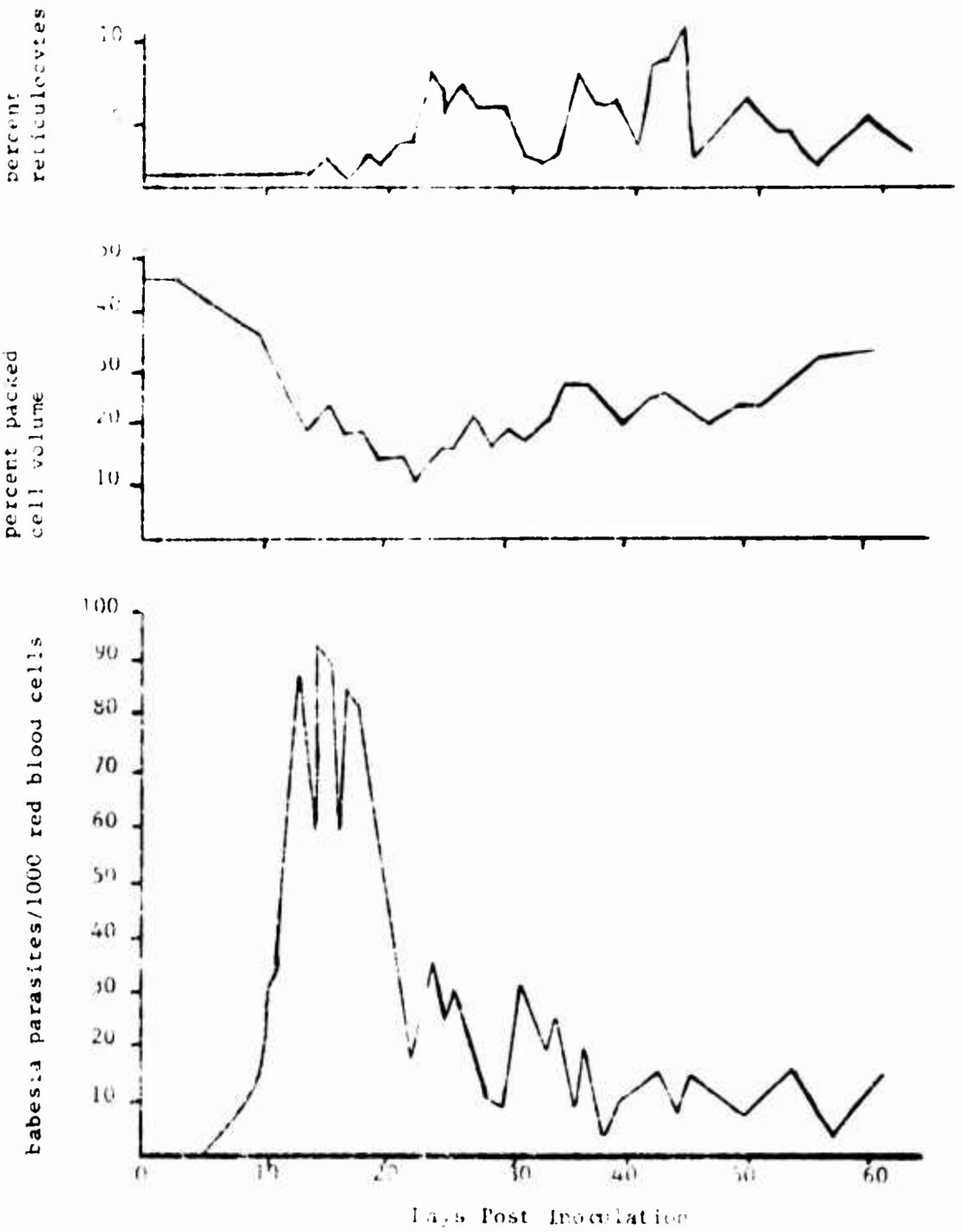
White Blood Cells. The total [illegible] remained within the normal limits. A slight [illegible] was observed on the differential count.

Serum Constituents. The BUN's [illegible] exceed 30 mg./100 ml. in any of the infected dogs. The cell's were [illegible] normal ranges (10-50 units/100 mls.) with the exception of [illegible] 70 units/100 ml. value from a dog which subsequently had all normal values. Six of the infected dogs had very slight transient rises in serum bilirubin (0.8-1.6 mg./100 ml.) which lasted from [illegible] days [illegible] normal.

Urine. The [illegible] the acute stages of disease from a fine yellow [illegible] which would stain the dog's hair and cage. [illegible] 3-4+ dye was observed in the 6 dogs which had elevated serum bilirubin. Urine pH, protein, glucose, and sediment were normal.

Clinical Signs. During the period of severe anemia the dogs were anorexic and listless. They were easily tired and displayed varying degrees of dyspnea when exercised. The mucous membranes were blanched. Splenomegaly was observed in all dogs. Hepatomegaly was not detected. A jugular pulse was present in [illegible] 9 acutely ill dogs. No signs of [illegible] were observed, and although food intake was reduced [illegible] the dogs continued to eat and drink even

Fig. 16 Comparison of the mean percent reticulocytes, mean percent packed cell volumes, and mean babesia parasites/1000 red blood cells of 9 laboratory Beagles infected with Babesia gibsoni.



when acutely ill. Weight loss was evident in all infected dogs.

Rectal temperatures of individual dogs fluctuated greatly during the period of [illegible]. The average rectal temperature from d1 to 12 [illegible] an individual high of 104.6 F.

Pathology. [illegible] were limited to the infected dogs. [illegible] and some spleens were [illegible] loss. The splenic contours of the infected [illegible] bulged prominently. Splenic [illegible] to the two animals sacrificed at [illegible] elements very prominent [illegible] numbers of immature reticulo-endothelial [illegible] white pulp. Splenic changes [illegible]-day experiment were not as [illegible] was recognized in all sections; [illegible] disrupted by the hematopoietic [illegible].

Hepatic stimuli of the two animals sacrificed during acute disease contained [illegible] myeloid and erythroid cells. In some areas [illegible] sinusoids due to proliferating cells. [illegible] centrolobular. An occasional focal disseminated [illegible] lying 2 to 5 hepatocytes was also seen. [illegible] was seen in these areas. Hepatic changes were minimal in the other dogs. An occasional cluster of RE cells marked the site of a small necrotic focus.

D. Bloodborne

The appearance of parasites in the blood within 5 days PI was not unexpected. The incubation or prepatent periods for experimental *B. gibsoni* infections have varied greatly depending on the route of inoculation and number of parasites.[26] Incubation times have ranged from 2 to 52 days. Transmission studies employing the main vector of *B. gibsoni*, the tick *Haemaphysalis bispinosa*, have resulted in prepatent periods of 12 to 42 days. These tests probably more closely approximate natural disease.

Anemia was the most striking clinical signs of infection. Its onset, however, developed gradually over a three-week period. By the time overt disease was evident in the infected dogs peak parasitemias had already been reached and the recovery phase began within 7-10 days. In natural infections of *B. gibsoni* where parasite counts were presented similar findings were reported.[27,28]

The recovery from the anemia was slow. Although the dogs appeared clinically normal when the study was terminated at day 90 PI, the mean PCV was 11% below the preinfection level. Occasionally parasitic exacerbation occurred in individual dogs during recovery with a concomitant drop in PCV's and RBC counts.

A very active hematopoietic response during the acute and recovery phases of the infection was evident from thin blood films and histopathological examinations. Morphologically the anemia was a macrocytic, hypochromic one typical of hemolytic disease. Reticulocytes began to appear in the peripheral blood of the infected dogs on day 12-18 PI and persisted to the termination of the experiment. If 4 days from the time of stimulation to the appearance of reticulocytes in the circulation are allowed, hematopoietic stimulation occurred in individual dogs when the erythrocyte count exceeded approximately 2 million RBC's per cmm. or a PCV of 20%.

A persistence of *B. gibsoni* parasitemia for over 60 days following acute parasitemia is, as far as is known, probably responsible for the anemic dogs' slow recoveries. Immunity in *B. gibsoni* infection results from the establishment of a low-grade parasitemia, and persistence of over 38 months has been reported.[4]

No hepatic or renal impairment were evidenced by the SGPT, bilirubin, and BUN tests. The very slight rises in serum bilirubins in 6 dogs occurred during acute disease and can be attributed to the hemolysis of RBC's. In an extensive study by others[25] of the influence of *B. gibsoni* on the serum enzymes of dogs, a fatal experimental infection demonstrated liver involvement as measured by the SGPT, serum glutamate oxaloacetate transaminase, and sorbitol dehydrogenase test. However, in the same study another fatally infected dog and two dogs who recovered spontaneously from infection had no evidence of liver impairment. Also in the same study, damage to the spleens of infected dogs was evidenced by a marked increase in lactate dehydrogenase.

The darkly colored urine seen in the infected dogs has been reported by others.[29] Some correlation between severity of anemia and the tendency for the urine to be dark was observed. No correlation between the presence of urobilinogen and color was noted, however. In marked contrast to severe *Babesia canis* infections, hemoglobinuria was not detected. We attribute the dark urine color to bile pigments which could not be detected with our test system.

Splenomegaly in infected dogs was a consistent finding in our study. Enlargement of the spleen in hemoprotozoon diseases is a normal physiological response due to increased activity in removing parasites from RBC's. Hepatomegaly has been reported as being present in 70% of *B. gibsoni* infections;[30] however, we were unable to detect enlarged livers in any of our infected dogs.

Clinical signs other than anemia, splenomegaly, and dark colored urine were vague. Anorexia, listlessness, fatigue and blanching of mucous membranes can all be attributed to anemia. Although low-grade [illegible] of approximately 104 F were observed during acute infection, [illegible] were frequently within the normal [illegible] been able to correlate change in body temperature with change in parasite numbers (Fig. 17). Icterus was not observed in any of the dogs.

[illegible] genesis of many babesia infections of animals is attributed [illegible] plugging of capillaries of organs with parasitized cells, cell debris, and free parasites. This clogging causes anoxia, the accumulation of [illegible] and hemorrhage of capillaries and tissue. This mechanism has been reported for B. canis infections.

[illegible] of B. gibsoni infection does not appear to involve [illegible] syndrome. Clinical disease and pathology in [illegible] must be related strictly to a progressive [illegible] and the destruction of parasitized RbC's in the reticulo-endothelial system.

5. [illegible] Babesia gibsoni Infections in Laboratory Beagles

A. Introduction

A study to define the signs, clinical pathology, and pathology of B. gibsoni infections in laboratory Beagles has been reported (see current Annual Reports). In this study dogs were inoculated with infected blood and allowed to progress through the acute disease and then recover. At the peak of infection, all dogs were severely depressed and anemic. Although no deaths occurred, dogs with untreated infections took several months to recover.

Traditionally, the treatment of B. gibsoni has been with the organic arsenical compounds. Treatment studies employing these drugs were usually undertaken in clinical situations in enzootic areas and detailed blood studies were not presented. Proven parasitic cures were never achieved. Recently two chemotherapeutic agents, diminazene aceturate and phenamidine isethionate, have been reported to be effective in reducing B. gibsoni parasitemias in controlled laboratory studies.[25,31] Neither drug was capable of producing sterile parasitic cures, however. In recent years several new chemotherapeutic agents have been made available for the treatment of babesiosis, but have as of yet been untried in canine B. gibsoni infections.

B. Materials and Methods

Twenty mature Beagles were used. The dogs were divided into 5 groups of 4 dogs. Groups of 4 dogs each were used to test the 4 drugs listed

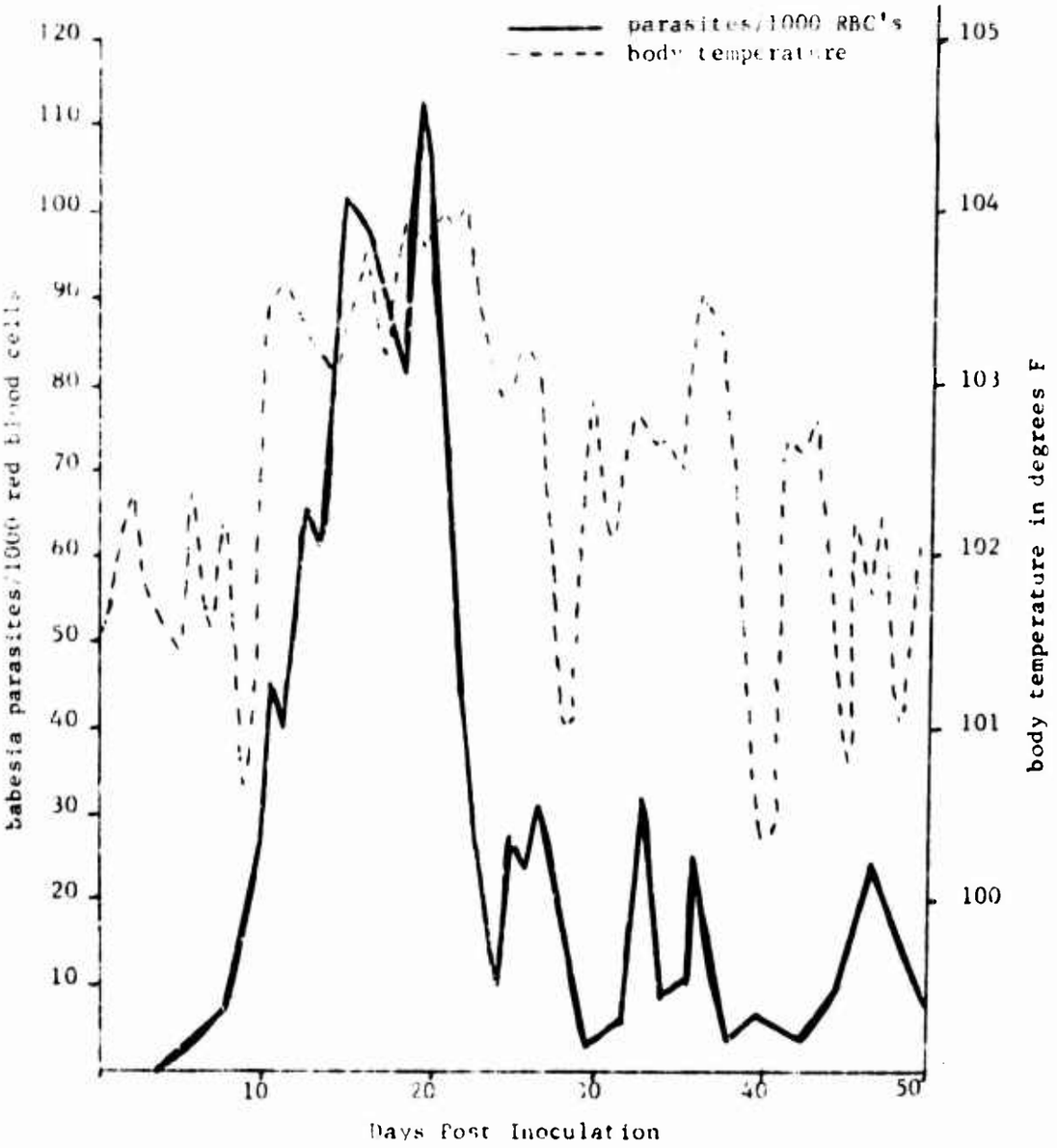


Fig. 17 Comparison of daily fluctuations in body temperature and parasites/1000 red blood cells of one laboratory Beagle infected with Babesia gibsoni.

below and one group of 4 dogs served as controls.

Baseline data were collected on all dogs for 2 weeks prior to infection. Each dog was examined and rectal temperature recorded daily. Blood and urine analysis was made according to the following schedule:

a. Daily

(1) Hematocrit
(2) Reticulocyte count
(3) Parasites per 1000 RBC's

b. Three times a week

(1) SGPT
(2) BUN
(3) Bilirubin
(4) Total WBC count
(5) Total RBC count
(6) Hemoglobin

c. Once weekly

(1) Urine pH
(2) Urobilinogen
(3) Urine protein
(4) Urine occult blood
(5) Differential WBC count

One splenectomized Beagle was inoculated intravenously with 5 ml. of infected blood from a carrier dog. After a parasitemia of 181 parasites/1000 RBC's was reached, blood was drawn from the splenectomized dog and 5 ml. of the infected blood inoculated into each of the experimental dogs.

The following drugs and dosages were tested for their effectiveness in treating Babesia gibsoni infections:

a. Berenil (Diminazene aceturate) Farbwerke Hoechst Ag.: 3 mg./lb. body weight administered twice, 48 hours apart.

b. Diapron (Amicarbalide) May and Baker Ltd.: 3 mg./lb. body weight administered twice, 24 hours apart.

c. Ganaseg (Diaceturate 4,4'-Diazoaminodibenzamidine) E. R. Squibb & Sons: 4.5 mg./lb. body weight twice, 24 hours apart.

d. Phenamidine Isethionate, May & Baker, Ltd.: 7.5 mg./lb. body weight twice, 24 hours apart.

Drugs were administered during acute disease when the parasitemias were 75 parasites or more/1000 RBC.

C. Results

Final analysis of the data has not been completed. However, preliminary results indicate the four drugs tested are effective in reducing B. gibsoni parasitemias. Diminazene aceturate and phenamidine isethionate produced the most dramatic reductions in parasitemias. Exacerbations of parasite numbers with concomitant reductions in packed cell volumes and red blood cell counts occurred 7 to 14 days following the decreases of parasitemia due to treatment. However, peak parasitemias observed during the exacerbations were of a much smaller magnitude than the parasitemias of the acute, pretreatment stages. Ganaseg was toxic at the dosage used in the study, and one dog died as a result of treatment with it.

D. Discussion

Diminazene aceturate and phenamidine isethionate were the most effective chemotherapeutic agents tested. Both drugs should be useful in controlling high parasitemias of acute disease until premunity can be established. Neither drug, however, was capable of producing "sterile" or parasitic cures.

The exacerbations of parasitemias following treatment indicate that treated B. gibsoni infections should be followed with periodic blood examinations to ensure that severe relapses do not occur.

III. Summary and Conclusions

1. Pathogenicity study on Ehrlichia canis, the causative agent of TCP, conducted in intact and splenectomized German Shepherd dogs. Although the response of individual dogs to infection with E. canis varied, all dogs exhibited fever, thrombocytopenia, anemia, leukopenia, and elevated erythrocyte sedimentation rates. Clinical signs consisted of conjunctivitis, ocular and nasal discharge, anorexia, weight loss, and hemorrhage. Mortality was 63 percent in the mature German Shepherds and 100 per cent in the immature German Shepherds. Splenectomized dogs showed the same clinical and hematological changes as intact controls; however, the thrombocytopenia was less severe in the early stages.

2. Experimental E. canis infections in newborn Beagle pups were characterized by nasal and ocular discharges, clinical signs of anemia, and poor weight gain. All infected pups survived the disease.

3. Electron microscopic studies were made on lung tissue of dogs acutely affected with TCP. These studies revealed the presence of morulae of E. canis in endothelial cells. The morulae consisted of groups of round and oval elementary bodies, 0.5 - 1.5 μ in diameter,

within a cytoplasmic vacuole. The elementary bodies had the same general features of rickettsiae and large particles of the Chlamydia group. A trilaminar cell wall and a plasma membrane were evident in all elementary bodies.

4. Tetracycline HCl was evaluated as a therapeutic and prophylactic agent for Ehrlichia canis infections in Beagle and German Shepherd dogs. Oral administration of tetracycline at the rate of 30 mg. per pound of body weight for 14 days resulted in remission of clinical signs; however, 2 of fifteen dogs remained carriers. All dogs cleared of the infection were reinoculated with the homologous strain of Ehrlichia canis and infections were reestablished, thus demonstrating the lack of resistance to reinfection. Tetracycline was also shown to be an effective prophylactic agent when administered daily at the rate of 3 mg. per pound of body weight.

5. Preadult Rhipicephalus sanguineus ticks fed on dogs [illegible] acute E. canis infections are subsequently capable of transmitting the disease transtadially. Sufficient evidence to prove or disprove transovarial transmission of E. canis by R. sanguineus has been gathered.

6. Primary canine monocyte cultures were used for in vitro propagation of two rickettsial agents infectious for the dog, E. canis and N. helminthoeca. The study suggests that it would be possible to propagate other members of the Order Rickettsiales in a similar manner. From a practical standpoint the monocyte culture technique offers a potential source of concentrated antigen which is required to develop a diagnostic test for TCP. The technique also offers a means whereby the basic physical and biochemical properties of E. canis can be studied. Such studies would provide the basis for a more precise taxonomic classification of E. canis.

7. Clinical disease and pathology of B. gibsoni infected dogs are the result of a slow progressive anemia caused by the destruction of parasitized RBC's in the reticulo-endothelial system. The disease is clinically characterized by a macrocytic, hypochromic anemia; splenomegaly; darkly colored urine; and a variable low-grade fever. The only outstanding histopathological feature of the disease is the extramedullary hematopoiesis found in the spleen. The drugs diminazene aceturate and phenamidine isethionate appear to be the drugs of choice for treating clinical B. gibsoni infection; however, neither drug is capable of producing radical, parasitic cures.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 183, Diseases of military animals in Southeast Asia

Literature Cited.

References:

1. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1968 - 30 June 1969.

2. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1969 - 30 June 1970.

3. Wilkins, J.H., Bowden, R.S.T., and Wilkinson, G.T.: A new canine disease syndrome. Veterinary Record 81:57-58, 1967.

4. MacVean, D.W.: That new dog disease. Malaysian Kennel Review 66, 1968.

5. Spence, J.B., Giam, C.H., and Theis, J.H.: A new canine disease syndrome. Veterinary Record 81:328-329, 1967.

6. Walker, J.S., Rundquist, J.D., Taylor, R., Wilson, B.L., Andrews, M.R., Barck, J., Hogge, A.L., Jr., Huxsoll, D.L., Hildebrandt, P.K., and Nims, R.M.: Clinical and clinicopathologic findings in tropical canine pancytopenia. J.A.V.M.A. 157: 43-55, 1970.

7. Huxsoll, D.L., Hildebrandt, P.K., Nims, R.M., Ferguson, J.A., and Walker, J.S.: Ehrlichia canis - the causative agent of a hemorrhagic disease of dogs? Veterinary Record 85:587, 1969.

8. Huxsoll, D.L., Hildebrandt, P.K., Nims, R.M., Amyx, H.L., and Ferguson, J.A.: Epizootiology of tropical canine pancytopenia. J. Wildlife Diseases 6:220-225, 1970.

9. Crago, C.C.. St. Croix, Virgin Islands. Personal communication.

10. Anderson, D.R., Hopps, H.E., Barile, M.F., and Bernheim, B.E.: Comparison of ultrastructures of several rickettsiae, ornithosis virus, and Mycoplasma in tissue culture. J. Bact. 90:1387-1404, 1965.

11. Carmichael, J., and Fiennes, R. N.: Rickettsial infections of dogs. Veterinary Record 54: 3-4, 1942.

12. Malberbe, W. D.: The diagnosis and treatment of Rickettsiosis in dogs. J. South African Vet. Med. Assoc. 19: 135-138, 1948.

13. Bool, P. H., and Sutmoller, P.: Ehrlichia canis infections in dogs on Aruba (Netherlands Antilles). J.A.V.M.A. 130: 418-420, 1957.

14. Buckner, R. G., and Ewing, S. A.: Experimental treatment of canine Ehrlichiosis and Hemobartonellosis. J.A.V.M.A. 150: 1524-1530, 1967.

15. Farrell, R. K.: Canine rickettsiosis in Current Veterinary Therapy 1966-1967 Small Animal Practice, (R. W. Kirk, ed). W. B. Saunders Co., Philadelphia, Pa. 285-288, 1966.

16. Horsfall, F. L., and Tamm, I.: Viral and rickettsial infections of man, 4th ed., J. B. Lippincott Co., Philadelphia, Pa. 1965.

17. Buckner, R. G., and Ewing, S. A.: Canine Ehrlichiosis in Current Veterinary Therapy IV Small Animal Practice (R. W. Kirk, ed.) W. B. Saunders Co., Philadelphia, Pa. 679, 1971.

18. Barker, L. E.: Determination of antibiotic susceptibility Rickettsiae and Chlamydiae in BS-C-7 cell cultures, in antimicrobial agents and chemotherapy--1968. American Society for Microbiology, Ann Arbor, Michigan, 425-428, 1969.

19. Neitz, W. O., and Thomas, A. D.: Rickettsiosis in the dog. J. South African Vet. Med. Assoc. 9: 166-174, 1938.

20. Hines, L. R.: An appraisal of the effects of long-term chlortetracycline administration. Antibiotics and chemotherapy 6: 623-641, 1956.

21. Donatien, A., and Lestoquard, F.: Etat actual des connaissances sur les rickettsioses animales. Arch. Inst. Pasteur Algerie 15: 142-187, 1937.

22. Ewing, S. A., and Philip, C. B.: Ehrlichia-like rickettsiosis in dogs in Oklahoma and its relationship to Neorickettsia helminthoeca. A.J.V.R. 27: 67-69, 1966.

23. Burgdorfer, W.: Hemolymph test a technique for the detection of rickettsiae in ticks. Am. J. Trop. Med. & Hyg. 19: 1010-1014, 1970.

24. Nyindo, M.B.A., Ristic, M., Huxsoll, D.L., and Smith, A.R.: Studies in tropical canine pancytopenia: in vitro cultivation of the causative agent - Ehrlichia canis. Am. J. Vet Res. In press.

25. Ruff, M.D., Fowler, J.L., Matsuda, K., and Fernau, R.C.: Babesia gibsoni: Influence of infection on serum enzymes of dogs. 406th Med. Lab. To be published.

26. Groves, M.G., and Dennis, G.L.: Field and laboratory studies of canine infections with Babesia gibsoni. Experimental Parasitology. In press.

27. Groves, M.G., and Yap, L.F.: Babesia gibsoni in a dog. J.A.V.M.A. 158:689-694, 1968.

28. Groves, M.G., and Vanniasingham, J.A.: Treatment of Babesia gibsoni infections with phenamidine isethionate. Veterinary Record 86:8-10, 1970.

29. Rao, M.A.N.: Piroplasm gibsoni Patton. Indian Journal of Medical Research 14:785-800, 1927.

30. Seneviratna, P.: Studies of Babesia gibsoni infections of dogs in Ceylon. Ceylon Veterinary Journal 13:107-110, 1965a.

31. Bauer, F.: Chemotherapy of Babesia gibsoni infection in the dog. Zentralbl. Vet. Med. 14B:170-178, 1967.

Publications:

1. Huxsoll, D.L., Hildebrandt, P.K., Nims, R.M., Amyx, H.L., and Ferguson, J.A.: Epizootiology of tropical canine pancytopenia. J. Wildlife Diseases 6:220-225, 1970.

2. Huxsoll, D.L., Hildebrandt, P.K., Nims, R.M., and Walker, J.S.: Tropical canine pancytopenia. J.A.V.M.A. 157:1627-1632, 1970.

3. Huxsoll, D.L., Hildebrandt, P.K., Nims, R.M., and Walker, J.S.: Tropical canine pancytopenia in Current Veterinary Therapy IV (R.W. Kirk, ed.) W.B. Saunders Co., Philadelphia, Pa. 677-679, 1971.

4. Walker, J.S., Rundquist, J.D., Taylor, R., Wilson, B.L., Andrews, M.R., Barck, J., Hogge, A.L., Jr., Huxsoll, D.L., Hildebrandt, P.K., and Nims, R.M.: Clinical and clinicopathologic findings in tropical canine pancytopenia. J.A.V.M.A. 157:43-55, 1970.

5. Nims, R.M., Ferguson, J.A., Walker, J.L., Hildebrandt, P.K., Huxsoll, D.L., Reardon, M.J., Varley, J.E., Kolaja, G.J., Watson, W.T., Shroyer, E.L., Elwell, P.A., and Vacura, G.W.: Epizootiology of tropical canine pancytopenia in Southeast Asia. J.A.V.M.A. 158:53-63, 1971.

6. Ristic, M., Conroy, J.D., Siwe, S., Healy, G.R., Smith, A.R., and Huxsoll, D.L.: Babesia species isolated from a woman with clinical babesiosis. Am. J. Trop. Med. & Hyg. 20:14-22, 1971.

7. Burghen, G.A., Beisel, W.R., Walker, J.S., Nims, R.M., Huxsoll, D.L., and Hildebrandt, P.K.: Development of hypergammaglobulinemia in tropical canine pancytopenia. Am. J. Vet. Res. 32:749-756, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

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10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
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72 CURRENT	[illegible]	21

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

23. (U) To determine and characterize causative agents of infectious diseases occurring in military dogs during their induction and training; to determine how the infections are spread; and to develop effective control measures. Studies on agents associated with acute respiratory disease and diarrheal diseases are specifically emphasized. The potential importance of infectious agents newly found in dogs are evaluated.

24. (U) Conventional epidemiologic and microbiologic technics are employed and new procedures will be developed as needed.

25. (U) 70 07 - 71 06 Viral etiology of disease in recruit military dogs was studied further. Parainfluenza SV-5 continues to be the predominant agent responsible for canine respiratory infections at recruitment center (Lackland AFB, Texas) and scout dog training center (Ft. Benning, Ga.). More than 85% of newly procured were found to be susceptible to SV-5 infections. A transmissible agent has been recovered from sentry dogs involved in an outbreak of a diarrheal disease in Germany. Further studies on the properties of 3 new canine rhino-type isolates have been carried out. A new small, naked virus has been recovered from a canine semen specimen. A selective media containing antibiotics and indicators has been developed to facilitate the isolation and identification of Brucella canis. The cultural technic is now being evaluated to determine if it could supplant current laborious and costly isolation methods. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul-30 Jun 71.

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Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 184, Diseases of recruit military animals

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Description.

Objectives are first to determine the etiology and epidemiology of respiratory disease in military dogs and to develop methods of control and prevention, and secondly to identify and characterize viruses of military dogs and laboratory animals which may be potential zoonotic agents or interfere with the utilization of the animals in the research programs or diagnostic services at WRAIR. Specific studies were done on parainfluenza infections, etiology of a gastrointestinal disease outbreak in military dogs and on characteristics of rhino-like viruses and corona viruses isolated from dogs.

Progress.

1. Respiratory Disease in Military Dogs.

From 1966 to 1968, epizootics of respiratory disease occurred in military dogs at the induction and training centers. The disease episodes disrupted the deployment and training of the dogs. Parainfluenza SV-5 virus was recovered from the affected dogs and the virus was found to be highly communicable[1]. In subsequent years SV-5 infections have occurred in more than 75% of the dogs at the induction and training centers[2]. This report summarizes continued observations on the occurrence of SV-5 infections in military dogs.

Previous observations indicated that 90% (618 of 685 of newly procured dogs were serotest susceptible to SV-5 infection (Annual Report 1969-1970). During the past year, similar observations were made. Eight-seven percent, or 249 of 285 dogs received at the Lackland Air Force Base (LAFB) induction center were susceptible to SV-5. Dogs from California again had the highest rate of positive reactors. In the past year, 33% or 15

of 46 dogs from California were SV-5 serotest positive. The 22 remaining positive dogs came from 17 states representing all areas of the United States. The findings clearly indicate that newly procured dogs are highly susceptible to SV-5 infection and that the virus infects dogs throughout the United States.

Following procurement at LAFB, dogs were shipped to Ft. Benning for scout dog training. Dogs from LAFB completing training at Ft. Benning continued to have a high incidence of SV-5 antibody. In 1970, 90% or 48 of 53 dogs and all 14 dogs departing in 1971 had SV-5 antibody. The dogs departing Ft. Benning in 1970 had arrived in 1969 and 19 of 27 of these dogs converted at Ft. Benning. All 24 dogs arriving at Ft. Benning from LAFB in 1970 and 1971 had SV-5 antibody. Thirteen of these dogs were serotest positive upon arrival at LAFB. The last dog in this group arrived in April 1970 and was serotest positive when processed at Ft. Benning in August 1970. The findings in 1970-71 are consistent with previous observations that SV-5 infections begin at the procurement center and spread to nearly all the dogs.

In addition to the dogs received at Ft. Benning from LAFB, dogs also are received from the "Biosensor Research Team" (BRT) at Edgewood Arsenal. Following receipt of these dogs, they are quarantined apart from the dogs procured at LAFB. Subsequently the dogs from both sources may be trained together. From March 1970 to February 1971 serum specimens were prepared from 14 dogs upon arrival at Ft. Benning. Each of these dogs did not have SV-5 antibody. The findings indicate SV-5 infections are not occurring at BRT facilities at Edgewood Arsenal and that these dogs are susceptible to infection. In contrast to the dogs from LAFB, only 1 of 29 BRT dogs completing training in 1970-71 at Ft. Benning had serological evidence of SV-5 infection. The time and place of infection of the serotest positive dog is unknown. The failure of nearly all the BRT dogs to convert suggests that SV-5 infections were not occurring in the units in which the dogs were trained.

The absence of SV-5 infections at Edgewood and the potential infection and respiratory disease during training may warrant the quarantine of these dogs at Edgewood. Potential SV-5 vaccines for dogs are under commercial development and after suitable safety and potency tests, field trials of the vaccine in the dogs at Edgewood may be of value. Further observations on the epidemiology of SV-5 infections of military dogs will be continued.

2. Virus Studies of a Gastrointestinal Disease Outbreak in Military Dogs in Germany.

During February and March 1970 an epizootic of diarrheal disease occurred in 60 of 63 dogs at the USAFE Patrol Dog Training School in Wiesbaden, Germany. An investigation into the epidemiology and etiology of the epizootic was conducted by CPT A. Hall, USAF and T/SGT J.E. Kielty (Report #70-3, Dept of the Air Force, 4th Epidemiologic Flight (USAFE), APO New York 09332, 30 June 70). Microbiologic and toxicological investigations failed to incriminate the etiologic agent. Although viruses were not recovered in monkey, baby hamster kidney and several human cell cultures, the authors made a presumptive diagnosis of viral gastroenteritis. In January 1971, a second outbreak of diarrheal disease occurred in these dogs and fecal and serum specimens were collected and shipped to WRAIR for virus studies.

Fecal specimens from 3 affected dogs were treated with antibiotics, centrifuged and inoculated into primary dog kidney (PDK), continuous canine thymus (CCT) and the Walter Reed canine (WRC) cell cultures. In addition an untreated mixture of the 3 stool suspensions was fed to a 4-month Beagle dog. The dog did not develop any signs of disease.

A transmissible agent was recovered from 1 fecal specimen in PDK and CCT cell cultures. The agent, 1-71, was first detected in PDK cells on the sixth day of cultivation. On passage in PDK cells, giant cells were evident and hematoxylin and eosin stained infected cultures contained multinucleated giant cells with some cells having what appeared to be destroyed nuclear fragments. The infected cultures did not hemadsorb guinea pig red blood cells. These characteristics were similar to those of the canine corona-like virus L-198R isolate described in the previous annual report (1969-70). The 1-71 agent was not neutralized by canine distemper and SV-5 antiserum or by serum obtained 23 days after feeding the original fecal suspension to the puppy. The agent was reisolated in PDK cell cultures and a 1:4 neutralization titer was found in the 30-day convalescent serum of the dog providing the isolate. In addition, 3 of 4 other convalescent dogs had 1:4 - 1:16 neutralization titers to the isolate. The 1-71 isolate has been purified by three terminal dilutions and seed virus pools have been prepared. Detailed characterization and serological studies are in progress to compare the isolate with other known canine viruses. At present, the relationship of the isolate to the diarrheal disease is unknown. Pathogenicity tests in dogs for this purpose are planned.

3. Recovery of a New Canine Rhino-like Virus from a Laboratory Dog.

Puppy production of the WRAIR beagle colony at Cumberland, Virginia has been seriously affected by comparatively large numbers of still-born pups. In swine and other species, parvoviruses have been associated with stillbirths and neonatal diseases[2]. The parvovirus of swine has been recovered from the semen of boars which may result in infection at time of conception[4]. In the past year, a parvovirus of dogs was described by this laboratory and antibody to this virus was found in the serum of beagles of this colony[3]. To determine if the recently described canine parvovirus or other viral agents were involved in the loss of puppies, semen specimens were collected from 3 dogs at the Cumberland colony and from a laboratory dog at WRAIR.

From the semen of the laboratory dog a transmissible agent, A358, was recovered in the Walter Reed Canine (WRC) cell line. The agent produced an entero-virus like cytopathic effect similar to the canine rhino-like viruses described in the previous annual report (1969 [illegible]). The agent was chloroform stable and passed through a 50 nm filter which retained infectious canine hepatitis (ICH) virus. Growth of the virus was not inhibited by 5-Iodo-Deoxyuridine indicating that the virus contained RNA. The agent was inactivated at pH 3 [illegible]. Thus the properties of this agent are similar to the rhino-like viruses described in the previous annual report. Antiserum to the previously described canine rhino viruses 1198T, A128T, 3-68 and A128Thr did not significantly neutralize with the isolate. Antiserum to the isolate is being prepared in rabbits and guinea pigs. Further studies of the A358 isolate are in progress.

4. Further Studies on the Properties of Canine Rhino-like Virus (CRV) Isolates.

The previous annual report summarized initial findings on 4 apparently new canine rhino-like viruses (CRV), designated 3-68, L-1198T, A128T, and A128Thr. Each virus contained RNA, readily passed through a 50 nm membrane filter, was chloroform and ether resistant, and was inactivated at pH 3.0. A fifth similar isolate (A358) was described in the preceding section of this report. These findings would place the agents in the picornavirus group, more specifically with the rhinoviruses. During the past year further studies were carried out to compare the properties of the CRV with the other rhinoviruses of man and animals. Specific studies were carried out on the acid lability at pH 3.0, 4.0 and 5.0 and the ability of molar magnesium chloride ($MgCl_2$) to stabilize the viruses at 50°. In addition, serological studies were conducted to determine the antigenic relationships of the first 4 isolates. Antiserum to the fifth isolate is in preparation.

The effect of pH 3.0, 4.0 and 5.0 on the stability of the CRV was determined (Table 1). A known acid resistant virus (polio type I) and acid labile virus (Echo 28) was included for control purposes. After incubation for one hour at 37C at pH 3.0 all CRV isolates and Echo virus 28 were completely inactivated. At pH 5.0 only 1 test virus (A128Thr) and Echo virus 28 were inactivated. At pH 4.0, 2 CRV were stable and 3 were labile. Polio virus was stable at each pH tested. With one exception (A128Thr) the CRV viruses differ from the human rhinoviruses in acid lability and resemble the vesicular exanthema virus of swine (VEVS) and feline picornaviruses (FP)[8]. The exceptional isolate (A128Thr) also differs from the other CRV in its ability to multiply in tissue cultures from a variety of species other than from dogs.

Picornaviruses differ in their ability to resist heat inactivation at 50C in presence of molar magnesium chloride ($MgCl_2$). Under these conditions most human and some swine picornaviruses are stable (e.g., polio virus, echo viruses), whereas many picornaviruses of lower animals are inactivated (e.g., foot-and-mouth disease virus, vesicular exanthema virus of swine, and feline picornaviruses). For comparative purposes, the effect of molar $MgCl_2$ on the stability of the 5 CRV isolates was examined (Table 2). Poliovirus type I was employed in the tests as a reference virus. Only 1 test virus, A128Thr was stabilized by $MgCl_2$. The remaining 4 CRV resembled VEVS and FP in not being stabilized by $MgCl_2$.

The results of cross CRV neutralization tests with immune rabbit and guinea pig serums are summarized in Table 3. Minor heterologous reactions of 1:16 to 1:64 of the homologous titers occurred between A128T and L198T antiserums and the respective antigens. The remaining rabbit antiserums did not cross react. The guinea pig antiserum were more specific; cross reactions were not observed between A128T and L198T. A very minor neutralization of L198T virus at 1:4 was produced by 3-68 serum. The findings indicate that 4 CRV isolates are antigenically distinct and suggest that the dog is infected with multiple serotypes, as in the case of human and other animal infections with this group of viruses. Further studies on the structure, buoyant density and other characteristics of these viruses are in progress.

5. Further Studies of a Canine "Corona-like" Virus L198R.

The previous annual report summarized the findings on the recovery of an agent with characteristics of a coronavirus. The virus was antigenically distinct from the coronaviruses of chickens (infectious bronchitis) mice (mouse hepatitis) and rats (rat coronavirus). The canine isolate also differed from other coronaviruses in producing cytopathic effects in only certain canine cell cultures and a lack

Table 1. The Effect of Selected Acid pH's on the Stability of Canine Rhino-like Virus Isolates

Virus	Control Virus Titer Phosphate Buffer pH 7.0	Reduction in Virus Titer After 1 Hour at 37°C in Buffer			
		Phosphate pH 5.0	Citrate pH 5.0	Citrate pH 4.0	Citrate pH 3.0
L198T	6.9	+0.1*	+0.4	3.9	≥4.9
A128T	6.5	0.5	0.2	4.0	≥4.5
3-68	6.0	+0.5	+0.5	0.5	≥4.0
A358	5.5	0.7	0.2	1.0	≥3.5
A128Thr	6.8	3.5	≥4.8	≥4.8	≥4.8
Reference Viruses					
Polio I	6.3	0.0	+0.2	0.3	+0.5
Echo 28	3.9	>2.4	>2.4	>2.4	>2.4

*Control virus titer minus test virus titer.

Table 2. The Effect of Molar Magnesium Chloride on the Stability of Canine Rhinovirus Isolates at 50°C

Virus	1 M $MgCl_2$	Virus Titer After 1 Hour at	
		5°C	50°C
L198T	0	8.0	3.7 (4.3)*
	+	7.7	4.3 (3.4)
A128T	0	7.3	4.8 (2.5)
	+	7.3	2.3 (5.0)
3-68	0	6.8	3.3 (3.5)
	+	6.8	2.8 (4.0)
A358	0	5.8	2.5 (2.3)
	+	5.8	<2.0 (≥3.8)
A128Thr	0	7.1	<1.5 (≥5.6)
	+	6.5	6.5 (0.0)
Polio I	0	6.3	<1.5 (≥4.8)
	+	6.1	6.5 (+0.4)

*Change in virus titer at 50°C.

Table 3. Cross Neutralization Antibody Tests of Canine Rhinovirus Isolates

Antiserum* / Strain	[1]/Antibody Titer with Virus 3-68	L198T	A128T	A128Thr
Rabbit Serums				
3-68	≥1024	<4	<4	Not Tested
L198T	<4	256	4	Not Tested
A128T	<4	16	256	Not Tested
A128Thr	<4	<4	<4	1024
Guinea Pig Antiserums				
3-68	≥16,000	4	<4	<4
L198T	<4	4096	<4	<4
A128T	<16	<16	1024	<4
A128Thr	<16	<16	<4	256

*Pre-immunization serums did not neutralize the homologous virus at a 1:4 dilution.

of pathogenicity in laboratory rodents. The report extends these findings with further observations on the structure of the virus, and additional serological studies with a porcine coronavirus.

For examination of viral structure a 500 ml pool of virus was prepared in a continuous canine thymus cell line. The virus was concentrated by ultrafiltration (Amnicon) with a XM 100A membrane and centrifuged at 41,000 G. The concentration procedure increased the titer from 7.0 $\log_{10}$ $TCID_{50}$/ml to 9.0 $\log_{10}$ $TCID_{50}$/ml. The concentrated viral suspension was placed on grids stained with 2% phosphotungstic acid and examined in a Siemens electron microscope. Electron microscopy was done by W. Engler at the Armed Forces Institute of Pathology. The negatively stained virus particles appeared to be typical coronavirus particles. The virions were pleomorphic surrounded with radiating spikes which had terminal knobs. The overall lengths of the virions varied from 110-200 nm with an average of 150 nm. The spikes were approximately 2.2 nm long and the terminal knobs approximately 1.2 nm in diameter. These findings provide further evidence for placing the L198R isolate in the coronavirus group.

During the past year transmissible gastroenteritis (TGE) virus of pigs has been proposed as a member of the coronavirus group[7]. Antiserum to TGE virus in canine serums has been reported by Norman and co-workers[6]. Therefore an attempt was made to compare the canine isolate with TGE virus of swine. Anti-TGE swine serum was obtained from Dr. Ristic of the University of Illinois and Dr. Norman at the National Animal Disease Laboratory, Ames, Iowa. Each antiserum neutralized the homologous TGE virus at a titer of 1:1024 and neutralized the canine L198R isolate at 1:16. Attempts to produce high titered L198R serum in rabbits and guinea pigs to date have not been successful. However, anti-L198R rabbit serum neutralized L198R and TGE viruses at a 1:4 dilution. The pre-immunization serum did not have any activity.

In an attempt to determine the intectivity of L198R for swine and to produce a potent antiserum, serotest negative swine to L198R and TGE were fed one million $TCID_{50}$ of L198R virus. The virus was not recovered from rectal swab specimens of the infected pigs and the pigs did not develop signs of disease. Neutralizing antibody did not develop to either L198R or TGE viruses 28 days after feeding. In addition each pig was given 5 intramuscular booster inoculations of 5.0 ml each at 7 day intervals. Twenty-one days after the last booster dose the pigs still did not develop L198R antibody. The serological studies indicate that I198R and TGE viruses are related. However, the determination of the precise relationship will require potent antiserum to both viruses. L198R virus differs from TGE in cell culture host range as L198R virus did not multiply in swine kidney cells and L198R did not multiply in susceptible swine. An attempt to produce potent L198R serum in serotest negative puppies is in progress. Further comparative studies of L198R, TGE are in progress.

Summary and Conclusions.

1. Respiratory Disease in Military Dogs.

Respiratory SV-5 infections continue to occur in military dogs at the Lackland Air Force Base induction center and at the Ft. Benning scout dog training center. Eighty-seven percent of newly procured dogs at the Lackland induction center are serotest susceptible to SV-5 infection. More than 90% of these dogs completing training at Ft. Benning have serological evidence of SV-5 infections. The dogs provided by the Biosensor Research Team at Edgewood Arsenal, Maryland are serotest susceptible to SV-5. Consideration should be given to vaccinating these dogs against SV-5 infections.

2. Virus Studies of a Gastrointestinal Disease Outbreak in Military Dogs in Germany.

In 1970 and 1971 episodes of diarrheal disease have occurred in military dogs in Germany. From the fecal specimen of 1 dog a transmissible agent has been recovered. The agent appears to be similar to the canine corona-like virus recovered last year. Further studies of this agent are required to determine its pathogenicity for dogs.

3. Recovery of a New Canine Rhino-like Virus from a Laboratory Dog.

An apparently new rhino-like virus was recovered from a semen specimen of a laboratory dog. The agent produced cytopathic effects in the Walter Reed canine line. The agent was antigenically distinct from previously described canine viruses. Further characterization studies of this agent are in progress.

4. Further Studies on the Properties of Canine Rhino-like Virus (CRV) Isolates.

Further studies were carried out on the properties of 5 CRV isolates. The CRV were all acid labile at pH3.0 and with 1 exception (A128Thr) were acid stable at pH5.0. With the exception of the same isolate (A128Thr) all the isolates were not heat stabilized by molar magnesium chloride. Four of the 5 CRV isolates resemble the vesicular exanthema virus of swine and feline picornaviruses in these properties. Further studies on the morphology, buoyant density, and other properties of the CRV isolates are in progress.

5. Further Studies of a Canine "Corona-like" Virus L198R.

The canine corona-like, L198R virus was examined in the electron microscope. Following negative staining typical coronavirus particles were evident. The virions were approximately 110-200 nm in length surrounded by spikes with terminal knobs. Anti-transmissible

gastroenteritis (TGE) swine serum neutralized L198R virus at 1/64th of the homologous titer. The canine virus was not infectious for serotest negative pigs to TGE and L198R.

The findings support the classification of L198R virus in the coronavirus group.

The L198R virus is antigenically related to TGE virus. However, further experiments are required to define the extent of this relationship. Studies on the pathogenesis of L198R in dogs are in progress.

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 184, Diseases of recruit military animals

Literature Cited.

References:

1. Binn, L. N., Lazar, E.C., Rogul, M., Shepler, V.M., Swango, L.J., Claypoole, T., Hubbard, D.W., Asbill, S.G., and Alexander, A.: Upper respiratory disease in military dogs: Bacterial, mycoplasma, and viral studies. Am. J. Vet. Res. 29:1809-1815, 1968.

2. Binn, L.N., and Lazar, E.C.: Comments on epizootiology of parainfluenza SV-5 in dogs. J.A.V.M.A. 156:1774-1777, 1970.

3. Binn, L.N., Lazar, E.C., Eddy, G.A., and Kajima, M.: Recovery and characterization of a minute virus of canines. Infection & Immunity 1:503-508, 1970.

4. Cartwright, S.F., and Huck, R.A.: Viruses isolated in association with herd infertility, abortions and stillbirths in pigs. Vet. Rec. 81:196-197, 1967.

5. Johnson, R.H.: A search for parvoviridae. Vet Rec. 84:19-20, 1969.

6. Norman, J.O., McClurkin, A.W., and Stark, S.L.: Transmissible gastroenteritis (TGE) of swine: Canine serum antibodies against an associated virus. Can. J. Comp. Med. 34:115-117, 1970.

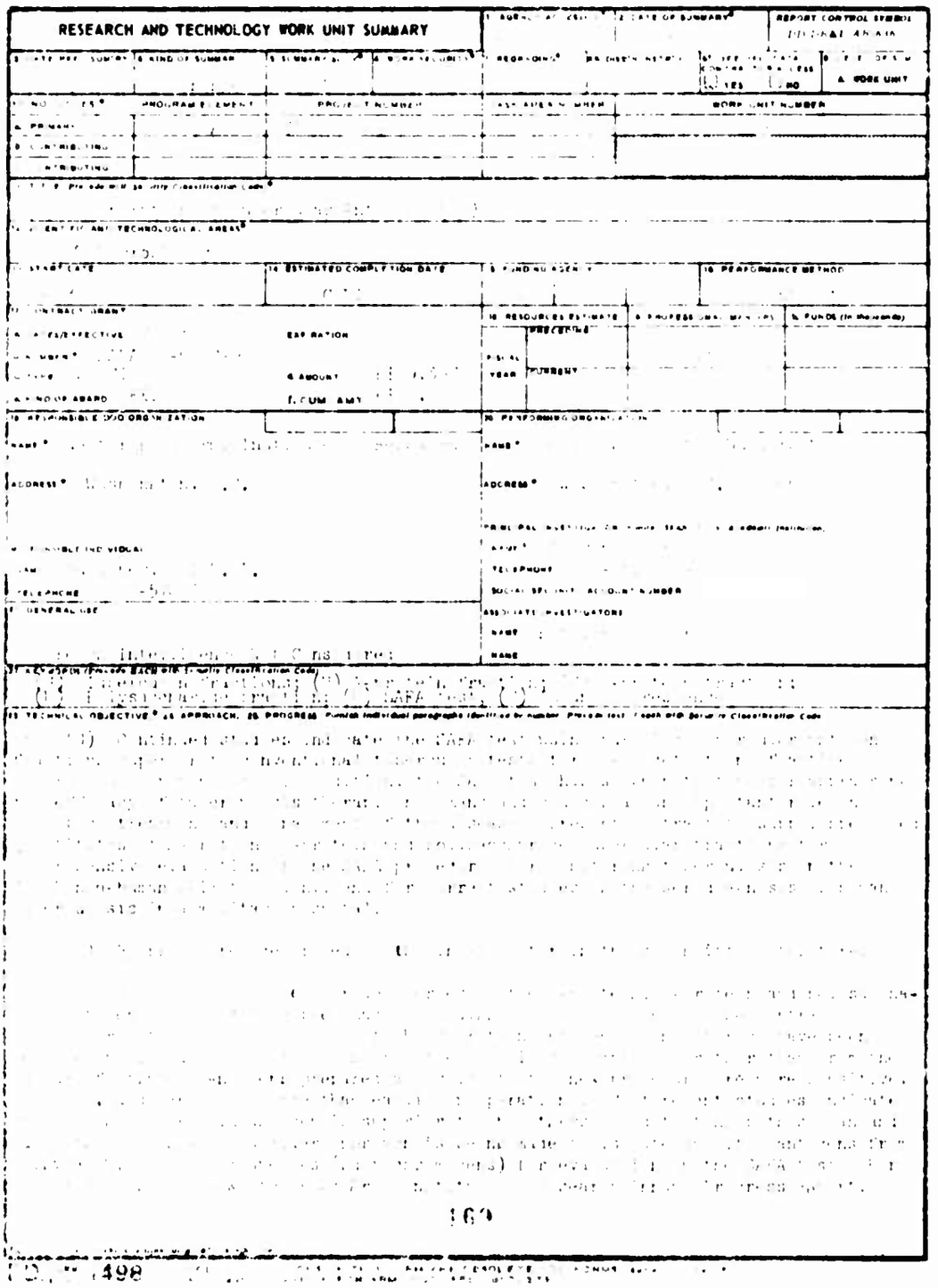
7. Tajima, M.: Morphology of transmissible gastroenteritis of pigs. Arch. f.d. ges. Virus 25:337-351, 1968.

8. Wawrzkiewicz, J., Smale, C.J., and Brown, F.: Biochemical and biophysical characteristics of vesicular exantehma virus and the viral ribonucleic acid. Arch. f.d. ges. Virus 25:337-351, 1968.

Publications:

1. Goodwin, B.S., and Binn, L.N.: Upper respiratory viral diseases of the dog. Current Veterinary Therapy IV, Small Animal Practice, edited by Kirk, R.W., W.B. Saunders Co. Phila. Pa. p.643-644, 1971.

2. Lazar, E.C., Swango, L.J., and Binn, L.N.: Serologic and infectivity studies of canine SV-5 virus. Proc. Soc. Exptl. Biol. & Med. 135:173-176, 1970.



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

REPORT CONTROL SYMBOL
DD-DR&E-AR(AR)

YES NO

PROGRAM ELEMENT

PROJECT NUMBER

WORK UNIT NUMBER

PROGRAM

CONTRIBUTING

DISTRIBUTING

START DATE

ESTIMATED COMPLETION DATE

PERFORMANCE METHOD

CONTRACT/GRANT

EAP RATION

AMOUNT

FUNDING AGENCY

RESOURCES ESTIMATE

PROGRESS (Not to exceed 200 words)

FUNDS (In thousands)

PRIOR FY

CURRENT FY

BUDGET FY

RESPONSIBLE DOD ORGANIZATION

NAME

ADDRESS

RESPONSIBLE INDIVIDUAL

NAME

TELEPHONE

PERFORMING ORGANIZATION

NAME

ADDRESS

PRINCIPAL INVESTIGATOR

NAME

TELEPHONE

SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

NAME

NAME

GENERAL USE

(U) TECHNICAL OBJECTIVE (U) APPROACH (U) PROGRESS

Project 3A061101A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 189, Preparation of tubercular antigens

Investigators.
Principal: Lewis F. Affronti, Ph.D.
Associate: Earl H. Fife, Jr., M.S.

Description.

This work unit consists of a contract to prepare specific tuberculin antigen fractions and to supply the Department of Serology, WRAIR, with specified amounts of each antigen. Fractionation procedures developed by the principal investigator are used to isolate A-protein antigen from filtrates of M. tuberculosis cultures, C-protein antigen from the bacterial cells, and polysaccharide antigen from the cell walls of the tubercle bacilli. These antigens are used by investigators in the Department of Serology in studies on the serodiagnosis of simian and human tuberculosis, and for screening for tuberculosis in the animals of the nonhuman primate colony, WRAIR

Progress.

In accordance with the terms of the contract, regular deliveries of the A-protein, C-protein and polysaccharide antigen fractions have been made to the Department of Serology, WRAIR During the present reporting period, the quality of the C-protein antigen was significantly improved by preparing the fraction from the tubercle bacillus rather than the culture filtrate. The present antigen is considerably more stable, more sensitive, and gives much less nonspecific fluorescence in tests with sera from nontuberculous donors. In recent comprehensive studies on simian tuberculosis, the new C-protein was superior to the A-protein and polysaccharide antigens for early detection of active disease. The three antigens now are being evaluated for their efficacy in detecting human tuberculosis and for appraising therapeutic response.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY: 71 07 01	REPORT CONTROL SYMBOL: DD-R&E AR 636					
3. DATE PREV SUM: 70 07 01	4. KIND OF SUMMARY: D. CHANGE	5. SUMMARY SYS	6. WORK SECURITY	7. REVISION	8. INACTIVE DATE	9. SPECIFIC ACTIVITY: YES	10. LEVEL OF EFFORT: A. WORK UNIT

11. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	021
b. CONTRIBUTING				
c. CONTRIBUTING				

12. TITLE (Provide with Security Classification Code)
(U) Tropical Disease Bulletin Information Retrieval System (21)

13. SCIENTIFIC AND TECHNOLOGICAL AREAS
002600 Biology; 004200 Computers

14. START DATE	15. ESTIMATED COMPLETION DATE	16. FUNDING AGENCY	17. PERFORMANCE METHOD
69 07	CONT	DA	B. CONTRACT

18. CONTRACT/GRANT
a. DATES/EFFECTIVE: 71 06 EXPIRATION: 71 08
b. NUMBER: DADA 17-69-C-9171
c. TYPE: U. CPFF
d. AMOUNT: 0
e. KIND OF AWARD: EXT
f. CUM. AMT.: 63,108

19. RESOURCES ESTIMATE

	PRECEDING	20. PROFESSIONAL MAN YRS	21. FUNDS (In Thousands)
FISCAL YEAR	71	1	33
	CURRENT 72	0.1	0

22. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012
RESPONSIBLE INDIVIDUAL
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TELEPHONE: 202-576-3551

23. PERFORMING ORGANIZATION
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. citizen (optional))
NAME: Voccola, H. W.
TELEPHONE: 301-587-3531
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME: Schafer, June A.
NAME:

24. GENERAL USE
Foreign Intelligence Not Considered

25. KEYWORDS (Provide KWIC and Security Classification Code)
(U) Information Retrieval; (U) Information Handling; (U) Computer Programming; (U) Text Processing

26. TECHNICAL OBJECTIVE 27. APPROACH 28. PROGRESS (Furnish individual paragraphs identified by number. Provide end of each with Security Classification Code.)

23 (U) To develop a computer system to create, maintain and index abstracts from the Tropical Disease Bulletin, and to develop manipulative capabilities such as the updating of the master file and sorting in certain fields within the file.

24 (U) Most of the programs have been rewritten in FORTRAN so as to provide greater flexibility and enable us to use the WRAIR CAC 3300.

25 (U) 70 07 - 71 06 All programs have been recorded to run on the WRAIR CAC 3300. Methods for updating the data base have been investigated. Experimentation with various screen-bit sets has been initiated. For technical report see WRAIR Annual Progress Report 7 July 70 - 30 Jun 71.

29. (Available in unclassified user's hands)

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

Project 3A061101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 00, In-House Laboratory Independent Research

Work Unit 190, Tropical Disease Bulletin Information Retrieval System

Investigators
Principal: Harry W. Voccola
Associate: June A. Schafer

Description

The purpose of this effort is two-fold. The first objective is to develop the software capable of handling the input created by the encoding of the Tropical Disease Bulletin and to manipulate it so as to correct errors, build the file, reformat index tapes suitable for handling by the regular Biological Abstracts system. The second objective is to develop a search technique capable of handling full text, fractions of words within text, and ultimately, manipulation procedures involving the discovery of synonyms without the use of a thesaurus.

During the past year the TEMAC and MAP programs used to build and correct the files needed by this project have been converted to FORTRAN for use on the CAC 3300. In addition FORTRAN programs for use on the 3300 have been written to search the files and format search output. This conversion will enable us to carry out the second objective of this project at the WRAIR computer installation. In addition, the use of a higher level language will facilitate any additional programs conversion that might be necessitated by equipment configuration changes at WRAIR. The entire system is correctly being documented.

Summary and Conclusions

With the accomplishments of the conversion good progress has been made toward the goal of bringing this project in-house. Preliminary experiments indicate that the bit screen approach to Full-text searching should be productive. If further experimentation confirms this conclusion the capability for handling textual data should find broad applicability within the Army Medical Service.

PROJECT 3A071102B71C
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
A 0A [illegible]	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. CRGN INSTR'N	9. SPECIFIC DATA CONTRACTOR ACCESS	10. LEVEL OF SUM
07 07 01	[illegible]	U	U	NA	NA	☒ YES ☐ NO	A. WORK UNIT

12. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A761102B71A	00	169
b. CONTRIBUTING				
c. CONTRIBUTING	(600 141SA(C))			

13. TITLE (Provide with Security Classification Code)
(U) Parasitic Diseases (U)

14. SCIENTIFIC AND TECHNOLOGICAL AREAS
002.00 Biology

15. START DATE	16. ESTIMATED COMPLETION DATE	17. FUNDING AGENCY	18. PERFORMANCE METHOD
[illegible]	[illegible]	DA	C. In-House

19. CONTRACT GRANT [illegible]
a. DATES/EFFECTIVE
b. NUMBER
c. TYPE
d. KIND OF AWARD
EXPIRATION
e. AMOUNT
f. CUM AMT

20. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
PRECEDING FISCAL YEAR 71	3	75
CURRENT FISCAL YEAR 72	3	

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

RESPONSIBLE INDIVIDUAL
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TELEPHONE: 202-576-3551

22. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
Division of T&I
ADDRESS: Washington, D. C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. academic institution)
NAME: JAMES, B. B., Col. MC
TELEPHONE: 202-576-3505
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: A. P. MOON DA
NAME:

PII Redacted

23. GENERAL USE
Foreign intelligence not considered

24. KEYWORDS (Provide Key Words and Security Classification Code)
(U) Parasite; (U) Schistosomiasis; (U) Pathology; (U) Primate; (U) Chemotherapy

25. TECHNICAL OBJECTIVE. 26. APPROACH. 27. PROGRESS (Furnish individual paragraphs identified by number. Provide 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 the 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) 70 07 - 71 06 Eighteen Aotus trivirgatus monkeys were infected with either Schistosoma haematobium, S. mansoni or S. japonicum. The most severe lesions were in S. japonicum monkeys. More tissue damage was produced by S. japonicum eggs than other species and more eggs were produced. Twenty-three other primates of 5 species (rhesus, pigtail, green, owl and tree shrew) were infected with S. haematobium. All species were susceptible. Green monkeys were the most susceptible and tree shrews were the least. Both parasite species and host species are important in pathogenesis of the disease. A nitrovinylfuran had considerable antischistosomal activity against S. mansoni and S. japonicum in rhesus monkeys. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Jul 70 - 30 Jun 71.

28. (Available to contractors unless classification dictates)

Project 3A061102B71A COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 102, Parasitic Diseases

Investigators:
Principal: E. H. Jadun, Ph.D., Lib. Doc
Associate: J. G. Bourseois; E. Bueding; A. W. Weaver; LTC D. G. Erickson, MC; MAJ E. L. Hickman, MC; F. von Lichtenberg

1. Schistosoma haematobium infections in five species of primates.

Since Fairley's description of lesions in the bladder and ureters of crived monkeys experimentally infected with Schistosoma haematobium, several species of primates have been studied as experimental hosts of this parasite. Stanley found worms only in the mesenteric veins and liver of one Macaca mulatta exposed. McKenzy and Moore found eggs in the feces and urine of one infected monkey (M. mulatta), but found no worms during a subsequent post-mortem examination. Kuntz and Malakatis infected Cercopithecus sp. and Papio hamadryi; Hsu et al. infected M. mulatte with S. haematobium; Jordan and Goatly described infections in five Cercopithecus aethiops centralis; and Jordan et al. reported that baboons were relatively susceptible and developed severe urinary lesions. Experimental infections in mangabeys and in chimpanzees were described by Sons. The susceptibility of other primate species to this parasite is currently under investigation. Recently, Jadun et al. reported that chimpanzees (Pan satyrus) experimentally infected with S. haematobium developed lesions of the urinary system which are remarkably similar to those reported for man. However, the large size, scarcity, and relatively high cost tend to restrict the use of this host model.

The great range of susceptibility reported in different hosts led to the present investigation in which infections from an Iranian strain of S. haematobium were compared in five species of primates. The animal's degree of susceptibility was judged from the percentage of parasites developing in the host, the growth and maturation of the worms, the location of worms in the host, the length of prepatent period, the ability of the parasites to survive, the relative amount of egg excretion, the distribution and viability of eggs in the host tissues in various organs and the overall significance of infection.

Twenty-four primates were used: six Macaca mulatta (rhesus monkey), six M. nemestrina (pigtail monkey), two Cercopithecus sabaeus (green monkey), six Papio sp. (baboon) and seven Aotus trivirgatus (owl monkey).

S. haematobium (Iranian strain) cercariae were obtained from 31 Bulinus sericinus. All animals, except the owl monkeys, were exposed to the same cercarial suspension by placing cercariae into hand-held glass rings placed for 30 minutes on a clipped and rinsed area of abdominal skin. Six owl monkeys were exposed on another day and owl monkey No. 2 was exposed separately. As shown in Table 2, doses varied from 100 to 1,000 cercariae per animal given in a single exposure. Hamsters were exposed to 200 cercariae from the same cercarial pool as infectivity controls.

Fecal examination before exposure revealed no schistosome eggs. Weekly examinations of feces and of centrifuged urine specimens were begun 5 weeks after exposure. The fecal samples were examined by the AMS III technique. The viability of eggs was determined by the miracidial hatching technique. Hematocrit determinations, white blood cell counts, and differential counts were conducted. Serum electrophoresis, total protein, glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase determinations were made. The presence of antibodies was detected by the fluorescent antibody technique. Necropsy was performed 25 or 27 weeks after exposure except with owl monkey No. 2 which was killed after 16 weeks. After injection of heparin, each animal was bled, and the pelvic, mesenteric and hepatic vessels were perfused separately, as described by Sadun et al. Worms were measured unfixed.

All major organs were dissected free after perfusion and examined for gross pathologic changes. Samples were collected for histopathologic studies and for tissue egg counts. Tissues for histopathologic examinations were fixed in buffered 10% formalin and were subsequently stained with Delafield's hematoxylin and eosin. Egg assays were performed by potassium hydroxide digestion.

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 Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences - National Research Council.

Serologic, hematologic and biochemical studies

Monthly fluorescent antibody (FA) tests were performed from the time of exposure until the end of the experiment for all animals except the tree shrews and the owl monkeys. The course of antibody protection was quite similar for all animals (Table 1). No antibodies were detected prior to and one month after exposure to infection. Two months following exposure antibodies were present in all animals except one rhesus and two pigtail monkeys. Three months after infection all but one (No. 664) of the animals tested had developed fluorescent antibody titers ranging from 1:16 to 1:256. By the end of the experiment all but one monkey (No. 664) were still positive with titers ranging from

1:16 to 1:256. This monkey had the greatest worm burden at necropsy, but demonstrated a positive FA test (1:16) only during the fourth month. No consistent and significant abnormalities were observed in the hematologic and biochemical observations.

Parasitologic and pathologic observations

The results are summarized in Tables 4, 5, 6, and 7. Mean worm recovery in the hamsters was 24 percent (4 males: 1 female). Eggs in feces and/or urine of all infected animals appeared normally.

Rhesus monkey

All animals of this group became infected, and the percent of recovery of adults varied from 5 to 9%. Most parasites were recovered from the liver and the branches of the mesenteric venous system. A disproportionate number of male to female worms was observed (approximately 10 to 1), but most females were paired with males in the mesenteric veins. In all but two animals, eggs were found in the feces 15 to 17 weeks after exposure, but they were present only for a few weeks. Eggs were detected in the urine of two animals on one occasion after 23 or 24 weeks of infection, but eggs were never seen in the feces on one of these (No. 709). Organ egg assays revealed eggs primarily in the large intestine and the liver, but a few eggs were found in the small intestine, lungs and bladder. None of the monkeys of this species had marked gross abnormalities. Two of the animals had no gross lesions. In the others erythematous spots were seen in the colon

Table 1

Results of Hemagglutination Antibody Tests on 6 Monkeys Serum at Monthly Intervals After Exposure to Schistosoma haematobium

Monkey No.	Reciprocal of titers at monthly intervals following exposure						
	0	1	2	3	4	5	6
Rhesus							
580	0	0	64	16	64	256	64
582	0	0	16	256	64	64	64
664	0	0	0	0	16	0	0
718	0	0	0	256	256	1024	256
78	0	0	1024	64	1024	64	64
709	0	0	256	256	256	64	16
Pigtail							
560	0	0	0	256	64	16	16
108	0	0	0	64	256	16	64
Cynomolgus							
51	0	0	16	256	256	256	64
81	0	0	64	256	64	1024	256

* Sera from two shows of 1:2 not tested

Table 2

Worm Recoveries in Monkeys Exposed to Schistosoma haematobium

Animal no.[a]	No. cercariae	Worms recovered: Inferior vena cava		Mesenteric veins		Liver		Total		%	Worm length (mm)	
		Male	Female	Male	Female	Male	Female	Male	Female		Male	Female
Rhesus												
856	100	0	0	13	1	25	0	38	1	39	ND[b]	ND
860	100	0	0	10	3	23	0	33	3	36	ND	ND
664	500	0	0	132	25	115	1	247	26	55	ND	ND
718	500	7	0	42	3	53	1	102	4	21	ND	ND
78	1,000	0	0	34	7	7	0	41	7	5	ND	ND
709	1,000	2	2	22	5	23	1	47	8	15	ND	ND
Pigtail												
109	500	2	1	73	9	119	1	194	11	41	8.9	15.2
108	1,000	0	0	106	7	137	0	243	7	25	9.4	16.0
Green												
71	500	8	6	83	7	12	0	103	13	25	10.6	17.1
81	500	0	0	86	11	0	0	86	11	19	10.2	15.9
Tree shrew												
6	1,000	0	0	31	5	0	0	31	5	4	ND	ND
Owl												
2	500	6	3	126	56	42	12	174	71	49	ND	ND
10	575	0	0	0	0	0	0	0	0	0	ND	ND
17	230	0	0	0	0	0	0	0	0	0	ND	ND
24	575	0	0	0	0	0	0	0	0	0	ND	ND
29	230	0	0	42	35	14	1	56	36	40	11.3	15.8
30	575	0	0	17	7	15	4	32	11	8	6.3	7.9
75	230	0	0	0	0	0	0	0	0	0		

[a] Average monkey weights (kg): rhesus, 2.6; pigtail, 6.0; green, 3.5; treeshrew, 0.4; owl, 0.9.

[b] ND = not done

[c] Only one of six tree shrews exposed became infected.

occasionally, and a few white areas were seen in the liver. Discoloration of the bladder mucosa was observed in two animals, but histologic examination did not reveal bilharzial lesions. Microscopic examination revealed moderate numbers of eggs and granulomas in the liver and colon (Fig. 1) and, rarely, in the lung. Granulomas were usually composite. In addition, portal fields were diffusely infiltrated with inflammatory cells, predominantly lymphocytes and eosinophils. Some of the portal veins showed endophlebitis. One animal also had endophlebitis of a pancreatic vein.

Pigtail monkey

Both of the animals exposed to cercariae became infected. Nearly all adult worms recovered at necropsy were recovered from the inferior vena cava. As in the rhesus, a disproportion of male to female worms was observet (25:1). Eggs were detected in the stools of the animals 14 weeks after exposure, but were never found in the urine. Organ assays

Table 3

Fecal and Urine Egg Recovery in Monkeys Exposed to Schistosoma haematobium

Monkey no.	Feces				Urine			
	Week first detected	Peak week	NEPG[a] at peak	No. weeks eggs seen	Week first seen	Peak week	NEPM[b] at peak	No. weeks eggs seen
Rhesus								
856	14	14	1	1	0	0	0	0
860	13	18	9	5	23	23	1	1
664	13	16	20	5	0	0	0	0
718	17	17	2	2	0	0	0	0
78	0	0	0	0	0	0	0	0
709	0	0	0	0	24	24	1	1
Pigtail								
109	14	17	6	6	0	0	0	0
108	14	14	9	2	0	0	0	0
Green								
71	12	14	9	5	24	25	1	2
81	0	0	0	0	24	25	1	3
Owl								
2	ND[c]	ND	ND	ND	ND	ND	ND	ND
10	6	6	1	2	0	0	0	0
17	7	12	8	4	0	0	0	0
24	7	12	4	3	0	0	0	0
29	6	15	13	17	0	0	0	0
30	6	6	10	1	0	0	0	0
75	6	13	11	6	0	0	0	0

[a] NEPG — number of eggs per gram
[b] NEPM — number of eggs per milliliter
[c] ND — not done.

revealed that most of the eggs were in the large intestine and liver with some also in the lungs, small intestine and bladder. The adult worms recovered were well developed, mature and of normal length.

Although both monkeys showed erythema of the bladder mucosa at necropsy, no lesions were seen by microscopic examination. The remaining organs were normal when examined grossly, except for esophagostome lesions of the colon and a larval cestode in the mesentery.

The bilharzial lesions seen by microscopy were similar to those of the rhesus; granulomas were seen in the lower colon (Fig. 2) and liver, and portal endophlebitis was marked in one of the animals (Fig. 3).

Green monkey

Both animals of this species became infected. The percent recovery of worms was 19 and 25. The worms were mature, well developed and longer than those recovered from the other species. Although most worms were recovered from the mesenteric veins, a considerable number were also recovered from the inferior vena cava in one animal. Eggs were found in the feces of one of the two animals 12 weeks after exposure and in

Table 4

Number of Schistosoma haematobium *Eggs Found in Various Organs in Relation to the Number of Female Worms Recovered*

Monkey no.[a]	No. female worms	Eggs found × 10^3						
		Liver	Lung	Small intestine	Large intestine	Bladder	Total eggs	Eggs per female
Rhesus								
856	1	.6	0	0	9.7	0	10.4	10.4
860	3	9.5	0.3	0.1	[illegible]	0.4	22.2	7.4
664	26	10.8	1.7	0.2		0	27.1	1.0
718	4	0	0	0		0	0	
78	7	2.7	0	0		0	2.7	0.4
709	8	3.9	0	0		0.1	4.2	0.5
Pigtail								
109	11	6.4	0.5	0.5	[illegible]	0.2	41.6	3.8
108	7	17.9	2.7	0.9	22.9	<0.1	44.6	6.4
Green								
71	13	3.7	2.2	<0.1	21.7	4.4	31.9	2.3
81	11	7.2	0	0.2	3.9	0	11.4	1.0
Owl								
2	71	ND[b]	ND	ND	ND	ND	ND	ND
10	0	0	0	0	0	0	0	
17	0	0	0	0	0	0	0	
24	0	0	0	0	0	0	0	
29	36	325	4.5	0	273	3.2	606	16.8
30	11	2.6	0.5	0	0	0	3.1	0.3
75	0	0	0	0	0	0	0	0

[a] Tree shrew tissues not digested

[b] ND – not determined

the urine of both animals 24 weeks after exposure. While most of the tissue eggs were found in the large intestine, many eggs were also present in the bladder of one animal.

No significant gross lesions were seen in either monkey, except for a foreign body in the bladder of one animal which was accompanied by histologic evidence of non-specific inflammation.

Microscopic examination demonstrated scattered eggs and granulomas in the bowel, liver and lung, but not in the bladder. Pseudotubercles were relatively scant and tended to contain only a single egg. Mild, focal endophlebitis was seen in the portal veins.

Tree shrew

Only one animal of this species was infected. The percent recovery of worms was low (4%), and the worms were found exclusively in the mesenteric veins. As in the other species, the number of male worms recovered greatly exceeded the number of females. No data are available on the egg recoveries from these animals.

No significant gross lesions were observed. A few eggs and granulomas were seen in the colon and liver of the infected animal.

FIG. 1. Rhesus monkey, colon. Composite granulomas in the colonic submucosa; the eggs are degenerate, the lesion is in the healing stage with minimal concentric fibroblastic proliferation and peripheral mononuclear cells. H & E. × 280.

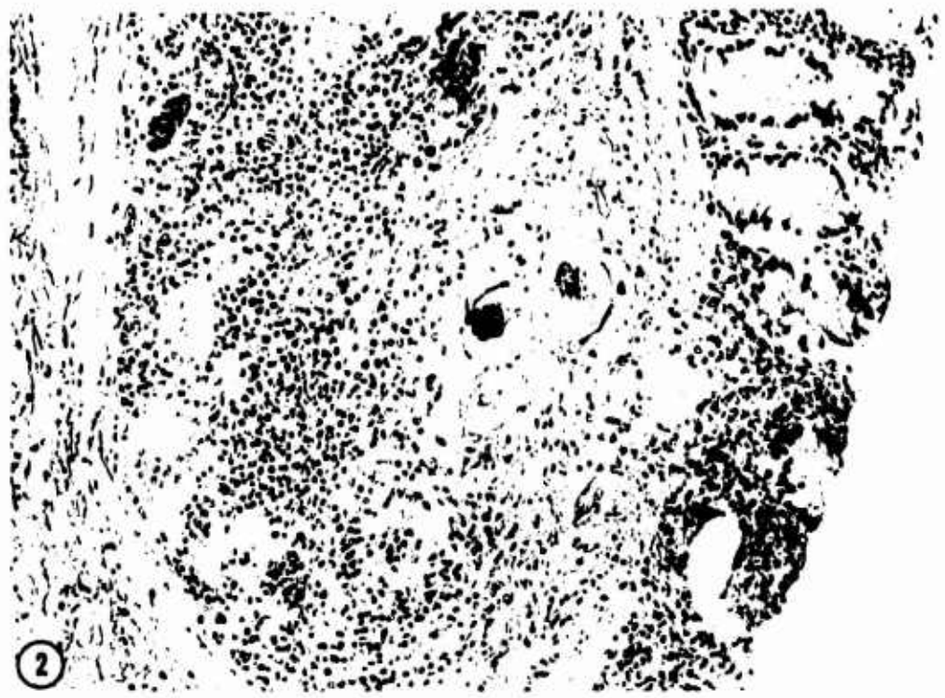


FIG. 2. Pigtail monkey, colon. Earlier composite granuloma with egg shells and degenerate eggs in the epithelioid cell stage, somewhat more superficial, with inflammation of the lamina propria. H & E. × 280.

Table 9

Histopathologic Studies on Five Species of Primates Exposed to Schistosoma haematobium *Infection*

Species	Granulomata in organs studied			
	Liver	Large intestine	Bladder	Lungs
Rhesus				
856	0	+ +	0	+
860	0	+	0	0
661	+	+	0	ND
718	+	0	0	0
78	+	+	0	0
709	+	0	0	0
Pigtail				
109	0	+ + +	0	0
108	+	0	0	0
Green				
71	+	+	+	+
81	+	+	0	0
Tree shrew				
4	+	+	ND	0
3	0	0	0	0
Owl				
10	0	0	0	0
17	0	0	0	0
24	0	0	0	0
29	+ +	ND	+ +	+ +
30	+ +	ND	0	0
75	0	0	0	0

0 = none found; ND = not done; + = scanty; + + = abundant; + + + = very abundant

Owl monkeys

As evidenced by eggs recovered from the feces, all seven animals became infected. However, only three of the seven had worms at necropsy. The percent recovery of adult worms varied from 8 to 49. The worms appeared normal in size in one animal (No. 29) but were stunted in another (No. 30) in which the females had no eggs in utero. Most worms were recovered from the mesenteric and intrahepatic veins. However, in one monkey of this series, nine worms were also recovered from the inferior vena cava. Early in the infection (6-7 weeks) eggs were found in the feces of all animals studied, but no eggs were found in the urine. The highest concentration of eggs in the tissues was in the liver and large intestine.

No gross lesions were observed in 5 of the 7 animals. One monkey had pseudotubercles visible on the liver surface, and 1 - 2.5 mm slightly elevated reddish patches in the bladder, rectum and cecum. Microscopic examination of this animal demonstrated mostly composite granulomas in the liver (Fig. 4), lung (Fig. 5) and bladder (Fig. 6). Liver involvement was relatively intense, with diffuse portal inflammatory infiltration, and prominence of reticuloendothelial cells, but

without portal endophlebitis. Numerous eosinophils were seen in the granulomas and portal spaces (Fig. 4). The lung granulomas were large, poorly delimited and mostly composite (Fig. 5). The lesions of the bladder submucosa were similar; the subepithelial layer adjacent to granulomatous lesions was also mildly and diffusely inflamed (Fig. 6).

Although all of the five primate species studied became experimentally infected with S. haematobium, their susceptibility and the course of infection varied considerably. In general, the pigtail, the green and rhesus monkeys were more susceptible in that worms persisted until necropsy and viable eggs were passed. Most of the worms were found in the portal circulation, and most of the eggs and granulomas were observed in the intestine and liver. However, even in these animals, eggs were found in the urine only occasionally, and they appeared late in the course of the infection and persisted for only a few weeks. The tree shrew demonstrated the most evident natural resistance to this parasite. No eggs were found at any time in the feces or urine of these animals.

It is of interest to note that the green monkeys were not significantly more susceptible to infection than were the rhesus monkeys, though they are more susceptible to S. mansoni than are the rhesus.

Our observation may be influenced by the relatively small number of female worms present, since light infections with S. mansoni do not show as marked a tendency toward self-cure as do heavier infections. However, the observation that a greater percentage of the worms in the green monkeys were found in the mesenteric veins rather than in the liver, as was the case in the rhesus, and that an over-all greater percentage of female worms persisted in the green monkeys, may indicate a more suitable host-parasite relationship with them.

An important indication of natural resistance might be an altered ratio of male to female worms, since the latter may be more vulnerable to the host response. It is noteworthy that, whereas the overall male to female ratio in the pigtail was about 24:1, and in the rhesus, about 10:1, in the green monkey it was about 8:1. This ratio in the owl monkeys was about 2:1, but they were not exposed to the same cercarial pool as were the other species. However, the predominance of male worms in S. haematobium infections in experimental animals is common, and the sex ratio may even vary depending on the time of exposure to the same batch of cercariae; thus our results may be merely a reflection of this phenomenon.

Spontaneous regression of lesions, elimination of eggs in tissues and a tendency toward self cure occurred in the owl monkeys. Although all the Aotus monkeys had eggs in their feces during the period of study only 2 of the 6 exposed to the same cercarial pool had worms present at necropsy, and the worms in one of these were stunted and the females had no eggs in utero. The infection in these animals was characterized by composite granulomas with occasional endophlebitis of the portal veins. In those owl monkeys in which infection persisted, liver

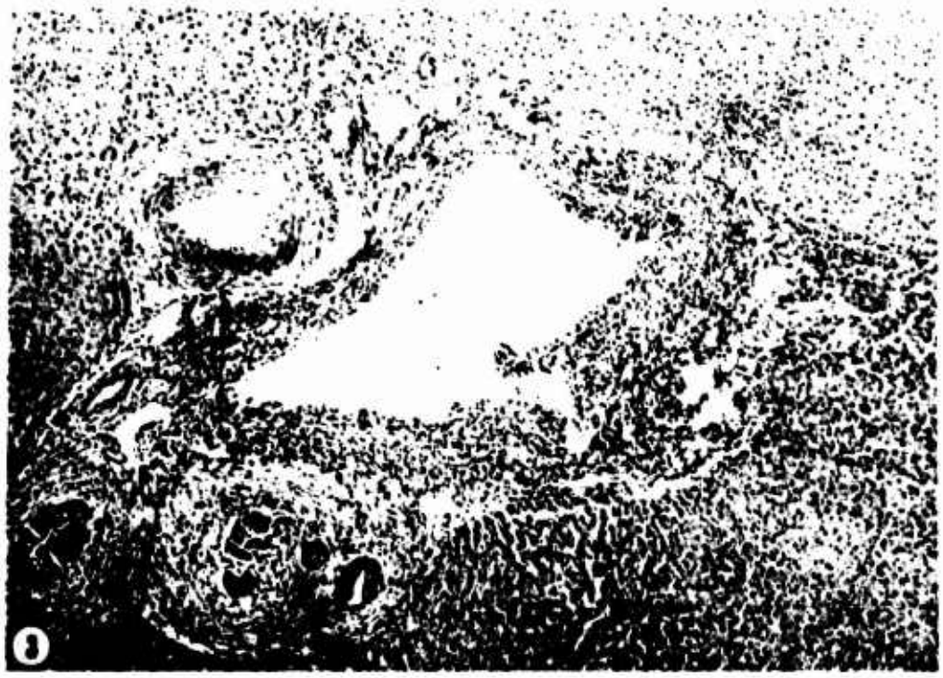


FIG. 3. Pigtail monkey, liver. Portal vein with marked endophlebitis. Note diffuse portal inflammatory cell infiltration, intact artery, bile ducts, and parenchyma. H & E. × 120.

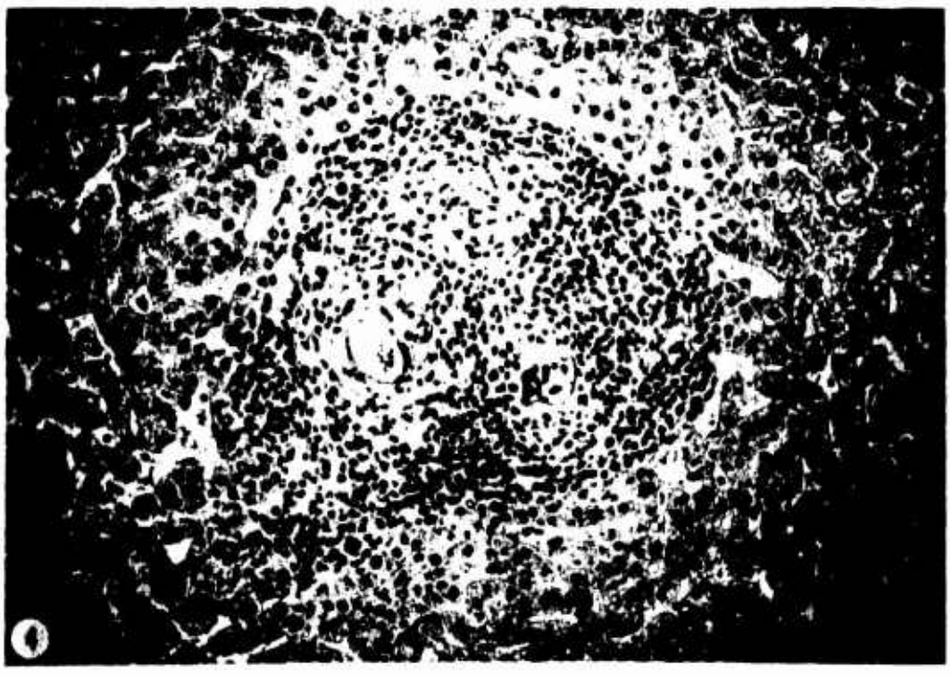


FIG. 4. Owl monkey, liver. Typical composite granuloma; many of the inflammatory cells are eosinophils. There is prominence of Kupffer cells. H & E. × 280.

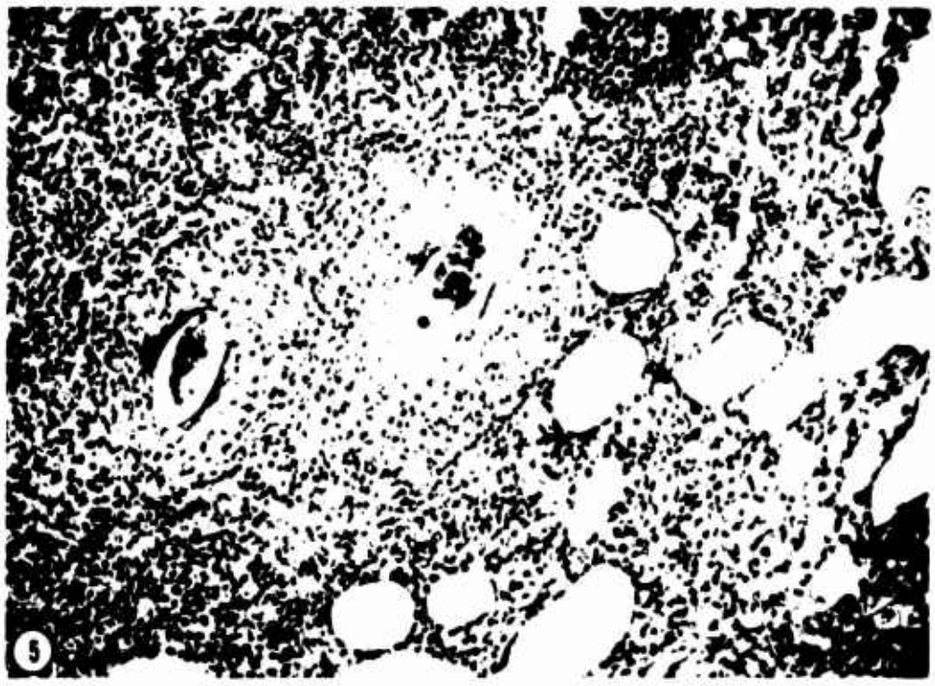


Fig. 5. Owl monkey, lung. Composite granuloma showing two eggs with mature miracidia surrounded by epithelioid cells, eosinophils and lymphoid cells. The infiltration is poorly defined and extends into adjacent alveolar walls. H & E. × 280.

Fig. 6. Owl monkey, urinary bladder. Composite granuloma of subepithelial layer of bladder. The adjacent bladder tissue is mildly inflamed and edematous but the mucosa is intact. H & E. × 120.

involvement was relatively intense with diffuse portal inflammatory infiltration and prominence of reticuloendothelial cells, but without portal endophlebitis. A more detailed analysis of the pathology in this species is being reported separately as a part of a comparative study of three species of human schistosomes in the owl monkeys.

The number of eggs in tissues per female worm for all species (Table 4) was much lower than that in chimpanzees. This may represent differences in the rate of egg production or egg destruction, or both. Cercopithecus and rhesus monkeys apparently destroy eggs deposited in tissues much more rapidly than do chimpanzees.

The present observations suggest that the percent recovery of worms within a few months after exposure to primary infection is not completely reliable as a measure of host susceptibility, since those factors favoring successful migration and maturation of schistosomes are not necessarily the same as those favoring longevity of the parasite and sustained patency of infection. In this respect these observations are essentially similar to those obtained in comparing the susceptibility of 10 species of primates to infection with S. mansoni.

No direct correlation could be observed between susceptibility and the natural habitat of the primates studied. The histologic characteristics of the granulomas were variable as to stage, size and number of eggs per granuloma within the same species and none of them showed lesions distinctive from any other. Variations seemed to be mainly a function of time and intensity of oviposition.

2. Comparison of Schistosoma haematobium, S. mansoni and S. japonicum infections in the owl monkey, Aotus trivirgatus.

Recent observations in the chimpanzee have demonstrated significant differences in the topography, type and severity of lesions resulting from experimental infections with the 3 human schistosome species. Most comparative studies in the past were conducted in small laboratory animals such as mice and hamsters, or in less susceptible hosts where pathologic patterns are different from those observed in man. When primates were studied, comparisons were limited to only 2 parasite species. A comparison of the granulomas around the eggs of the 3 species of human schistosomes was conducted by Warren and Domingo by injecting eggs into the tail vein of mice. They observed that the granuloma formations around S. mansoni and S. haematobium eggs were similar, whereas those around S. japonicum eggs were different and resembled a "foreign body response." Thus, observations by various workers suggest that varying granulomatous reactions to various schistosomes may be a function of the host species as well as of the parasite species.

A search for hosts which might serve as models for human schistosomiasis has been conducted in our laboratories for more than decade. In the course of these studies we observed that the owl, or night monkey

(Aotus trivirgatus) was susceptible to infection with all 3 human schistosomes. This report summarizes observations on the relative susceptibility, course of infection and host response of the owl monkey to the 3 species of human schistosomes.

The Aotus trivirgatus monkeys used were obtained from Colombia through primate dealers and were healthy, active adolescents weighing approximately 900 gm at the time of exposure. Fecal examination prior to exposure revealed no schistosome eggs. Necropsies of a large number of animals of this species used in our malaria research have never yielded evidence of natural schistosome infections.

Cercariae for exposures were obtained from Bulinus sericinus, Biomphalaria glabrata, and Oncomelania nosophora snails infected with S. haematobium (Iranian strain), S. mansoni (Puerto Rican strain) or S. japonicum (Japanese strain), respectively. Animals were exposed while anesthetized with phencyclidine after the right lateral abdominal skin had been shaved and washed with dechlorinated water. Exposures to S. mansoni and S. haematobium cercariae were accomplished by pipetting the desired volume of cercarial suspension into 2.5 x 1.5 cm glass rings hand-held on the skin for 30 minutes. Monkeys were exposed to S. japonicum by placing counted cercariae on the skin with a loop of monofilament nylon. Seven animals were exposed to 230-575 S. haematobium cercariae (Table 6), 6 to 100-1,000 S. mansoni cercariae (Table 7) and 5 to 50-500 S. japonicum cercariae (Table 8). Albino mice or hamsters were exposed to the same cercarial suspensions as infectivity controls. All animals exposed to a given schistosome species were exposed to the same cercarial suspension except for S. haematobium animal No. 2 and S. mansoni animals No. 685 and 689 which were exposed on different days. Fecal examinations for all animals and urine examinations for animals exposed to S. haematobium were begun five weeks after exposure and continued weekly until necropsy. Fecal specimens were concentrated by the Ritchie III technique or the formalin-ether-buffered-alcohol technique. Urine specimens were centrifuged before examination for S. haematobium eggs. The viability of eggs was determined by miracidial hatching.

During necropsy the schistosomes were collected by perfusion. The portal vessels were perfused separately in animals infected with S. haematobium. Worm numbers, distribution and length were determined. Worms were measured unfixed.

All major organs were dissected free after perfusion, gross pathologic changes were noted and specimens were collected for tissue-egg assays and for histopathology. Tissues for histopathology were fixed in neutral buffered 10% formalin and stained with Delafield's hematoxylin and eosin. Selected specimens were also stained with Massons's trichrome. Tissue-egg assays were made after digestion by 4% potassium hydroxide. Mean granuloma size in the liver was measured by techniques described by von Lichtenberg.

Parasitologic observations

Schistosoma haematobium: Worms were found in 3 of the 7 monkeys exposed to this parasite when perfused 16 or 28 weeks after exposure (Table 6). Worms were recovered from the pelvic area in only one animal. The worms in animal No. 30 were only about one-half the length of those in No. 29 (males, 6.3 mm; females, 7.9 mm, vs. 11.3 and 15.8 mm, respectively) and the females had no eggs in utero, in contrast to those in No. 29 which had numerous well-formed eggs in utero. S. haematobium eggs were recovered from the feces of all 7 animals by the seventh week of infection, but eggs were never recovered from the urine. Eggs from the feces hatched normally in dechlorinated water. Fecal egg recovery was sporadic in all animals except No. 29, and eggs were no longer detectable in 5 of the monkeys 16 weeks after exposure. No eggs were recovered from the tissues of the 4 animals which had no worms remaining at necropsy. A few eggs were found in the tissues of monkey No. 30, thus attesting both to the former maturity of the infection and to the host's ability to eliminate both adults and eggs. Tissues from monkey No. 2 were not digested, but many eggs were seen in press preparations of colon and bladder. In monkey No. 29, which continued to have eggs in the feces until the time of necropsy, the cecum and colon had the greatest number of eggs per gram of tissue (Table 9). The liver and large intestine had approximately equal numbers of eggs. Eggs were present both in press preparations of bladder mucosa and in KOH digests of the bladder, although eggs had never been detected in the urine. No diarrhea or other evidence of disease was seen in the monkeys during the course of this infection. Worm recovery in hamsters used as infectivity controls was approximately 7 percent 18 weeks after exposure, and one-third of the worms recovered were females.

Schistosoma mansoni: All six monkeys exposed became infected and had eggs in the feces 7 weeks after exposure. The mean number of eggs per gram of feces reached a peak between 13 and 22 weeks and tended to level off thereafter. The eggs hatched normally in dechlorinated water. Even at the peak of egg output (270 eggs per gram of feces) the animals appeared healthy without any diarrhea or other obvious manifestation of disease. Worm recoveries at 33 and 35 weeks (Table 7) ranged from 37 to 63 percent of the cercarial exposure. The 2 monkeys exposed later than the other 4 (Nos. 029 and 068) and examined after 9 weeks had 24 and 39 percent of the cercarial exposure present as adults. Worm recoveries in mice used as infectivity controls were 47 and 38 percent, respectively, for the two exposure dates. Varying cercarial exposures from 100 to 1,000 cercariae did not significantly affect the percent of worms maturing. There was no evidence that worms were eliminated or stunted in the older infections. The average length of worms recovered from animals perfused 33 or 35 weeks after exposure was 10.5 mm for males and 11.2 mm for females; those examined after 9 weeks averaged 8.4 and 9.2 mm, respectively. Most worms were found paired in the mesenteric veins. Tissue digests demonstrated that the liver contained about 60 percent of the eggs in chronic infections (Table 10) and

Table 6 *Worm recoveries in oral monkeys exposed to* S. haematobium *cercariae*

Animal No.*	No. cercariae	Necropsy (weeks)	Worm recoveries: Inferior vena cava Male	Inferior vena cava Female	Mesenteric veins Male	Mesenteric veins Female	Liver Male	Liver Female	Total Male	Total Female	Total Both sexes	Total Percent
17	230	28	0	0	0	0	0	0	0	0	0	0
75	230	28	0	0	0	0	0	0	0	0	0	0
29	230	28	0	0	42	35	14	1	56	36	92	40
2	500	16	6	3	126	56	45	12	174	71	245	49
10	575	28	0	0	0	0	0	0	0	0	0	0
24	575	28	0	0	0	0	0	0	0	0	0	0
30	575	28	0	0	17	7	15	4	32	11	43	8

* Monkey No. 2 exposed on a different day than the others.

Table 7 *Worm recoveries in oral monkeys exposed to* Schistosoma mansoni *cercariae.*

Animal No.	No. cercariae	Necropsy (weeks)	Worm recoveries: Mesenteric veins Male	Mesenteric veins Female	Liver Male	Liver Female	Total Male	Total Female	Total Both sexes	Total Percent
22	100	33	20	18	4	1	24	19	43	43
16	500	33	105	96	9	5	114	101	215	43
42	500	35	77	74	20	16	97	90	186	37
13	500	35	153	155	3	2	156	160	316	63
685	300	9	18	25	14	16	32	41	73	24
659	1,000	9	89	100	83	115	172	215	387	39

Table 8 *Worm recoveries in oral monkeys exposed to* Schistosoma [illegible] *cercariae.*

Animal No.	No. cercariae	Necropsy (weeks)	Worm recoveries: Mesenteric veins Male	Mesenteric veins Female	Liver Male	Liver Female	Total Male	Total Female	Total Both sexes	Total Percent
1*	50	33	1	2	0	0	1	2	3	6
2	500	19	50	48	14	12	64	54	122	24
3	500	7	67	67	0	2	67	69	136	27
4	250	8	27	33	6	4	41	27	68	28
5*	250	8	5	2	14	13	19	15	34	14

* Animal [illegible] exposed on [illegible]

Table 9 *Number of* Schistosoma haematobium *eggs found in organs of oral monkeys after 28 weeks*

Animal No.	No. females	Eggs	Mean number of eggs per worm (and eggs per female) in: Liver	Colon	Ovaries	Total recovered	Eggs per female worm per week
24	36	0.5 (0.61)	12.1 (46.53)	21.5 (67.8)	1.0 (10.4)	6.0 (12.2)	7.15
30	11	< 0.2	< 0.1	0	0	0	

* Eggs per female worm per week [illegible]

67 percent in acute infections. Few eggs were found in the lungs, and those seen were only in older infections. The average number of eggs per female worm per week was 49 in acute infections and 90 in chronic infections.

Schistosoma japonicum: This parasite produced severe disease and ultimately caused the death of all 5 animals exposed. Eggs were found in the feces of all animals 7 weeks after exposure and hatched normally in dechlorinated water. Bloody-mucoid, diarrheic stools were common during the entire infection in those monkeys which survived only 10 weeks, and, even in animal No. 1 (Table 5) which had only a few schistosomes, patches of blood and mucus were usually present in the feces throughout the course of infection. Except for monkeys No. 1 and No. 5 which were perfused more than 24 hours after death, worm recoveries were relatively uniform. The worms were usually found paired in the mesenteric veins. Worm recovery in mice used as infectivity controls was 60 percent. Worms in the monkeys perfused 7 weeks after exposure had an average length of 10.1 mm for males and 12.7 mm for females. Tissue egg assays revealed that most of the eggs were located in the colon (Table 11). The liver also had a high concentration of eggs. In monkey No. 3, 34% of

Table 10 *Number of* Schistosoma mansoni *eggs found in organs of owl monkeys.*

Animal No.	No. females	Duration (weeks)	Mean number of eggs per gram of tissue (×10³) and per cent of eggs*					Eggs per female worm per week
			Liver	Ileum	Colon	Cecum	Small intestine	
22	19	13	0.1 (1.5)	2.0 (58.8)	0.8 (11.9)	1.1 (7.4)	1.1 (20.4)	108
16	101	13	0.2 (0.7)	5.0 (55.2)	1.8 (21.2)	2.9 (7.9)	2.5 (13.0)	60
12	89	15	0.6 (1.9)	9.1 (66.5)	2.0 (6.0)	10 (20.7)	2.5 (8.0)	99
13	160	15	0.6 (1.1)	9.5 (31.7)	5.7 (10.7)	11 (38.9)	7.2 (14.6)	91
		Avg	(1.3)	(53.1)	(13.0)	(18.7)	(14.0)	90
685	11	9	0 (0)	0.2 (61.9)	0.08 (15.3)	0.1 (11.4)	0.05 (11.5)	23
689	215	9	0 (0)	3.1 (72.4)	0.1 (13.0)	1.3 (4.1)	1.1 (10.2)	75
		Avg	(0)	(67.2)	(14.2)	(7.9)	(10.9)	49

* Figures in parenthesis are the per cent of the total eggs found in organs digested

Table 11 *Number of* Schistosoma japonicum *eggs found in organs of owl monkeys.*

Animal No.	No. females	Duration (weeks)*	Mean number of eggs per gram of tissue (×10³) and per cent of eggs*				Eggs per female worm per week
			Liver	Colon	Cecum	Small intestine	
1	2	31	10.7 (21.8)	70.4 (71.7)	1.3 (0.6)	5.7 (5.9)	13,837
2	61	10	11.2 (9.8)	161 (86.8)	2.1 (0.4)	6.6 (2.9)	3,136
3	69	7	7.1 (14.0)	38.2 (44.5)	18.9 (7.6)	34.2 (33.9)	2,571
4	47	8	5.5 (7.2)	209 (86.4)	17.3 (3.5)	2.7 (0.9)	7,277
5	15	8	1.6 (11.0)	79.0 (86.2)	0.5 (0.3)	1.5 (2.5)	7,108
		Avg†	(10.5)	(76.0)	(3.5)	(10.1)	5,171

* Figures in parenthesis are the per cent of the total eggs found in organs digested
† Average for acute infections, weeks 2 to 5

all tissue eggs counted were from the small intestine, and, as was true in all S. japonicum infected monkeys, most of the eggs found in the small intestine occurred in the proximal one-third. On a proportionate basis, more eggs were found in the liver in the animal (No. 1) which survived 33 weeks than in the others.

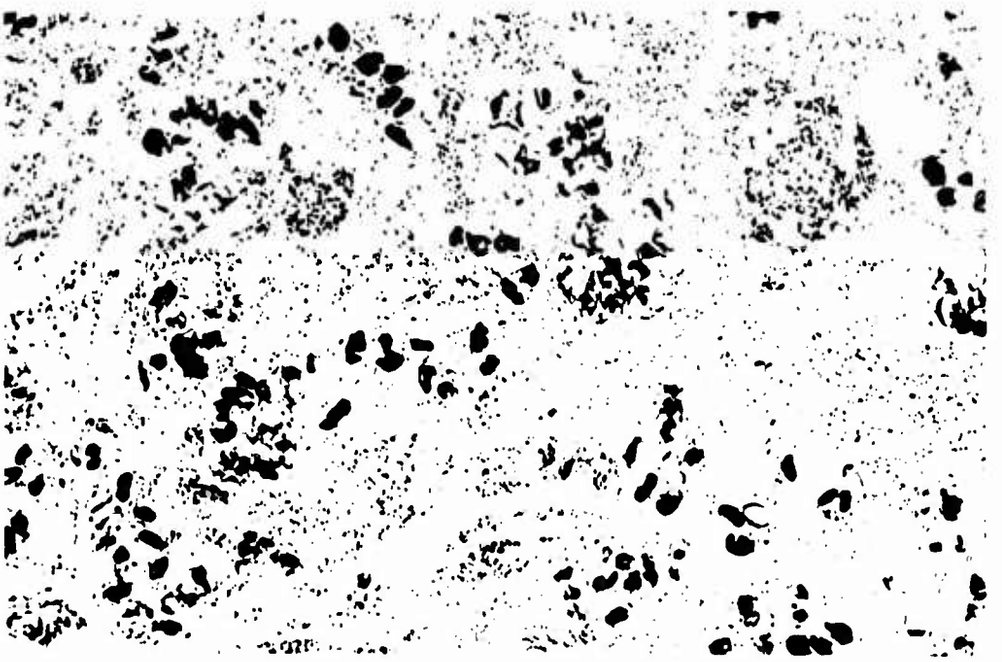
The average number of tissue eggs per female worm per week is probably higher than the true value because of the difficulty in recovering all of the worms from the two animals perfused 24 hours after death after considerable autolysis had occurred. When the number of eggs recovered by tissue digestion was compared with the number of female worms, the values of S. mansoni chronic infections averaged 90 while that for S. haematobium and S. japonicum were 715 and 13,857, respectively. For acute infections the S. mansoni averaged 49 while S. japonicum averaged 5,173.

Pathologic observations: In general, pathologic lesions of note were confined to the liver and gut, with lesser lesions being found in the lung.

Gross pathologic findings: Gross lesions were found at necropsy in only one animal (No. 29) exposed to S. haematobium. Whitish pseudotubercles were visible on the liver surface, and slightly elevated hemorrhagic polypoid-type patches 1-2.5 mm in diameter were present in the bladder, cecum and retosigmoid colon.

All animals infected with S. mansoni had numerous nodular whitish granulomas 0.5-1.5 mm in diameter over most of the surface of the liver and throughout the parenchyma. Animals examined more than 9 weeks after exposure had scattered nodular pseudotubercles (0.5-3 mm in diameter) throughout the gut. These were most common in the cecum and proximal third of the colon. Monkeys examined 9 weeks after exposure had small granulomas on the liver surface, but no other significant gross lesions. In schistosomiasis japonica there were numerous 1-3 mm whitish granulomas on the liver surface and throughout the parenchyma. The distal third of the colon was severely altered, and the wall was thickened to 5 mm and hard. In some animals the proximal third of the small intestine also showed granulomatous nodules. In one animal there was mesenteric lymph node enlargement, and in another, a one cm inflammatory nodule in the omentum.

Microscopic pathologic findings: The histopathologic examination revealed that the distribution of eggs was similar to that determined by tissue egg assays. In general, the most severe lesions were observed in monkeys infected with S. japonicum and the least severe in those with S. haematobium (Table 12). The pattern of egg deposition in the gut of monkeys infected with S. japonicum and S. haematobium [illegible], with large numbers of eggs occurring in some [illegible] in others (Fig. 7). In S. mansoni infections there was a [illegible] pattern of lesions in the gut. While submucosal [illegible] was a prominent feature with S. japonicum and S. [illegible]. It was



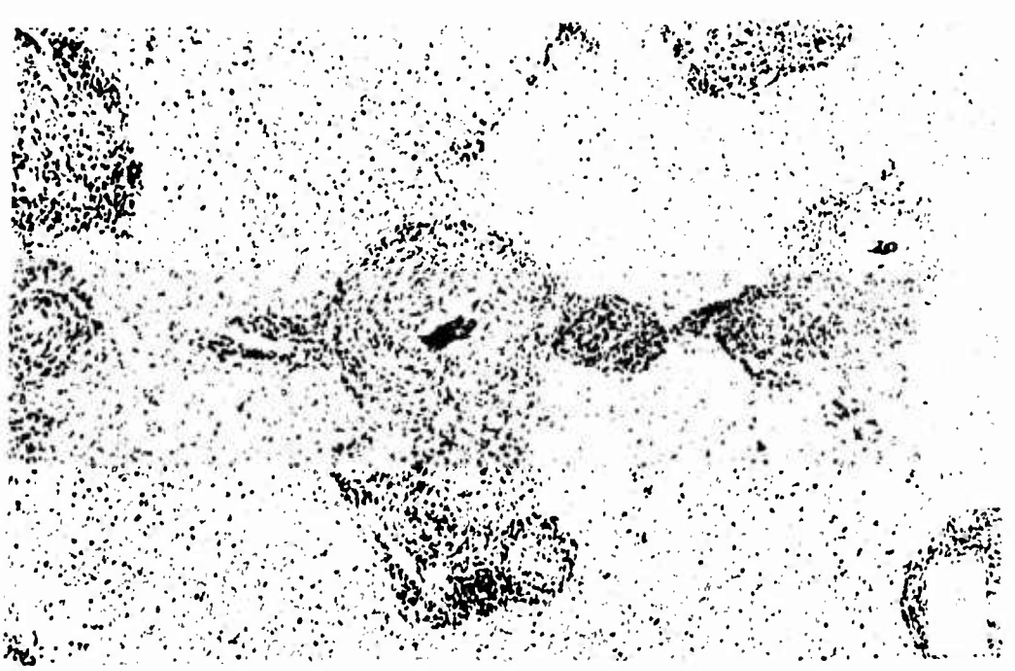


Figure 7. *S. mansoni*, small intestine. H & E. × 50. Massive encapsulation of the lamina propria and submucosa with diffuse inflammatory infiltration forming a "patch." Most eggs are in groups of up to 20, forming confluent or coalescing granulomas. The mucosal architecture is altered by autolysis.

Figure 8. *S. mansoni*, liver. H & E. × 140. Numerous granulomas, showing a single egg in the center of each. The granulomas are concentric and well delineated. Also shown is diffuse portal inflammation and fibrosis, characteristic of the portal veins. The parenchymal architecture is normal.

less marked with S. mansoni infections, though eggs were found in the lamina propria and in the submucosa with all 3 species.

Liver involvement was relatively extensive with all 3 parasite species. In the monkeys infected with S. japonicum and S. mansoni, brown pigment was observed in the Kupffer cells and in the splenic macrophages. Most of the liver seen in the animals infected with S. mansoni (Fig. 8) contained only one egg per lesion. About 50 percent of S. japonicum granulomas contained a single egg, 31 percent had two eggs and 19 percent had 3 or more eggs (Fig. 9). This characteristic was also evident in the gut submucosa and, ectopically, in an omental nodule. Lung granulomas in this infection generally contained a single egg. S. haematobium infected monkeys characteristically had composite granulomas containing up to 20 eggs each in all the organs studied (Fig. 10), including the lungs, while single egg granulomas were relatively scarce. The mean diameter of liver granulomas (Table 12) containing a single egg was greatest in S. mansoni infections, both in the acute and in the chronic stage (Figs. 11, 12, 13). However, the mean diameter of granulomas containing more than one egg in S. japonicum and S. haematobium chronic infections was equal to or greater than single egg granulomas of S. mansoni chronic infections. A few composite granulomas in S. japonicum chronic infections attained 1,080 microns in diameter, and many measured 400-500 microns. The largest granuloma measured in chronic S. mansoni infections was 460 microns. Thus, considering all the granulomas in chronic infections, the mean diameter was greater for S. japonicum than for S. mansoni or S. haematobium.

Table 12 *Pathologic observations in gut mucosa of monkeys infected with three schistosome species*

Species	No. of monkeys	No. female worms	Worms collected	Egg index	Small index	Liver	Liver	Liver	Liver	Remarks
S. haematobium	29	36	28	GR	ND	+	+	+	+	
	30	11	28	ND	ND	+	0	0	0	
	17	0	28	0	0	0	0	0	+	
	75	0	28	0	0	0	0	0	0	
	24	0	28	0	0	0	0	0	0	
	10	0	28	0	0	0	0	0	0	
S. mansoni	43	160	35	+	+	+	+	ND	0	
	16	101	33	GR	GR	+	+	ND		
	12	89	35	GR	GR	+	+	ND	+	
	685	41	9	+	0	+	0	0	0	
	689	215	9	+	0	+	0	ND	+	
S. japonicum	1	2	33	+	0	+	0	ND	+	
	2	61	10	+	+	+		ND	0	
	3	69	7	+			+	ND	+	
	1	37	8			+	0	ND		
	5	15	8			+	0	ND		

0 = None ... ND = Not done; GR = ...

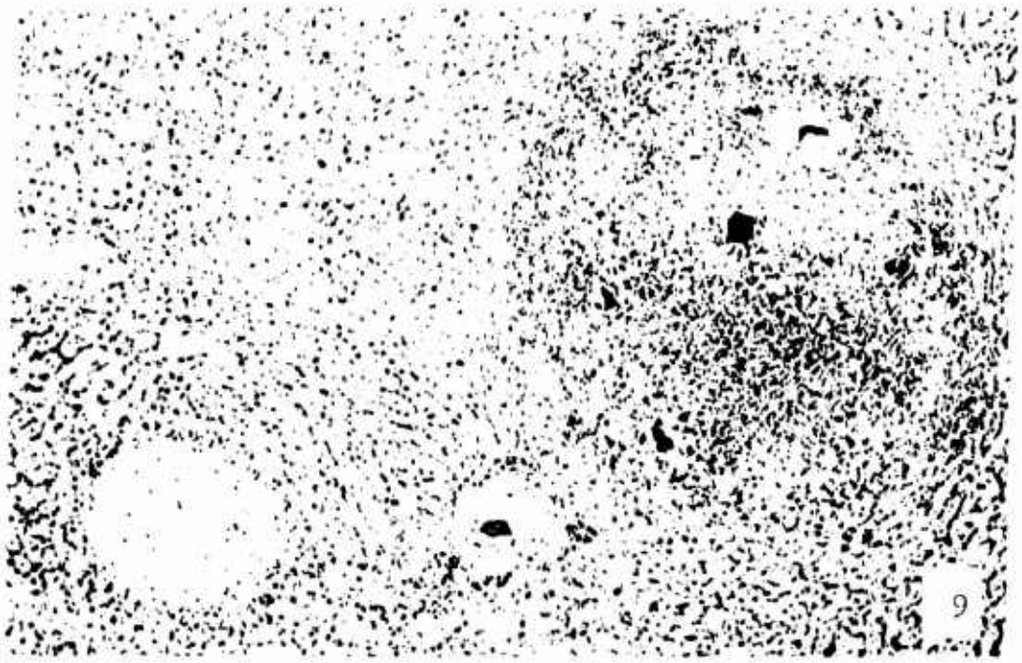


Figure 9 [illegible]

Figure 10 [illegible]

Figure 11 *S. japonicum* liver, H & E, × 350. Fixed tissue, embedded in paraffin and stained with hematoxylin and eosin. Granulomatous reaction surrounded by a peripheral cellular zone. Note the eosinophilic and granulomatic aggregates surrounded by epithelioid cells, giant cells, and fibroblasts in a fibrillar ground substance, and a wide infiltrative zone of cells, there is a concentration of eosinophils and lymphocytes which show perivascular cuffing. Connective tissue proliferation is also present.

Figure 12 *S. mansoni* liver, H & E, × 350. Pseudotubercle around a mature ovum of *S. mansoni* with a central miracidium, surrounded by epithelioid cells in a radial concentric fibrillar connective tissue capsule. Lymphoid cell halo which merges with the diffuse portal infiltrate. The liver parenchyma is normal.

Although the cellular composition in the liver granulomas varied in individual lesions, large numbers of eosinophils were more frequently seen in the granulomas formed around S. haematobium eggs (Fig. 13) than in those around the eggs of the other 2 species. In animals infected for 33 or 35 weeks with S. mansoni, a particularly well defined and delineated type of granuloma, often with central epithelioid cells, a few concentric fibroblasts and a peripheral round cell halo was observed (Fig. 12). A distinctive "edematous" type of granuloma was seen in 4 monkeys which died between 7 and 10 weeks after exposure to S. japonicum and in 2 monkeys killed 9 weeks after exposure to S. mansoni. The cells of the peripheral portion, mostly young fibroblasts mixed with a few polymorphonuclear and mononuclear cells, were widely separated by ground substance, giving the appearance of a loose, relatively acellular lesion (Fig. 11, 14). The peripheral encroachment of these granulomas upon adjacent structures such as bile capillaries and liver end plates was most striking in the largest, composite granulomas (Fig. 14). This "edematous" pattern could be observed occasionally even in lesions in the lung with S. japonicum (Fig. 15). Central necrosis and neutrophil aggregates were generally more pronounced and frequent in infections with S. japonicum than with the other 2 species (Fig. 11, 14). The Hoeppli phenomenon was seen only once in an intestinal lesion of a monkey infected with S. mansoni.

Eggs were found inside portal radicles much more frequently in S. japonicum infections than in infections with the other 2 species, and in several animals, infection with S. japonicum was accompanied by portal thrombophlebitis (Fig. 16). Fibrin clots, inflammatory cell aggregates and granulomas were commonly seen inside of the portal veins, and in one animal a definite adventitial inflammation of the central veins was also visible. Diffuse inflammatory infiltration of the portal connective tissue with predominantly lymphoid cells and eosinophils was seen in all 3 species. Mild focal fibrosis of portal fields was seen around involuting and healed granulomas and was most prominent in chronic infections with S. japonicum (Fig. 9). However, fibrous enlargement of portal fields as in pipe-stem fibrosis was not observed in any of these monkeys infected with one of the three schistosome species.

Table 13 *Mean diameters of liver granulomas for three species of schistosomes in Aotus monkeys.*

Species	Stage	Mean granuloma diameter (microns)[a]		
		1 egg	2 eggs	3 or more
S. *mansoni*	acute	391	ND	ND
	chronic	285	ND	ND
S. *japonicum*	acute	237	332	500
	chronic	179	376	284
S. *haematobium*	acute	ND	ND	ND
	chronic	161	ND	312

Acute = 7 to 10 weeks; chronic = 30 to 35 weeks.
[a] Values from a single animal.
ND = not done

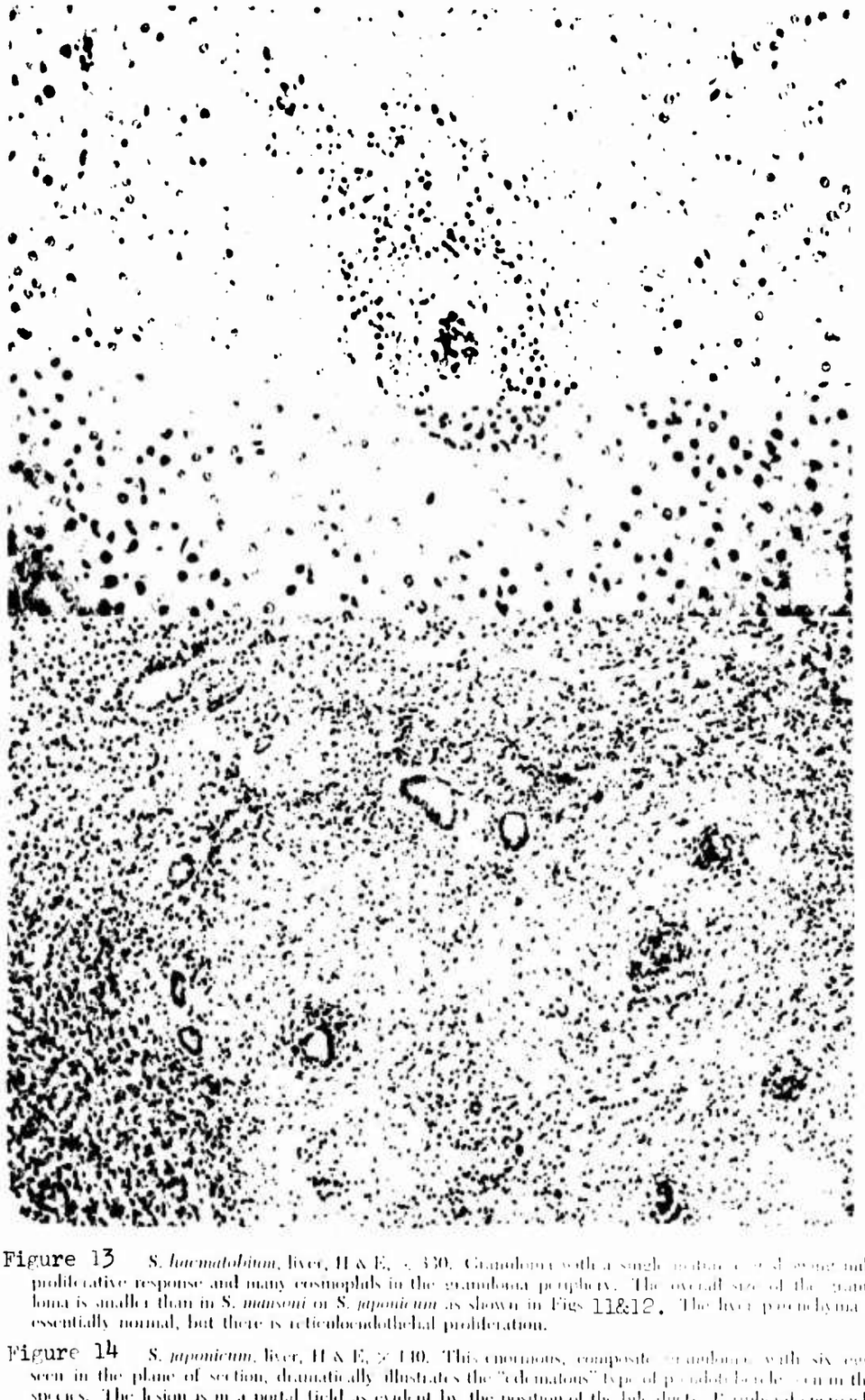


Figure 13 *S. haematobium*, liver, H & E, × 330. Granuloma with a single ovum and a mild proliferative response and many eosinophils in the granuloma periphery. The overall size of the granuloma is smaller than in *S. mansoni* or *S. japonicum* as shown in Figs 11&12. The liver parenchyma is essentially normal, but there is reticuloendothelial proliferation.

Figure 14 *S. japonicum*, liver, H & E, × 140. This enormous, composite granuloma with six eggs seen in the plane of section, dramatically illustrates the "edematous" type of granuloma seen in this species. The lesion is in a portal field as evident by the position of the bile ducts. Peripheral encroachment on the liver end plates with infiltration by inflammatory cells is shown around the granuloma.

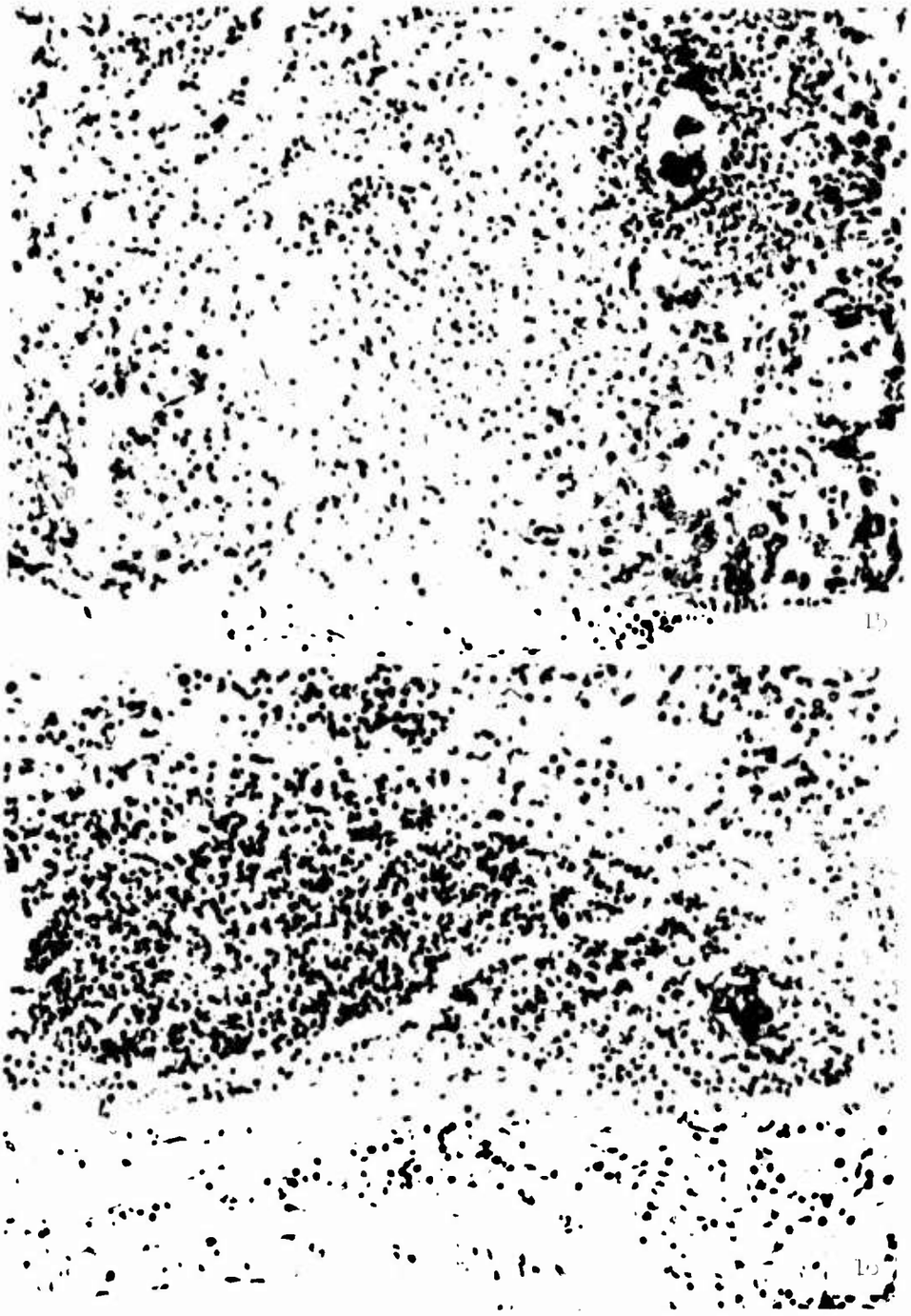


Figure 15. *Streptococcus* (Lancefield Group A). Mouse's Duchenne, × 3.0. Smear from the exudative stage of streptococcal peritonitis. Note the increased fibrin and the phagocytosis of streptococci by polymorphonuclear leukocytes. The smaller dark cells are fibrin deposits.

Figure 16. *Streptococcus viridans* (Lancefield Group H). Mouse's H. & E., × 3.0. Centrilobular hepatic necrosis. Liver cell and Kupffer cell nuclei are pyknotic and fragmented. On the right a thrombosed vessel is seen in the lumen surrounded by leukocytes. The vessel wall is heavily infiltrated, below the zone of pyknotic hepatic necrosis is seen.

In addition to the above described abnormalities which are directly attributable to schistosomiasis, other lesions were observed. One animal infected with S. mansoni had an unidentified helminth in the lung, apparently in a thickened alveolus with no other tissue reaction. Pulmonary edema and congestion were [illegible] in [illegible] animals succumbed to S. japonicum infection. Pyelonephritis was present in 4 out of 16 Aotus examined, and its degree and type varied independently of the severity of bilharzial infection; this lesion was also found in some of the animals which had no worms remaining at necropsy after exposure to S. haematobium. The pyelonephritis was mild, focal, and chronic in 7 animals; severe, chronic, active in another; and severe and acute in another one.

The owl monkey was susceptible to infection by the 3 species of schistosomes pathogenic in man, but the course of infection for different species varied considerably. Infection by S. haematobium was least severe. All monkeys exposed to S. haematobium were infected and passed eggs in their stools, but schistosomes were absent from most animals at the time of necropsy, and there was a marked reduction in size and fertility of the worms in one monkey. None of the monkeys infected with S. haematobium exhibited any outward signs of disease. Thus this host seemed to adapt readily to this infection and reacted by attempting to eliminate it. With S. mansoni infection, a state of equilibrium seemed to be maintained despite heavy worm burdens. Even in those monkeys in which numerous eggs were being shed into the feces, there was no diarrhea or gross blood present in the stools. In contrast, bloody, mucoid stools and diarrhea were common among the monkeys infected with S. japonicum, and the infection was uniformly fatal for them. Tissue digests revealed massive S. japonicum egg concentrations in the large intestine consistent with the severe intestinal disease seen. Both in acute and chronic S. mansoni infections most of the eggs were found in the liver rather than in the intestine as observed with S. japonicum. However, when the tissue-egg retention patterns were compared on the basis of percent of eggs recovered, it was found that the proportion of the eggs in the liver was greater in acute (6 weeks) S. mansoni infections than was found in chronic infections (over 6 weeks). With S. japonicum infections this trend seemed to be reversed. Although there were too few animals on which to base valid conclusions, this difference may have been significant in producing the contrasting manifestations of the disease. A larger proportion of S. mansoni eggs were found in the gut wall later than was the case with the chimpanzee, green monkey and rhesus monkey. However, this may be the result of a shift in worms to this site in order infections to occur in rhesus monkeys. In S. mansoni and S. japonicum infections, fecal and tissue egg counts failed to provide evidence that the rate of oviposition decreased with time or that worms were lost or disintegrated to occur with chronic infections. More extensive studies on the rate of destruction of eggs in tissues and changes in viability with time, similar to those of Cheever and Lower, would be of considerable interest in this host.

The considerably greater rate of oviposition occurring in S. japonicum infections might alone account for its greater virulence in Aotus monkeys, but the pattern of oviposition and the host granulomatous reaction to the eggs of the different species may also be significant. Certainly, if numbers of eggs deposited were alone the predominant factor in the severity of disease, then the Aotus with S. haematobium should have exhibited greater effects than seen here. Although the number of animals was small and the duration of infection varied, our observations suggest that the amount of tissue damage produced by each S. japonicum egg was actually greater than that by each S. mansoni egg, and that it was least with S. haematobium eggs. This characteristic was reported by Meleney et al. for a variety of nonprimate experimental hosts, but was not observed in the egg injection experiments in mice reported by Warren and Domingo. However, it is known that granulomas induced by injection of eggs do not attain the size nor severity of those found in actual infections. In experiments in which attempts to transfer sensitivity to schistosome eggs were made, success was achieved with cells from bisexual infections, but not with cells from unisexual infections or animals injected with eggs and inhibition of the delayed hypersensitivity response which produces granulomas can be accomplished by immunosuppressive drugs, thymectomy and antilymphocyte serum in animals injected with schistosome eggs, but this is not successful in infected animals. Thus, some possibly important differences exist in the host response induced by injected eggs and that induced by actual infection.

The mode of oviposition by the different schistosomes which favors the formation of composite granulomas in animals infected with S. haematobium and S. japonicum may also be significant in determining virulence. S. mansoni females lay eggs singly while S. haematobium and S. japonicum females contain many eggs and deposit them in clusters. Similarly, differences in adult migrating habits may affect the production of lesions. We found a relatively uniform distribution of lesions along the gut with S. mansoni, but a patchy distribution was evident in S. haematobium and S. japonicum infections. Domingo and Warren reported a relatively uniform distribution of eggs throughout the small intestines of mice with S. mansoni infections. A patchy distribution of gut and bladder lesions similar to that seen with the Aotus in this study was a prominent feature of S. haematobium infections in the chimpanzee and S. japonicum infections in mice.

Since the Aotus liver was heavily involved in infections with all 3 species of schistosomes, a detailed histopathologic comparison of the granulomas in the same organ was possible. This showed that, while single-egg granulomas with S. mansoni were larger, the overall granuloma size for S. japonicum was greater due to the frequency of composite lesions. S. japonicum lesions were also more frequently necrotic, were frequently associated with eosinophilia, and tended to displace and destroy adjacent liver tissue. As in human infections single-egg S. haematobium granulomas in the Aotus were relatively small. The similarity of the large composite granulomas found here in the early stages

of both S. mansoni and S. japonicum infections contrasts with the results of egg-injection experiments performed with mice, but agrees with observations made in infected chimpanzees. Schistosome granulomas tend to be largest and most exudative in early and intense infections, as was confirmed here. However, the [illegible] pattern of these granulomas has not been noted previously in other experimental primate hosts studied, although many of these had comparable bilharzial infections. Variations in the cell composition and morphology of schistosome granulomas may be due to a variety of factors. In recent experiments the roles of lysophosphatide compounds and of a granuloma sensitizing factor were analyzed. Both these components appear to be involved in granuloma formation and it is likely that the eggs of different schistosome species might possess different proportions of these and of other active compounds. On the other hand, different host species may vary in the overall capacity of their lymphoreticular system to respond to stimuli or, more specifically, in their degree of reactivity to diffusible schistosome egg antigens. The large, exudative granulomas occurring in early S. japonicum and S. mansoni infections of owl monkeys may represent a particularly vigorous host hypersensitivity response to antigens released by these eggs. This would also account for the marked endophlebitis found in our S. japonicum infections. Evidently the intensity of response to eggs of different schistosome species may vary according to host species, infection intensity and stage.

In spite of heavy egg deposition in the Aotus liver with all 3 schistosome species, alterations in the portal fields were limited to focal inflammation and fibrosis; enlargement of portal fields, as observed in pipe-stem fibrosis in chimpanzees with S. mansoni was not seen in any of the infected owl monkeys. In this respect the Aotus resembled the majority of primate species previously studied with S. mansoni infections. Thus, evidence from experimental infections suggests that the occurrence of diffuse pipe-stem fibrosis is not only related to the size and destructiveness of the granulomas, but also requires a special host predisposition thus far found only in a few host species. Although delayed hypersensitivity in the mouse has been related to granuloma formation, and the latter to portal fibrosis, the role of an immunological factor in the development of pipe-stem fibrosis has not yet been demonstrated.

It is obvious from the literature, and from this study, that both the parasite species and the host species play important roles in the pathogenesis of bilharzial disease. Further comparative studies in hamsters and primates to define these interactions are in progress.

3. Antischistosomal activity of a nitrovinylfuran derivative in rhesus monkeys.

The antischistosomal activity of the nitrovinylfuran, trans-5-amino-3(2-(5-nitro-2 furyl)-vinyl)-1,2,4 oxadiazole, has been observed in mice and hamsters infected with Schistosoma mansoni and in mice infected with S. japonicum. The drug destroyed many adult worms, damaged the reproductive system of those female worms which escaped destruction, was nontoxic to the host in curative doses, and gave no evidence of worms developing resistance to it.

Studies conducted with several other nonantimonial compounds have shown that the antischistosomal activity in rodents does not necessarily parallel the activity in primates and that tests in infected monkeys are necessary before assessing the potential value of these drugs for the treatment of human schistosomiasis. The present studies were designed to evaluate the prophylactic and therapeutic activity of this compound when administered orally to rhesus monkeys experimentally infected with S. mansoni or S. japonicum.

Thirty-three rhesus monkeys (Macaca mulatta) weighing 2.0-4.5 kg at the time of exposure were used in these studies. All monkeys were weighed in at the beginning of drug administration, again one day after cessation of treatment, and on the day of necropsy. They were kept in individual cages and fed a diet of commercial monkey pellets.

S. mansoni cercariae (Puerto Rican strain) used for exposures were obtained from 200 Biomphalaria glabrata snails which had been infected with miracidia hatched from eggs obtained from livers of infected albino mice.

After anesthesia with phencyclidine hydrochloride (1 mg/kg intramuscularly), monkeys were prepared for exposure by clipping the hair on the abdomen and washing the skin with dechlorinated water. While the skin was still damp, 446 cercariae in 1 ml of water were pipetted onto the skin. This was allowed to remain undisturbed for 20 minutes before the animals were replaced in their cages. The 15 monkeys exposed to S. mansoni were allocated to four groups as follows: Group I - treated with SQ 18,506 at 200 mg per kg of body weight twice daily for five days beginning on the day of exposure; Group II - untreated controls; Group III - treated with 300 mg per kg of body weight twice daily for five days beginning 90 days after exposure; and Group IV - untreated controls. The drug was administered by gavage after it was mixed with approximately 20 ml of strained applesauce in a 30 ml syringe, since the absorption of SQ 18,506 is improved when given in this manner. The drug was given at 8 AM and 4 PM daily, and all monkeys were fed immediately thereafter in order to promote better absorption. The monkeys were observed frequently for weight loss, weakness, anorexia, lethargy or other possible side effects of the drug.

Stools were examined for schistosome eggs before exposure. Beginning five weeks after exposure, one-gram samples of stools were concentrated twice weekly by the formalin-ether-buffered alcohol, technique, and the number of eggs per gram of feces was determined.

Necropsies were performed 60 days after completion of treatment, and monkeys were perfused by the method of Hubbe, et al. The mesenteric-portal venous system was perfused separately from the intrahepatic venous system by the appropriate placement of ligatures. Schistosomes used for glycogen assays and for intra vitam staining were perfused from the veins with a buffered glucose-physiological-saline solution; those used only for worm counts and measurements were perfused with physiological saline containing 225 mg phenobarbital sodium per liter. The worms were measured unfixed. The glycogen concentration of male worms was determined by a specific enzymatic micromethod. Damage to the reproductive system of the female worms, determined by an intra vitam staining method, was recorded. The internal organs of all monkeys were removed and examined for gross pathologic lesions after perfusion. Samples of the liver and of each third of the large and small intestines were digested by KOH for determination of eggs per gram of tissue.

Schistosoma japonicum cercariae (Japanese strain) were obtained from 60 Oncomelania nosophora snails which were exposed to miracidia obtained by hatching eggs from livers of infected albino mice. The snails were crushed, checked microscopically for the presence of cercariae and rinsed into a beaker of dechlorinated water. By means of a 4 x 4 cm monofilament nylon loop, 105 cercariae were counted and placed on the damp skin of each monkey prepared as for S. mansoni exposures. The skin in the area of application was kept moist for 5-10 minutes before the animals were returned to their cages.

The [illegible] monkeys exposed to S. japonicum were allocated to four groups as follows: Group I - treated with [illegible] mg/kg of body weight twice daily for 10 days beginning on the seventh day after exposure; Group II - untreated controls; Group III - treated with 100 mg/kg of body weight twice daily for 10 days beginning on the 49th day after exposure; and Group IV - untreated controls. The drug was administered as in the S. mansoni experiment and the same techniques were used to assess the drug's efficacy in both experiments. Four additional uninfected monkeys (Group V) were given the drug, one for each of the regimens used in the two experiments.

Therapeutic efficacy was based on the following criteria: frequency pattern of egg excretion in the feces; the number and distribution of eggs in the liver and intestines; the worm, sex ratio, length and distribution of schistosome adults; the glycogen content of male worms; the presence of abnormalities of the female reproductive system and the errors pathologic changes in the viscera.

No obvious signs of toxicity were observed in any of the treated animals. Group I monkeys gained weight during treatment and demonstrated a net weight increase at the time of necropsy. Group II untreated monkeys gained less weight during the treatment period and exhibited a net mean weight loss at the time of necropsy. Group III monkeys demonstrated less weight loss than untreated infected controls (Group IV) at the end of the treatment period, and registered a net mean weight increase at the time of necropsy, while Group IV monkeys had continued to lose weight. Group VI monkeys lost an average of 252 grams more than did the Group VII infected controls during the treatment period. At the time of necropsy, however, monkeys in Group VI had gained weight, while the untreated control (Group VII) had continued to lose weight. Group VIII monkeys demonstrated a mean weight loss of 82 grams more than that for Group IX untreated controls at the end of the treatment period. These treated monkeys (Group VIII) exhibited a mean weight gain at the time of necropsy, while the untreated controls (Group IX) had continued to lose weight. The Group X monkeys (treated, uninfected controls) given 250 mg per kg twice daily for 5 days, gained weight during treatment. The uninfected monkeys given 500 mg per kg twice daily for 5 or 10 days lost 1[illegible] and 1[illegible] grams, respectively, during treatment.

Schistosoma mansoni: As indicated in Table 14, the monkeys treated for 5 days beginning on the day of exposure had approximately the same worm burdens as their untreated controls (Groups I and II). No significant difference in the distribution of worms between the mesenteric and liver circulation was observed, nor was there any difference observed in the ratio of male to female worms recovered. The worms appeared normal and fully developed. No consistent significant decrease in the eggs found in the feces or in the eggs found in various organs was observed, except for the fact that patency was delayed in the treated animals (Table 15). Gross pathologic lesions exhibited by both groups were typical of S. mansoni infections at this stage and were similar.

When SQ 1[illegible],560 was given 35 days after exposure to infection (Groups III and IV), a 90[illegible] reduction in the worm burden was observed. Moreover, most of the worms recovered appeared to be immature and stunted. Intra vitam staining revealed marked abnormalities in the reproductive organs of a high percentage of female worms. Chemical analysis showed a marked reduction in glycogen in the male worms. Stool examinations conducted at regular intervals up to the time of necropsy showed that the untreated control monkeys exhibited an increasing number of eggs up to the 9th week. The number of eggs found in the stools of the treated monkeys decreased rapidly after treatment, and from 3 weeks after therapy up to the end of the experiment, only occasional eggs were found in the stools of these animals [illegible]. A 9[illegible]% reduction in the number of eggs found by digestion of tissue organs was observed in treated monkeys as contrasted with the untreated controls (Table 15). Gross pathologic lesions were considerably less severe in monkeys treated [illegible] weeks after exposure than in untreated controls.

Table 14

The Prophylactic and Curative Effect of SQ 18,506 in Monkeys Exposed to 0.5 Schistosoma mansoni Cercariae

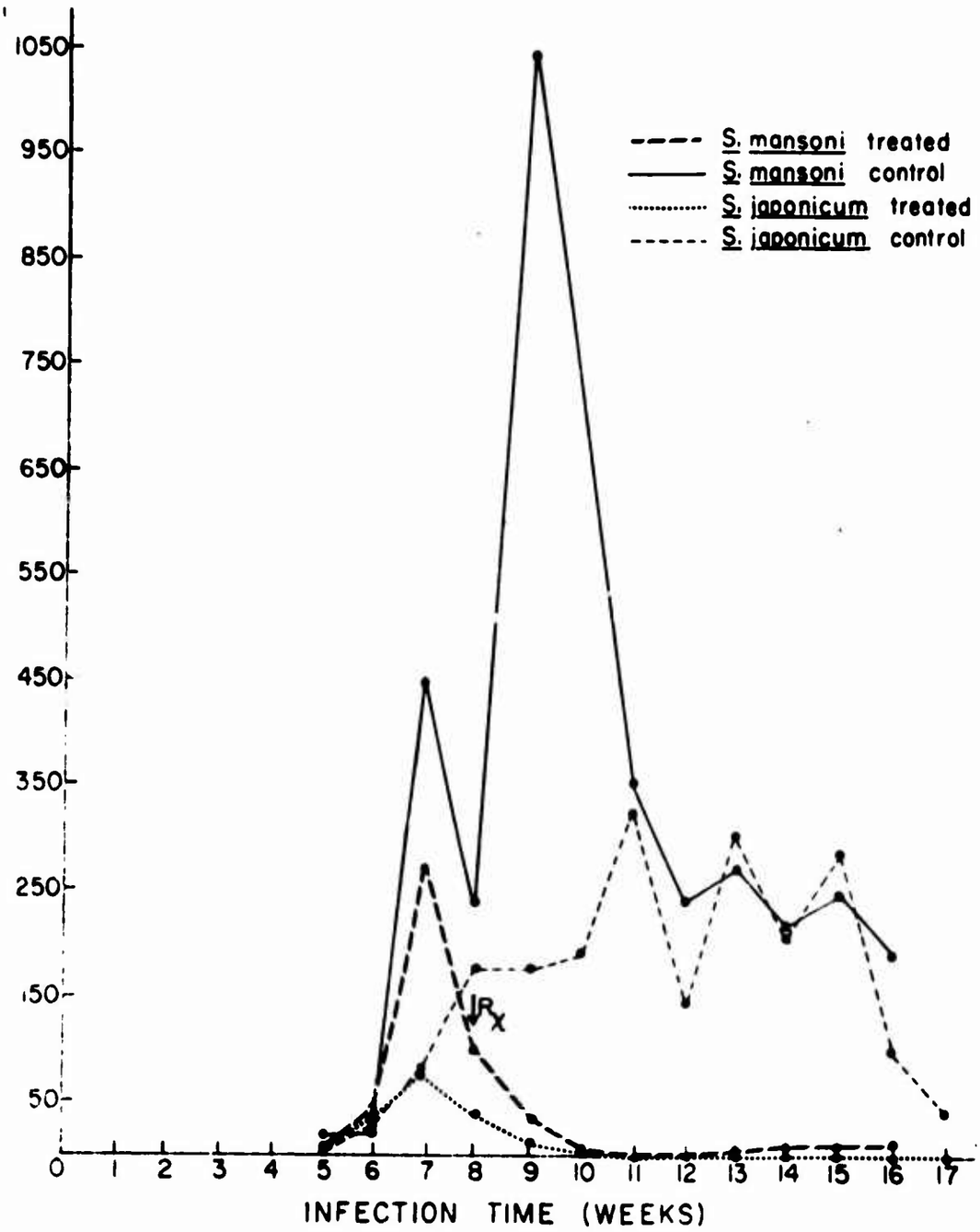
Group No.	Monkey No.	Dose mg/kg	Rx started (from day of exposure)	No. live worms recovered				Length of worms (mm)		Percent abnormal females
				Male	Female	Total	%	Male	Female	
I	1	250-bid 5 days	0	115	105	220	49	12.6	13.7	ND*
	2			139	127	266	60	ND	ND	
	3			159	147	306	69	12.4	13.3	
	4			111	101	212	48	13.3	13.9	
	5			104	89	193	43	12.2	14.0	
Mean				126	113	239	54	12.6	13.7	
II	6	-	No Rx	99	92	191	43	12.2	12.6	ND
	7			118	114	232	52	11.8	13.8	
Mean				109	103	212	48	12.0	13.2	
III	8	500-bid 5 days	56	2	30	32	7	ND	8.3	96
	9			16	13	29	7	8.5	8.4	28
	10			20	21	41	9	7.5	8.1	72
	11			2	6	8	2	ND	7.8	100
	12			0	1	1	<1	ND	ND	100
Mean				8	14	22	5	8.0	8.2	79
IV	13	-	No Rx	147	149	296	67	9.1	11.3	2
	14			101	103	204	46	9.9	12.7	4
	15			116	105	221	50	8.0	10.7	10
Mean				121	119	240	54	9.0	11.6	5
V	16	250-bid-5days		--	--	--	--	--	--	--
	17	500-bid-5days		--	--	--	--	--	--	--

Table 15

Number of S. mansoni Eggs Found in Various Organs in Relation to the Number of Female Worms Recovered and the Fecal Egg Excretion

Group No.	Monkey No.	Dose mg/kg	No. females	No. eggs/gm feces		No. eggs/organ x 10^3*			
				Maximum	Mean	Liver	Large intestine	Small intestine	Total
I	1	250-bid 5 days	105	448	191	93	522	301	916
	2		127	524	253	59	501	234	784
	3		147	520	271	37	738	41	816
	4		101	261	179	ND	ND	ND	ND
	5		89	365	191	79	308	97	484
Mean			113	424	217	67	517	168	750
II	6	-	92	127	31	59	656	31	746
	7		114	1160	317	65	626	67	714
Mean			103	644	174	62	641	49	730
III	8	500-bid 4 days	30	310	27	<1	16	13	30
	9		13	171	22	12	52	10	73
	10		21	693	79	23	101	47	171
	11		6	98	17	0	31	6	37
	12		1	399	69	0	83	53	136
Mean			14	334	43	7	59	26	99
IV	13	-	149	1415	311	73	570	134	777
	14		103	640	244	53	490	165	708
	15		105	2570	433	57	723	117	897
Mean			119	1542	329	61	594	139	794

Figure 19 Schistosoma mansoni and S. japonicum eggs excreted by rhesus monkeys before and after treatment with SQ 18,506



Schistosoma japonicum: Worm burdens in the treated monkeys were significantly reduced (Table 16). Worms of both sexes appeared stunted, and degenerative changes, particularly in the female reproductive organs, were clearly evident. The appearance of eggs in the feces of the untreated controls followed the previously described pattern. Briefly, a peak was reached at 11 weeks, then there was an irregular decline through the remainder of the experiment. In contrast, the passage of eggs in the treated monkeys was almost completely suppressed (Fig. 19). The number of eggs recovered in the various organs at necropsy was considerably lower among the animals of the treated groups (Table 17). Most of the eggs in tissues of treated animals were dark in color and appeared to be dead. Gross pathologic manifestations of infection in the treated monkeys were minimal or were much milder than in control animals. Typical lesions of the liver and intestines were present in untreated control monkeys.

The results, in general, show appreciable antischistosomal activity of SQ 18,506 in rhesus monkeys infected with either S. mansoni or S. japonicum. The doses used did not appear to be toxic to the monkeys. While the lowest dosage schedule used (250 mg/kg b.i.d. for five days) proved ineffective when administered beginning on the day of exposure to cercariae, chemotherapeutic activity was observable when the drug was administered twice a day in doses of 500 mg per kg of body weight beginning on either the [illegible] or fifth day following exposure to infection. Fecal egg excretion decreased rapidly within one week after the beginning of treatment and virtually ceased after 3 weeks. At necropsy the number of worms recovered from treated monkeys was considerably reduced and the few surviving worms were obviously damaged and appeared to be stunted. In particular, damage to the worms was revealed by frequent abnormalities in the female reproductive system and by a marked decrease in glycogen content in the males. A direct effect on the eggs was also observed. As indicated previously, the major drawback of this drug appears to be its poor absorption from the intestinal tract. Studies designed to increase the absorption and to maintain adequate blood and tissue levels are in progress. The antischistosomal activity in both S. mansoni and S. japonicum infections, together with low host toxicity, suggest consideration of further studies designed to test the potential value of this compound as a chemotherapeutic agent in human schistosomiasis.

Table 16

The Prophylactic and Curative Effect of SQ 18,506 in Monkeys Exposed to 105 Schistosoma japonicum Cercariae

Group No.	Monkey No.	Dose mg/kg	Rx started (from day of exposure)	No. live worms recovered				Length of worms (mm)		Percent abnormal females
				Male	Female	Total	%	Male	Female	
VI	18	500-bid 10 days	7	3	1	4	4	11.4	13.1	
	19			0	0	0	-	-	-	
	20			7	5	12	11	8.1	11.4	ND*
	21			26	12	38	36	9.9	12.8	
	22			10	9	19	18	8.8	11.9	
Mean				9	5	14	13	10	12	
VII	23	-	No Rx	20	13	33	31	13.5	19.4	ND
VIII	24	500-bid 10 days	56	4	3	7	7	ND	ND	67
	25			16	13	29	28			79
	26			2	1	3	3			100
	27			7	2	9	9			100
	28			0	0	0	-			ND
Mean				6	4	10	9			87
IX	29	-	No Rx	14	15	29	28	ND	ND	0
	30			17	16	33	31			0
	31			30	33	63	60			8
Mean				20	21	42	40			3
V	32	500-bid 10 days	--	--	--	--	--	--	--	--
	33			--	--	--	--	--	--	--

Table 17

Number of S. japonicum Eggs Found in Various Organs in Relation to the Number of Female Worms Recovered and the Fecal Egg Excretion

Group No.	Monkey No.	Dose mg/kg	No. females	No. eggs/gm feces		No. eggs/organ x 10^3*			
				Maximum	Mean	Liver	Large intestine	Small intestine	Total
VI	18	500-bid 10 days	1	0	0	11	<1	52	63
	19		0	0	0	0	0	0	0
	20		5	0	0	7	7	12	26
	21		12	20	13	45	87	181	312
	22		9	26	14	22	54	31	106
Mean			5	9	5	17	29	55	101
VII	23	-	13	165	98	76	69	571	716
VIII	24	500-bid 10 days	3	13	1	39	24	88	151
	25		13	232	35	193	237	86	516
	26		1	102	13	16	45	65	124
	27		2	30	4	29	17	8	54
	28		0	23	3	29	79	57	165
Mean			4	80	11	61	80	61	202
IX	29	-	15	1115	338	480	342	28	850
	30		16	237	101	138	785	220	1143
	31		33	450	94	266	258	716	1240
Mean			21	601	178	294	461	322	1077

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 165, Parasitic diseases

Literature Cited.

Publications:

1. Colwell, E. J., Armstrong, D. R., Brown, J. D., Duxbury, R. E., Sadun, E. H. and Leyters, L. J.: Epidemiologic and serologic investigations of filariasis in indigenous populations and American soldiers in South Vietnam. The Am. Jour. Trop. Med. & Hyg. 19 (No. 2):227-231, 1970.

2. Sadun, E. H.: Introduction of C. G. Panlit as Charles Franklin Craig Lecturer for 1969. The Am. Jour. Trop. Med. & Hyg. 19 (No. 3): 373-374, 1970.

3. Sullivan, T. J., Carnahan, V. A., and Cutting, F. T.: Filariasis without microfilaremia: A case report. Mil. Med. 135(No. 9), 1970.

4. Gore, R. W., Sadun, E. H. and Hoff, R.: Echinococcus granulosus and E. multilocularis: Soluble antigen fluorescent antibody test. Exper. Parasit. 28:272-279, 1970.

5. Sullivan, T. J.: Tropical Eosinophilia in the Republic of South Vietnam. The Amer. Jour. Trop. Med. & Hyg. 19 (No.6), 1970.

6. Erickson, D. G., Sadun, E. H., Lucia, H., von Lichtenberg, F. and Cheever, A. W.: Schistosoma haematobium: Infections in Five Species of Primates. Exper. Parasit. 29(No. 1):126-137, 1971.

PII Redacted

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

3. AGENCY ACCESSION	4. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6441	71 07 01	DD DR&E(AR)636

5. DATE PREV SUMRY	6. KIND OF SUMMARY	7. SUMMARY SCTY	8. WORK SECURITY	9. REGRADING	9a. OSD/R&T NOTE	9b. SPECIFIC DATA CONTRACTOR ACCESS	9c. LEVEL OF SUM
70 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	3A061102B710	00	166
b. CONTRIBUTING				
c. [redacted]	(DOGI412A(2)			

11. TITLE (Procede with Security Classification Code)

(U) Viral Infections of Man (09)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 010100 Microbiology 003500 Clinical Medicine

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	PROFESSIONAL MAN-YRS	FUNDS (In Thousands)
FY 71 (PRECEDING)	13	400
FY 72 (SUCCEEDING)	13	420

19a. RESPONSIBLE DOD ORGANIZATION

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20. GENERAL USE

Foreign Intelligence Not Considered

21. KEYWORDS (Provide with Security Classification Code)

(U) Virus Diseases; (U) Viruses; (U) Immunology; (U) Ecology; (U) Human Volunteer; (U) Respiratory Tract Infections; (U) Arbovirus Infections

22. TECHNICAL OBJECTIVE 23. APPROACH 24. PROGRESS (Provide brief abstract of work 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) Cortemporary 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) 70 07 - 71 06 Adenovirus type 4 (ADV-4) and ADV-7 vaccines were used for the first time at all 8 basic combat training posts beginning in January 1971. Surveillance data showed that during February and March these vaccines prevented over 11,000 cases of hospitalized acute respiratory diseases compared with the number of cases in past years when no vaccines were used. In September 1970 an ADV-21 vaccine was tested in 10 volunteers at Ft. Detrick; it was shown to be safe, and no vaccine virus was transmitted to susceptible control volunteers. Nine of 10 immunized volunteers shed virus in their stools and had neutralizing antibody responses. Using a broadly reactive ^{14}C labeled ADV-21 antigen, radioimmune diffusion studies showed development of ADV specific IgA and IgM serum antibodies in vaccinees. Seven of 9 vaccinees who shed virus in stools developed ADV specific IgA in stools but none developed IgA antibodies in their nasal secretions. Strains of denque-3 viruses of diverse geographic origins were compared by plaque reduction neutralization tests. DEN-3 strains from the Caribbean and Tahiti were similar but differed significantly from southeast Asian (SEA) strains; a subtype of DEN-3 is defined. Only the physical characteristics of soluble CF antigens could differentiate SEA strains of DEN-1 from prototype DEN-1. No tests could distinguish between type 2 strains. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1970 - 30 June 1971.

DD FORM 1498

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 166, Viral Infections of Man

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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.

1. Evaluation of Adenovirus Type 4 and 7, Live, Oral Vaccines in Man.

Preliminary results of studies conducted to determine the efficacy of adenovirus type 4 (ADV-4) and 7 (ADV-7) live, oral vaccines in a military population at Ft. Dix, N. J., during January through May, 1970, were reported in last year's Annual Report. Laboratory studies have now been completed and the results are reported below. The details of study design, vaccines, sampling and methods were previously presented and are presented again only superficially to acquaint the reader with those aspects of the study.

A. Study #6, Fort Dix Basic Combat Trainees, 1970. Part 1, Control of Acute Respiratory Disease in Recruits with Adenovirus type 4 and 7 Vaccines.

1. Review of Study Design, Vaccines, Sampling and Methods (previously reported).

The study was initiated in basic combat trainees at Fort Dix, N.J., on January 6, 1970. During December, 1969, the weekly ARD rate at

Fort Dix had ranged between 5-7/100 trainees/week and ADV-4 and ADV-7 had been isolated with similar frequency from trainees hospitalized with ARD.

After administrative processing at the Fort Dix Reception Center, trainees enter one of two basic combat training (BCT) brigades. All trainees reporting during a single week (one cohort) are formed into six training companies assigned to one brigade; those reporting during the following week (another cohort) form six companies in the other brigade. This schedule permitted selective immunization of those cohorts of trainees expected to enter the 3rd BCT brigade with both ADV-4 and ADV-7 vaccines and those expected to enter the 2nd BCT brigade with ADV-4 vaccine alone. The pedigree of the ADV-7 vaccine strain and formulation of this live, enteric-coated vaccine (Lot 16CV-02301, Wyeth) is identical to that described in previous annual reports; the vaccine contained between $10^{4.6}$ and $10^{4.7}$ tissue culture infectious dose$_{50}$ ($TCID_{50}$) per tablet when measured by conventional technique. Specifications of the live, enteric-coated ADV-4 vaccine (Lot 16CI-00801, Wyeth) were also described in previous annual reports; the vaccine for this study titered between $10^{3.6}$ and $10^{4.7}$ $TCID_{50}$ per tablet.

Trainees received adenovirus vaccine(s), concomitant with trivalent oral polio vaccines, within 72 hours after arrival on post. All trainees in five separate cohorts of the 2nd BCT brigade (5795 men) were immunized upon arrival with appropriate adenovirus vaccine(s) and respectively formed the two immunized groups in the study (Table 1). These totals exclude those few men who, for administrative reasons, were inappropriately immunized or not immunized at all.

Table 1. Immunization Status of Trainee Cohorts

Vaccine(s)	ADV-4		ADV-4, ADV-7	
	2nd BCT Brigade		3rd BCT Brigade	
Date Immunized	Cohort	No. Men	Cohort	No. Men
9-15 Jan	I	797		
16-22 Jan			II	965
23-29 Jan	III	1,129		
30 Jan- 5 Feb			IV	1,358
6-12 Feb	V	1,094		
13-19 Feb			VI	1,067
20-26 Feb	VII	1,332		
27 Feb- 5 Mar			VIII	1,254
6-12 Mar	IX	998		
13-19 Mar			X	1,151
Total No. of Men		5,350		5,795

Decision to hospitalize all trainees in the [illegible] cohorts rested with dispensary physicians at Fort Dix who were aware [illegible] study, but could not identify the nature of immunization [illegible]. Hospital admissions due to ARD among all trainees in immunized cohorts were recorded and weekly ARD rates calculated. One [illegible] companies which formed each weekly cohort was selected for intensive [illegible] trainees from such study companies who were hospitalized [illegible] were examined by one of the investigators within 24 hours [illegible] and studied for adenovirus disease. A throat washing for virus isolation and acute blood sample were obtained on admission, and a 2-week convalescent serum was drawn on all hospitalized study company trainees except for those absent without leave or those hospitalized during the final week of training.

Virus isolation and identification [illegible] were made using human embryonic kidney (HEK) cell cultures. Serum neutralizing (N) antibody titers to ADV-4 and/or ADV-7 in acute and convalescent serum pairs were determined in HEK cell cultures by [illegible] as described in previous annual reports. A hospitalized trainee from whose respiratory tract an adenovirus was recovered was considered to have been infected with that adenovirus. If virus was not recovered from throat washings, a 4-fold increase in N antibody to either ADV-4 or ADV-7 was considered evidence of infection with that type, provided the trainee was hospitalized after the 3rd week of training, i.e., 2 weeks post-immunization. An antibody increase without virus isolation from trainees hospitalized during the first three weeks of training was the expected consequence of adenovirus immunization. Thus, antibody responses in these individuals could not be considered evidence of adenovirus ARD. Trainees hospitalized after the 3rd training week with N antibody rises to both adenovirus types but without a recovered virus were considered to have an adenovirus infection of undetermined type.

2. Results.

An estimate of whether the method [illegible] hospitalized ARD yielded a study population that was representative of each brigade as a whole was obtained by comparing the gross ARD rates for companies studied intensively with those of their respective brigades as a whole. No significant difference in ARD rates between 2nd brigade study companies and the entire 2nd brigade was found (Table 2). However, 3rd brigade study companies did have a significantly lower total 8-week ARD rate (16.3) than the brigade as a whole (19.7). This difference was due to a higher admission rate in the entire 3rd brigade during the first three weeks of training (8.0 vs 6.0) when ARD admissions were infrequently associated with adenoviruses. ARD rates during the latter part of training (weeks 4-8) did not differ significantly between study companies (10.3) and the entire brigade (11.7). Thus, ARD hospitalizations in the study companies reflected accurately ARD hospitalizations for the entire brigade at the time of training when ARD was caused by adenoviruses.

Table 2. Crude ARD Rates of Study Companies and Entire Brigades

2nd Brigade	Study Companies	Entire Brigade
No. Trainees	805	5350
No. ARD Admissions	258	1623
ARD Rate/100		
Weeks 1-3 of training	10.8*	10.8
Weeks 4-8 of training	21.2*	19.5
Total ARD Rate/100/8 weeks	32.0*	30.3
3rd Brigade		
No. Trainees	911	5795
No. ARD Admissions	149	1144
ARD Rate/100		
Weeks 1-3 of training	6.0**	8.5
Weeks 4-8 of training	10.3*	11.2
Total ARD Rate/100/8 weeks	16.3***	19.7

* $p = > .05$
** Chi square = 6.57, $p = < .02 > .01$
*** Chi square = 5.81, $p = < .02 > .01$

The number of appropriately immunized trainees in the five study companies of each brigade and the total ARD hospitalizations are shown in Table 3. The ARD rate among recruits immunized with both ADV-4 and ADV-7 vaccines was half that of those immunized with ADV-4 alone.

Table 3. Crude ARD Rates of the Study Companies

Vaccine(s)	ADV-4	ADV-4, ADV-7
	2nd Brigade	3rd Brigade
Strength	805	911
No. ARD hospitalizations	258	149
Rate/100/8 weeks	32.0*	16.3

* Chi square = 58.3, $p = < .001$

The total hospitalizations associated with ADV-4 or ADV-7 and the type-specific adenovirus ARD rates for the two study groups are shown in Table 4. ADV-7 was isolated from throat washings of 149 trainees of the 2nd brigade and 8 trainees of the 3rd brigade. Isolation of ADV-7 was associated with four-fold or greater rises in homotypic N antibody in 135 of 142 (95%) trainees in both groups from whom both acute and convalescent sera were tested. Ten additional trainees in the 2nd

brigade and 1 in the 3rd brigade had serologic evidence of ADV-7 infection but no virus recovered from throat washings. The difference in ADV-7 ARD rates between the two groups was highly significant and represented a 95% suppression of ADV-7 disease in the brigade immunized with both adenovirus vaccines.

ADV-4 was isolated from 12 trainees in the 2nd brigade and 37 trainees in the 3rd brigade. Isolation of ADV-4 was associated with diagnostic N antibody rise in all 43 trainees from whom paired sera were obtained for testing. An additional 4 trainees from the 2nd brigade and 12 from the 3rd brigade fulfilled serologic criteria for ADV-4 infection in the absence of recovered virus. In contrast to ADV-7 ARD, ADV-4 disease occurred more frequently in the 3rd brigade trainees. An additional four 3rd brigade trainees were considered to have adenovirus ARD of undetermined type.

Thus, 175 admissions from the 2nd brigade study companies and 62 from those in the 3rd brigade could thus be associated with adenoviruses. The total adenovirus-associated ARD rates from study companies of the two brigades immunized with both adenovirus vaccines was one-third that of the brigade immunized with ADV-4 vaccine alone. No significant difference between rates of ARD due to other than adenoviruses was evident between the 2nd brigade (10.3 /100 /8 weeks) and the 3rd brigade (9.5 /100 /8 weeks).

A total of 5350 trainees entering the 2nd BCT brigade and 5795 trainees in the 3rd brigade were immunized with appropriate adenovirus vaccines. Illness attributable to the immunization was not detected in any of these trainees.

Table 4. Adenovirus-Associated Hospitalization Rates of the Study Companies

Vaccine(s)	ADV-4 2nd Brigade		ADV-4, ADV-7 3rd Brigade	
	Number	Rate/ 100/8 weeks	Number	Rate/ 100/8 weeks
Strength	805		911	
ARD hospitalizations				
ADV-7	159	19.8*	9	1.0
ADV-4	16	2.0**	49	5.4
Type undetermined	0	0.0	4	0.4
Total ADV ARD hospitalizations	175	21.8***	62	6.8

* Chi square = 170.4, p = < .001
** Chi square = 13.4, p = < .001
*** Chi square = 80.0, p = < .001

3. Discussion.

As expected, immunization with both adenovirus vaccines in this population at risk of disease from both adenovirus types was clearly superior to immunization with ADV-4 vaccine alone and led to a 50% suppression of total ARD hospitalizations in 3rd brigade study companies. These companies had 95% less ADV-7 hospitalizations than those in the 2nd brigade and this accounted for the difference in total ARD hospitalization rates between the two brigades. The degree of suppression of ADV-7 ARD by immunization with both vaccines is similar to that reported in previous annual reports in a smaller field trial and is of similar magnitude to the degree of suppression of ADV-4 ARD afforded by the standard ADV-4 vaccine (Edmonston, et al, 1966; Buescher, 1967; Vander Veen, 1968).

In contrast to the marked suppression of ADV-7 ARD admissions in the 3rd brigade, a higher rate of ADV-4 admissions occurred in this brigade. It should be emphasized that this study was not designed to determine to what extent the ADV-7 vaccine interfered with the protective efficacy of the ADV-4 vaccine but rather to detect whether a difference in ADV-4 ARD rates occurred between groups immunized with ADV-4 vaccine alone and with both adenovirus vaccines. Thus no measure of the decrease in protective efficacy of ADV-4 vaccine when given with ADV-7 vaccine was obtained. The difference in rates, however, suggests that simultaneous immunization with ADV-7 vaccine may interfere to some extent with the protective effect of the ADV-4 vaccine. No evidence of such interference was obtained in a previous study (Annual Report, 1970) in which insufficient numbers of vaccinees were tested to reveal the low degree of interference found in the present study.

The practical significance of vaccine virus interference in control of adenovirus ARD is unclear. The potency of the ADV-4 vaccine used in this study was low; all but one of the vaccine tablets tested titered less than $10^{4.0}$ $TCID_{50}$. The antigenicity of ADV-4 vaccines of similar titer have been disappointing in other studies (previous annual reports). It is possible that interference between the two vaccine viruses would be less manifest with a more potent ADV-4 vaccine, a hypothesis amenable to test in the future. In any case, the difference in ADV-4 ARD rates between the two study populations was small (3.4/100/8 weeks) when compared to the difference in ADV-7 ARD rates (18.8/100/8 weeks).

Whether total suppression of adenovirus associated ARD is more desirable than the level of suppression obtained in the 3rd brigade in this study (67%) remains uncertain since it is possible that some transmission of ADV-4 or ADV-7 in an immunized population may serve to prevent less common adenovirus serotypes of military importance (types 21 or 14) from emerging as a cause of ARD. Although adenoviruses other than ADV-4 and ADV-7 were not isolated from immunized trainees in this

study, the possibility of subsequent emergence of other adenoviruses cannot be dismissed. Our findings suggest, however, that these adenovirus types are less likely to be introduced or less likely to be efficiently transmitted in military trainee populations than ADV-7, which routinely emerged in military populations immunized with ADV-4 vaccine alone (Buescher, 1967; Rose et al 1970).

ARD hospitalizations associated with agents other than adenoviruses were not excessive in the 3rd brigade, although Influenza A_2 virus, rhinoviruses, and beta-hemolytic streptococci were recovered sporadically throughout the study from hospitalized trainees in this brigade. Details of these findings appear in Part II of this study (see below). Providing that suppression of ADV-4 and ADV-7 ARD does not lead commonly to emergence of other adenoviruses, immunization of trainees with live adenovirus vaccines to both types seems a promising means of control of ARD in military recruit populations.

B. Study #6, Fort Dix Basic Combat Trainees, 1970, Analysis of Acute Respiratory Disease in Recruits Immunized with Adenovirus Type 4 and 7, Live, Oral Vaccines.

It has been reported in Part I. A of this study that immunization of one brigade of trainees with ADV-4 and ADV-7 vaccines led to a 50% suppression of total ARD hospitalizations, compared with a brigade immunized only with ADV-4 vaccine. This established the protective efficacy of ADV-7 vaccine when given with ADV-4 vaccine to a population at risk to ADV-7 disease. A secondary objective of the study was to assess the role of respiratory pathogens other than ADV-4 and ADV-7 in the brigade of trainees immunized with both adenovirus vaccines.

1. Study Design

Five cohorts, comprising 5,795 trainees entered the 3rd BCT Brigade at Ft. Dix during the study period, and were immunized with ADV-4 and ADV-7 vaccines in the reception center within 48 hours after arrival on post. One training company from each cohort (each cohort contained a total of six companies) was selected for intensive study of all ARD hospitalizations, throughout their 8 week training period.

2. Vaccines and C. Sampling

These aspects were described in detail in the previous year's annual report, and reviewed in Part I. A of this year's report.

3. Methods

Virological, serological and bacteriologic methods were for the most part described in detail in the previous year's Annual Report. In addition to these methodologies, the following were also utilized.

a. Rhinovirus serotyping: Suspect rhinovirus strains, isolated from nasal washes obtained from hospitalized 3rd brigade trainees were tested for chloroform and acid sensitivity by standard methods. Those strains found to be chloroform resistant but acid sensitive were submitted to Dr. Edwin Lennette, Chief, Viral and Rickettsial Disease Laboratory, California, Department of Public Health, Berkeley, California, for final serotype identification.

b. Suspect strains of beta-hemolytic streptococci were grown in pure culture and submitted to Dr. Lewis W. Wannamaker, Director, Streptococcal and Staphylococcal Disease Commission, Armed Forces Epidemiology Board for final T and M protein serotype identification in his laboratory at the University of Minnesota. His laboratory employs standard procedures for determination of bacitracin sensitivity, T-slide agglutination pattern and M serotyping.

c. Isolation of rubella virus was attempted from throat washings obtained from all hospitalized individuals who exhibited any kind of a maculo-papular rash upon admission. Three African green monkey kidney tissue culture monolayers were inoculated with 0.3 ml throat wash each, then challenged on days 5 and 9 with ECHO-11 virus and observed for enterovirus cytopathic effect. Monolayers which were resistant to ECHO-11 challenge were considered to be infected with rubella virus. Representative isolates were confirmed by plaque neutralization using hyperimmune rubella antisera. Supernatant tissue culture fluid from all negative throat washes was subjected to one blind passage and challenge.

Rubella hemagglutination-inhibition antibody titers, employing dextransulfate treated sera were performed on acute and convalescent sera obtained from all suspect clinical rubella admissions as well as from all other admissions from whom no respiratory viral or bacterial pathogens were isolated.

4. Results

There were 911 trainees in the 5 intensively studied training companies; all were immunized with both ADV-4 and ADV-7 live, oral vaccines during the 8-week training period. Hospital admissions in this group, as shown in Table 5, were predominately due to respiratory infections.

Table 5. Hospital Admissions, 3rd BCT Brigade Study Companies

	Number	Rate/100/8 weeks
Hospital Admissions	149	16.4
Nonrespiratory Admissions	18	2.0
Rubella (15)		
Suspect typhoid immunization reactions (3)		
Total No. Respiratory Admissions	131	14.4
Uncomplicated ARD (113)		
ARD complicated by pneumonia (17)		

a. Nonrespiratory Admissions

Admission criteria governing hospitalization for acute respiratory disease vary only slightly throughout CONUS BCT posts, with temperature over 100°F. being the main determinant for admission. Despite this, individuals were admitted to the ARD ward with rubella who

were only mildly febrile (98.6-99.6°F.). Disease was clinically evident in 14 of these individuals and the diagnosis confirmed by isolation and antibody rise (9), isolation alone (1--no convalescent sera was available) and 4-fold or greater HAI antibody response alone (4). One individual who did not manifest clinical disease had a 4-fold HAI antibody response and, in the absence of infection by other respiratory pathogens, he was considered to have been admitted because of rubella.

Three individuals were admitted to the ARD ward within six hours following their initial typhoid immunization, all complaining of sore arms and all with fever. No evidence of infection by respiratory pathogens was found in these instances and they are thus also excluded from further consideration.

b. Uncomplicated Acute Respiratory Disease Admissions.

One hundred thirty-one men were admitted to the ARD wards with apparent bonafide acute febrile respiratory disease, yielding a rate of 14.4/100/8 weeks. Of these admissions, 17 were complicated by pneumonia (13%) and the remaining 114 (87%) were judged to be uncomplicated acute upper respiratory disease admissions. Table 6 shows the results of the virologic, bacteriologic and serologic evaluations of the 131 ARD hospital admissions. The lefthand column lists the various respiratory pathogens included in the evaluation of each case and the criteria used in assigning individuals to the different agent categories are given at the bottom of the table.

The table comprises three broad categories: First, those admissions which were associated with the recovery of and/or serologic response to a single respiratory pathogen; second, those admissions associated with recovery of two or more agents and/or serologic responses and finally those admissions which could not be associated with any identifiable agents, either by isolation or serologic tests.

Table 6. Uncomplicated ARD Admissions

Infection due to Single Agents	Week of Training 1	2	3	4	5	6	7	8	Total	Rate /100
A. ADV-7	0	0	1	1	1	4	0	1	8	
B. ADV-4	0	2	1	9	8	9	6	0	35	5.2
C. ADV, type undetermined	0	0	0	1	2	1	0	0	4	
D. Influenza A_2	5	1	0	0	0	1	0	0	7	
E. Rhinoviruses	1	2	4	1	2	0	0	0	10	2.2
F. Group A. streptococci	0	0	0	2	0	0	0	0	2	
G. Herpes Virus hominis	0	1	0	1	0	0	0	0	2	
H. Mycoplasma	0	0	0	0	0	0	0	0	0	
Infection due to Multiple Agents										
No. admissions	1	2	2	3	1	0	2	0	11	1.2
										8.6
Total admissions associated with infection(s)	7	8	8	18	14	15	8	1	79	8.6
Total admissions associated with unidentifiable agents	5	0	7	4	3	3	4	0	35	4.1
									114	

Infection criteria: A & B = isolate plus N antibody rise; N antibody rise alone after 3rd week.
C = N antibody rise after 3rd week to ADV-4-7
D = isolate and/or CF antibody rise.
E,F,G = isolate.
H = CF antibody rise

ARD admissions associated with single pathogens

Evidence for infection by a single agent was obtained from 68 of 131 ARD hospital admissions. As was discussed in the first section of the report, 47 of these 68 admissions were associated with adenovirus infection--8 with type 7, and 35 with type 4, and 4, type undetermined (but either type 4 or type 7). Criteria for adenovirus infection included isolation of the virus with a homotypic neutralizing antibody response, neutralizing antibody response alone after the 3rd week of training, isolation alone and finally for the 4 individuals with undetermined type infection, neutralizing antibody responses to both type 4 and 7 after the 3rd week of training. Neutralizing antibody responses to either ADV 4 or 7 during the first three weeks training could be the expected consequence of immunization and thus could not be considered as evidence for natural or wild adenovirus infection. Seven individuals had evidence of Influenza A_2 (Hong Kong 168) infections and, as expected the majority (6) occurred in the first two weeks of training. Influenza A_2 was isolated from 3 of the 7 cases and 6 of the 7 showed 4-fold or greater Influenza A_2 complement fixing (CF) antibody rises.

Rhinoviruses were the only agents isolated from 10 different individuals and of those isolates, 7 were typable with available rhinovirus immunotyping sera. The following types were obtained:

Table 7.

No. Rhinovirus (RV) Isolates	Immunotype
1	RV 2
2	RV 16
1	RV 34
1	RV 51
2	RV 53
3	Unidentified*

*Tested against 89 prototypes RV except the following immunotypes: RV 57, RV 62, RV 71, RV 72, RV 73.

Like Influenza, these agents were associated with hospitalizations that occurred early in training.

Isolation of a group A beta-hemolytic streptococcus as the sole respiratory pathogen accounted for two admissions from the same training company, during the same week. Neither strain was M typable but both were bacitracin sensitive and had a 12/13 T-agglutination pattern.

Two individuals proved to have herpes simplex as the sole respiratory pathogen and no individuals had mycoplasma CF antibody responses.

Thus, 68 of 114 uncomplicated admissions (61.4%) were associated with evidence for infection by a single respiratory pathogen. Nontheless, despite immunization with both ADV-4 and ADV-7 vaccines, adenovirus infections (primarily type 4) were associated with over twice as many hospitalizations (47) as nonadenovirus agents (21). Possible explanations for the excess number of ADV-4 admissions have been presented in earlier parts of this report--vaccine interference and/or a relatively impotent ADV-4 vaccine compared to the ADV-7 vaccine.

ARD admissions associated with multiple pathogens

Eleven of 114 uncomplicated ARD admissions (10.3%) were found to have evidence of infection by two or more respiratory pathogens. These findings are presented in detail in the next table.

Table 8. ARD Admissions Associated with Multiple Agents

Week of Training	No. of Admissions	Agents
1	1	(M. pneumoniae)*+ (Influenza A_2)
2	2	Influenza A_2 ϕ + Group A Strep, T-28, M-Neg %
		Rhinovirus (RV) type 34 + Group A Strep, T-11, M-neg
3	2	ADV-7 ϕ + RV type 53 + (Influenza A_2)
		ADV-4 + RV type unident #
4	3	ADV-4 + (Influenza A_2
		ADV-4 + (Influenza A_2)
		Group A Strep, T-12, M-12 + (ADV-4)
5	1	ADV-4+ RV type 43
6	0	
7	2	ADV-4 + RV type 53
		ADV-4 + (Influenza A_2)
8	0	

* Parentheses indicate serologic evidence of infection only.

ϕ Adenovirus infections were established by isolation and/or a 4-fold rise in homotypic neutralizing antibody; Influenza A_2 by isolation and CF rise.

% Group A Strep and Rhinovirus infections established by isolation alone.

\# Tested against rhinovirus immunotypes 1 through 89.

Combinations of infectious agents follow no particular pattern and these admissions were distributed throughout the training cycle. Ten of 11 admissions associated with more than one respiratory pathogen involved two agents and one admission was associated with evidence of simultaneous infections by three pathogens (ADV-7, Rhinovirus type 53 and Influenza A_2). Although Influenza A_2 was recovered the throat of one individual, evidence for infections in other individuals with Influenza A_2 relied solely on a 4-fold or greater rise in CF antibody. Influenza vaccine was routinely administered during the first or second week of training so it is possible that immunization could account for some of the CF responses. However, immunization is more often associated with hemagglutinating antibody responses rather than with CF responses which more likely occur with natural infection.

ARD admissions associated with unidentifiable agents

During the course of the study 35 admissions to ARD wards could not be associated with any identifiable respiratory disease agents, accounting for 23.5% of all admissions to ARD wards and 30.7% of those judged to be bonafide respiratory disease admissions. Twenty-one of the 35 admissions (60%) occurred during the first three weeks of training during a time when adenovirus associated ARD does not characteristically occur. As shown on the next table, this represents almost half (21/44) of all respiratory admissions that occurred during training weeks 1-3. During the latter weeks of training there were only 14 ARD admissions associated with unidentifiable agents, and these represent 20% (14/70) of all respiratory admissions during that period of time.

Table 9	Weeks of Training 1-3	4-8	Total-8 weeks
No. Respiratory Admissions Associated with Infectious Agents	23	56	79
No. Respiratory Admissions Associated with Unidentifiable Agents	21	14	35
Total No. Respiratory Disease Admissions	44	70	114

c. ARD admissions complicated by pneumonia

Seventeen individuals were initially admitted to ARD wards and later transferred to the pneumonia ward because of radiologic evidence of pneumonia. This represents 11.4% of all ARD admissions and 14.9% (17/114) of those actually judged to have respiratory disease. The

following table summarizes virologic, serologic and bacteriologic data obtained from these individuals upon admission to the ARD ward, prior to transfer to the penumonia ward.

Table 10. ARD Admissions Complicated by Pneumonia

	Week of Training							
Infectious Agents	1	2	3	4	5	6	7	8
ADV-4			1	(1)		(1)		
Influenza A_2	1					(1)		
Rhinovirus					1*			
Group A Strep					1#			
No identifiable agents	1	4	2	1	0	2	0	0
Total No. Admissions	2	4	3	2	2	2	0	0

Parentheses indicate serologic evidence of infection alone.
* Type unidentified, tested against rhinovirus immunotypes 1-89.
\# T agglutination pattern = 3/31/41, M, nontypable.

ADV-4 was isolated from one individual but in two instances only an ADV-4 neutralizing antibody response could be documented. On individual had an Influenza A_2 isolate as well as a CF antibody response and this admission occurred during the first week of training. An unidentified rhinovirus and a Group A streptococcus were isolated from two other individuals. No evidence of any infection was obtained in 10 cases. None of these admissions were associated with a 4-fold or greater rise in M. pneumonae CF antibody titer.

d. Summary of ARD Hospital Admissions, 3rd BCT Brigade

The following table summarizes the total number and per cent of total (for each of the various categories already described) of all hospital admissions classified for statistical purposes as ARD admissions in 3rd brigade trainees.

Table 11. Per Cent Distribution of ARD Admissions

Category	Number of Admissions	Per Cent
Non-ARD Admissions (rubella, short reactions)	[illegible]	[illegible]
ARD Admissions	13[illegible]	(87.9%)
ARD complicated by pneumonia	17	(11.4%)
Uncomplicated ARD	114	(76.5%)
ADV associated ARD	47	(31.5%)
Non-ADV associated ARD (Influenza, rhinovirus, group A strep, etc.)	21	(14.1%)
ARD associated with multiple agents	11	(7.4%)
ARD associated with unidentifiable agents	35	(23.5%)

Thus, in only 23.5% of all ARD hospital admissions in this group was a reason and/or possible cause for ARI inapparent.

e. Previously reported epidemiologic studies have shown that adenoviruses (types 4, 7 and to a lesser extent type 21) are the major cause of acute febrile respiratory disease requiring hospitalization of U. S. military trainees during basic combat training. During epidemic periods, and depending upon immunity rendered by vaccination, influenza viruses may also produce considerable respiratory disease requiring hospitalization. Although a host of other viral and bacterial pathogens (parainfluenza viruses, rhinoviruses, Coxsackie A-21, group A streptococci, M. pneumoniae, etc.) have caused sporadic disease and even some epidemics in military populations, none of these agents have demonstrated the pathogenic qualities (transmissability, virulence, etc.) ascribed to either adenoviruses or influenza viruses. Thus, when this first large group of trainees was immunized with ADV-4 and ADV-7 vaccines, it was essential to monitor closely the effects of immunization not only to determine vaccine efficacy, but also to see if, under these circumstances, other respiratory pathogens would emerge and exhibit similar capacity to provoke disease like that associated with adenovirus infections.

The results clearly indicate that under the conditions of this particular study no respiratory pathogens replaced the adenoviruses as major or even significant cause of hospitalized ARD. Adenovirus

associated ARD accounted for nearly one-third of the admissions and these were primarily due to ADV-4. As previously discussed (Part A) the ADV-4 vaccine was of marginal potency and clearly less effective than the ADV-7 vaccine.

As expected, small number of Influenza A_2 virus and rhinovirus associated ARD admissions occurred early in training whereas adenovirus associated ARD was found during the latter part of training. As for other potential viral and bacterial respiratory pathogens, no para-influenza virus isolates were found despite the use of appropriate isolation techniques. Coronavirus isolation techniques were not employed. Group A streptococci were found only occasionally, and serologic evidence of M. pneumoniae infection was demonstrated in only one individual in the entire study.

The 11 ARD admissions associated with evidence for the simultaneous occurrence of two or more respiratory infections point out the difficulties of establishing the etiologic basis for ARD hospitalizations in military training populations. Epidemiologic studies limited to the search for only one or two etiologic agents have failed to consider the problem of multiple infections and conclusions regarding causality of disease should be guarded. The availability of several recently developed virologic and serologic techniques not utilized in this study, could perhaps decrease further the number of ARD admissions associated with unidentifiable agents. Sixty per cent of these admissions (21/35) occurred during the first three weeks of training, and it is possible that the addition of rhinovirus and coronavirus serology (and coronavirus isolation) to the battery of tests employed would reduce this number even further. Obviously more numerous and more sensitive techniques will be required to define all the agents of disease in such a group if similar studies are to be contemplated for the future.

Few conclusions should be drawn from the isolation and serologic results obtained from the 17 ARD admissions complicated by pneumonia. It is well recognized that the mere presence of organisms in the upper respiratory tract does not always reflect the cause of lower respiratory tract disease. No upper respiratory infection could be demonstrated in 10 of these cases but no attempt was made to obtain sputum cultures from any of these individuals after they were transferred to the pneumonia ward. The etiologic spectrum of pneumonia in hospitalized recruits is virtually unknown except in those few instances where the evidence for M. pneumonia, in near epidemic proportions, has been obtained.

ADV-4 and ADV-7 vaccines seek to control but not eradicate the two most common causes of ARD requiring hospitalization in recruit populations. Given ADV-4 and ADV-7 vaccines of adequate and equal

potency, coupled with proper timing of administration and usage, control of disease caused by these two viruses can probably be achieved. The results of this study suggest that attempts to control non-adenovirus associated ARD will be more difficult because of the multiplicity of agents and the number that are yet unidentified. Furthermore, if these findings are substantiated by future studies, the question must be asked, "Is control of the various remaining causes of ARD in these populations, in view of low morbidity and absence of mortality, a realistic and practical goal?"

II. Adenovirus Surveillance Program 1970-71

Data is incomplete at the time of writing this report and complete analysis of FY 71 surveillance will be included in next year's report. In this report the use of adenovirus vaccines and their effect on crude ARD rates will be considered for each post (or if appropriate, groups of posts) based on data available beginning July 1, 1970, through March 27, 1971.

Ft. Ord: Beginning as early as July 1970, ARD rates at Ft. Ord exceeded 3/100/week and remained between 2.0 and 3.0 throughout August and September. Surveillance data indicated that 80% or more of disease was caused by ADV-7 and late in August it was decided to immunize incoming recruits at Ft. Ord with ADV-7 vaccine alone. The vaccine used (lot 16 CV-02401, Wyeth) contained $10^{3.8}$ - $10^{4.0}$ $TCID_{50}$ vaccine virus/tablet and was the same lot of vaccine used at Ft. Wood and Ft. Lewis earlier in the year (Feb-Apr). Immunization was initiated on 5 Sept and by 10 Oct all recruits in training were immunized. However, the ARD rates steadily increased from 2.5 on 10 Oct to 6.2 on 12 Dec and by that time, ADV-4 associated ARD was occurring with a frequency equal to ADV-7 disease. Subsequently, representative capsules of vaccine shipped to Ft. Ord were tested for potency simultaneously with tablets from the same lot that had been stored at WRAIR. These titrations showed that vaccine shipped for use at Ft. Ord contained $10^{2.4}$ $TCID_{50}$ virus/tablet, considerably less than $10^{3.8}$ $TCID_{50}$ obtained for vaccine stored at WRAIR. Loss of titer probably reflects improper handling during shipping in late August, since the vaccine wasn't refrigerated when shipped. Loss of potency in part explains lack of effect on ADV-7 associated ARD, but there was still enough effect to bring about the emergence of ADV-4, which by late November was causing two-thirds of adenovirus associated ARD.

New lots of both ADV-4 (16 CI-02901, Wyeth) and ADV-7 vaccines (16 CV-02701 and 16 CV-02801, Wyeth) were released for use in mid-January, 1971. These vaccines contained $10^{5.1}$ (ADV-4) $10^{5.2}$ (ADV-7) and $10^{5.6}$ (ADV-7) $TCID_{50}$ respectively. With the introduction of these vaccines ARD rates at Ft. Ord remained low, ranging between 1.6 and 2.1/100/week and during February adenovirus associated ARD accounted for less than 10% of the total.

Ft. Dix, Ft. Wood, Ft. Lewis

The decision to use available ADV vaccines at these posts beginning on 1 October 1970 was based on prior year's surveillance data. Each post had experienced high rates in November and December in two or more of the four years since surveillance was begun. The available vaccines were less than optimal. ADV-4 vaccine (Lot 16 CI-00801) contained $10^{3.5}$ - $10^{3.8}$ $TCID_{50}$/Tablet and ADV-7 (Lot 16 CV-02301) $10^{4.4}$ and

(16 CV-0240!) $10^{3.8}$ $TCID_{50}$. The experience with these vaccines at all three posts was somewhat similar, as was the experience with the new lots of vaccines after Christmas.

a. Ft. Dix

ARD rates dropped from 3.6 on 17 Oct to 1.6 on 12 Dec, apparently the result of immunization. By 14 Nov, 100% of trainees had been immunized with both old vaccines but the supply of ADV-4 became exhausted. Thus immunization was continued until after Christmas with ADV-7 vaccine alone. Beginning on 16 Jan incoming recruits received the new vaccines, but the ARD rates continued to rise from 1.3 (16 Jan) to 4.8 on 20 Feb because trainees in the latter weeks of training had received only the old ADV-7 vaccine. Subsequently the rate fell to 1.4 on 20 Mar and this may be attributed to immunization with new lots of vaccines.

b. Ft. Wood

Following immunization with old ADV-4 and 7 vaccines, the ARD rate fell slowly from 4.1 on 7 Nov to 2.1 on 19 Dec. After Christmas ARD rates climbed to 4.3 on 20 Feb after which they delined rapidly to 1.4 on 20 Mar. Immunization with new vaccines began in mid-January and as at Ft. Dix, the increased rates in Feb reflect disease in the latter weeks of training among men immunized with the old vaccines.

c. Ft. Lewis

Similarly at Ft. Lewis ARD rates declined from 4.0 on 24 Oct to 1.8 on 28 Nov following immunization with old ADV-4 and 7 vaccines. However, rates then rose abruptly, 3.5 on 5 Dec and 5.4 on 12 Dec at which time surveillance data showed that 68.5% of ARD was not attributable to adenoviruses. No non-adenovirus agents were isolated during that or the proceding week and there is no satisfactory etiologic explanation for the abrupt increase. After Christmas, rates at Ft. Lewis remained low, unlike Ft. Dix and Ft. Wood, and immunization with the new lots of vaccines undoubtedly helped maintain these low rates (Jan average - 1.3/week, Feb. - 1.6/week, and Mar - 1.6/week).

d. Ft. Campbell, Ft. Jackson, Ft. Knox, Ft. Polk

The southern posts, none of which had high ARD rates in the fall, began immunization with new vaccines on 16 January. At each post, vaccines were administered to all recruits in the first five weeks of training and thereafter to each weekly input. Bearing in mind that some of these posts received ADV-4 vaccine alone in Jan - Mar of one or more of the last four surveillance years, there was still a marked reduction in monthly ARD rates obtained for this year when both ADV-4 and 7 vaccines were used compared to monthly averages for the previous four years.

Table 12. Comparison of Monthly ARD Rates/100

		1967-1970 Four Year Average of Monthly ARD Rate/100	1971	% Reduction
Ft. Campbell	Jan	5.6	1.9	66.1
	Feb	22.1	6.2	72.0
	Mar	17.4	7.9	54.6
	Subtotal	45.1	15.0	67.8
Ft. Jackson	Jan	7.0	4.5	35.7
	Feb	14.8	8.0	46.0
	Mar	13.6	11.8	13.2
	Subtotal	35.4	24.3	31.4
Ft. Knox	Jan	2.0	1.6	20.0
	Feb	8.0	4.8	40.0
	Mar	10.5	5.0	52.4
	Subtotal	20.5	11.4	44.4
Ft. Polk	Jan	3.5	3.3	5.7
	Feb	7.4	4.2	43.3
	Mar	9.5	3.6	62.1
	Subtotal	20.4	11.1	45.6
Total 4 Posts		121.4	61.8	49.1

The table indicates that during Jan-Mar of 1971 the number of ARD admissions was reduced by one-half over the average of the four previous years. Per cent reductions ranged from a 67.8% at Ft. Campbell to a 31% at Ft. Jackson. It is of interest that at Ft. Jackson during March, rates hovered around 2.5 - 3.0/week despite the almost total absence of adenovirus associated ARD during that month. No other pathogens had been identified as of the writing of this report.

Thus, the Adenovirus Surveillance System has to date, failed to account for two episodes of increased ARD admissions: One at Ft. Lewis in December, 1970, and the other at Ft. Jackson during March, 1971. Neither increase was associated with recovery of adenoviruses, agents which the system was specifically designed to detect. Sensitive methodology for detecting other respiratory pathogens may have to be incorporated into the system in the future. Aside from these two instances, control of adenovirus disease (and hence, ARD in general) seems possible with availability of potent ADV-4 and ADV-7 vaccines coupled with appropriate timing of their administration to military training populations.

IIIA. Induced Gastrointestinal Infection of Man with Living Adenovirus Vaccine Type 21.

Study #1, Whitecoat Volunteers, September 1970.

The principle causes of Acute Respiratory Diseases (ARD) requiring hospitalization in Basic Combat Trainees (BCT) in CONUS are adenoviruses (ADV) Types 4 and 7. Living, enteric-coated, ADV Type 4 (L-AV-4) and ADV Type 7 (L-AV-7) vaccines have been demonstrated to be highly effective in the suppression of ADV-4 and ADV-7 associated hospitalized ARD as indicated in previous sections of this report. Other adenovirus serotypes that have been associated with ARD in military trainees are ADV-21 and ADV-14. In 1967, ADV-21 caused a significant amount of disease in trainees at Fort Dix, New Jersey. Extensive use of L-AV-4 and L-AV-7 vaccines may lead to the emergence of other adenovirus serotypes as major causes of ARD and ADV-21 is a very likely candidate.

A living, enteric, ADV Type 21 vaccine (L-AV-21) has not previously been evaluated because of the oncogenicity of ADV Type 21 strains in immunologically-incompetent newborn hamsters and the uncertainty of the relationship of adenovirus infections and neoplasia in man. Results of a recent serologic survey of cancer patient sera and matched controls for possible reactions with Adenovirus T antigens (supported by the Solid Tumor Virus group of the NCI, NIH) showed no indication of antibody activity in cancer patient or control sera to the known adenovirus T antigens by the complement fixation test. From these results, it was concluded that adenoviruses do not appear to be involved in production of significant numbers of human tumors, and permission to study efficacy and safety of living ADV Type 21 vaccines in military personnel was granted by the Vaccine Development Branch of NIAID and the AIDRB, OTSG.

The following study was designed to permit evaluation of the safety and immunogenicity of live, oral, enteric ADV Type 21 virus immunization in man.

1. Design of Study:

a. The Study Group: Volunteers were chosen from enlisted personnel participating in PROJECT WHITECOAT. A complete and comprehensive explanation of the study and its risks was given to the enlisted men by the Project Director in the presence of the principal investigators. Following this, each individual was interviewed personally and given an opportunity to ask additional questions and express their desire to participate. A consent statement, on file in the U. S. Army Medical Unit, Fort Detrick, Maryland, was signed by each volunteer.

The volunteer group consisted of 15 men found to be free of ADV-21 antibody (serum dilution <1:2) by tissue culture neutralization

test. Volunteers were housed in individual rooms on two closed wards for the duration of the study; each ward contained both volunteers who received L-AV-21 and those who received placebo enteric capsule. All volunteers shared common recreational and dining facilities. Detailed medical histories and physical examinations were performed on each volunteer on admission to the study wards. Complete hospital records were initiated and maintained on each volunteer. Initial medical evaluation also included an electrocardiogram, chest x-ray (PA and lateral), complete blood count, urinalysis and throat culture.

b. Vaccine Virus Used for Immunization: Adenovirus Type 21 (strain V-270) propagated in human embryonic kidney (HEK) cells was obtained by Wyeth Laboratories from National Institutes of Health. The strain was passaged two times in HEK, then through 11 passes in human diploid fibroblast culture (WI-38), lyophilized, mixed with an inert vehicle, and prepared into enteric-coated capsules (Lot CIX-02101). The capsules were shown to contain an average of $10^{6.3}$ $TCII_{50}$ upon titration of virus in HEK cell cultures. Virus obtained from the capsules was neutralized by hyperimmune ADV Type 21 antiserum in tissue culture neutralization tests. Volunteers not receiving L-AV-21 received an enteric-coated placebo capsule (enteric-coated placebo #4 containing lactose, Wyeth); this preparation was shown to contain no cytopathogenic agent when a liquid suspension of it was inoculated into HEK tissue culture tubes.

Ten volunteers received the adenovirus vaccine (L-AV-21) and five volunteers received the placebo tablet on study day 0.

2. Methods. Blood was obtained at 0800 hours on study days -5, -3, 0 and daily through day 14 and then on day 18 and 21 for white blood cell and differential count, hematocrit and platelet count. Blood was obtained at 0800 hours on study day -5, -3, 0, 4, 7, 14, and 21 for total, direct and indirect bilirubin, SGOT, SGPT, alkaline phosphatase and BUN. Urinalysis was obtained on admission to the study and daily thereafter until day 14, then on days 16, 18, and 21. The above laboratory tests were performed by standard laboratory procedures.

Blood was obtained on days -4, 0, 7, 10, 14, 18, 21, and 28 for serologic studies. Serum neutralization tests were performed on serum samples from the volunteers in HEK tube cultures using ADV Type 21 vaccine strain (V-270). Serum neutralization end-points were determined at a time when the test dose of virus showed 10 $TCID_{50}$ in HEK tube cultures. Adenovirus complement-fixation titers on 0 and 28 day serum samples were determined by standard micro-titer procedures against a commercial adenovirus CF antigen obtained from Microbiological Associates.

Throat washings and stool (or rectal swab) specimens were obtained on each volunteer on study days -4, -3, -2, -1, and day 0 through day 28. 0.3 ml aliquots of each throat wash and 0.3 ml

aliquots of a 10% suspension of each stool sample were inoculated into two HEK tube cultures. Tubes were incubated at 36°C and observed for cytopathic effect (CPE) for seven days. Isolates exhibiting characteristic ADV CPE were typed in tissue culture neutralization tests in HEK tube cultures with specific rabbit ADV type 21 antiserum. Those exhibiting herpesvirus CPE were typed with hyperimmune herpesvirus hominis antiserum, and those exhibiting enterovirus CPE were typed with hyperimmune enterovirus antisera. When either enterovirus or herpesvirus CPE was observed, an aliquot of the original material was treated with appropriate hyperimmune sera and observed for an additional 21 days.

3. Results

a. Patterns of Virus Shedding:

Stool Excretion. Figure 1, [illegible] the 10 volunteers receiving T-AV-21 excreted ADV [illegible] in the stools. ADV-21 shedding was demonstrated first on study day 4 and last on study day 21. Duration of fecal shedding varied between 4 days and 17 days with a mean of 10.1 days. None of the five volunteers receiving the placebo tablet excreted ADV in the stool prior to the study. All ADV isolated were typable as ADV Type 21. In addition, one immunized volunteer (#3) excreted ECHO virus type 6 in his stool from day -4 to day 12 of the study.

Nasopharyngeal Excretion. ADV-21 excretion was not demonstrated in throat washings of either immunized or placebo volunteers. Excretion of herpesvirus hominis was demonstrated in throat washings of three volunteers, all in the T-AV-21 immunized group (Volunteers #3 (day -2 through day 6), #5 (days -4 through -2), and #10 (day -3 and day 1).)

b. Antibody Response:

Immunized Volunteers. Table 3 details ADV-21 neutralization titers of volunteers immunized and placebo, in 0-day and 28-day serum samples against 10 $TCID_{50}$ of the ADV-21 vaccine strain V-270.

FIGURE 1 ADV 21 STOOL EXCRETION IN IMMUNIZED VOLUNTEERS

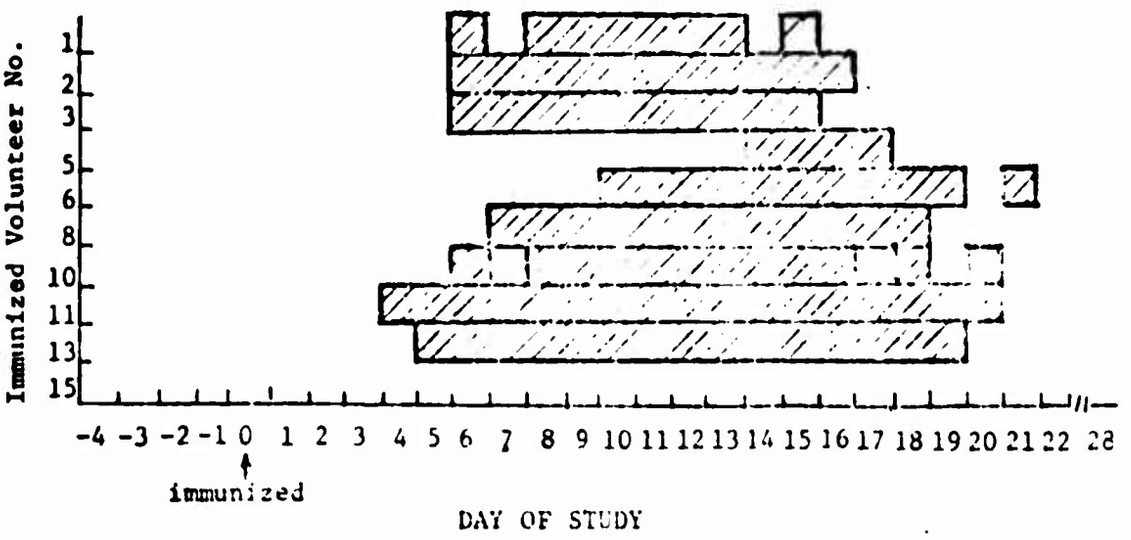


FIGURE 2

ADV-21 STOOL EXCRETION ON STUDY DAYS IN IMMUNIZED VOLUNTEERS

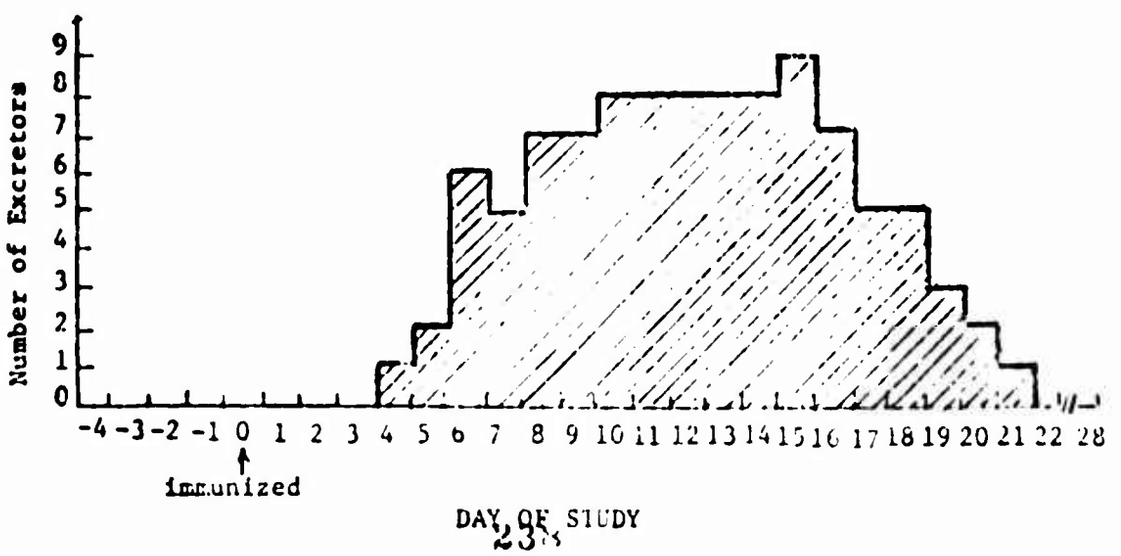


Table 13 Experimental ADV-21 Infection in Man: Neutralizing and Complement Fixing Antibody Responses After Virus Ingestion

Immunized Excretors ADV-21	Volunteer No.	Days ADV-21 Excretion	ADV-21 Neut Titer (Reciprocal) 0 Day	ADV-21 Neut Titer (Reciprocal) 28 Day	ADV CF Titer (Reciprocal) 0 Day	ADV CF Titer (Reciprocal) 28 Day
	1	8	<2	2	5	5
	2	11	<2	32	10	20
	3	10	<2	64	10	10
	5	4	<2	4	<5	<5
	6	11	<2	8	10	10
	8	10	<2	2	10	10
	10	13	<2	32	10	20
	11	17	<2	64	10	10
	13	15	<2	64	10	20
Immunized Non-excretor						
	15	0	<2	<2	20	10
Placebo	4	0	<2	<2	10	10
	7	0	<2	<2	10	20
	9	0	<2	<2	10	20
	12	0	<2	<2	20	10
	14	0	<2	<2	20	40

Neutralizing antibody was not detected in the 28-day serum of the five placebo volunteers or in the one immunized volunteer who failed to excrete ADV-21. Antibody responses of the nine immunized ADV-21 excretors were variable; two showed detectable N antibody only at a 1:2 serum dilution whereas two others had N titers of 1:64. Repeat neutralization tests using a wild ADV-21 isolated at Fort Dix, New Jersey in 1967 gave similar results.

In addition to an ADV-21 N antibody response, volunteer #3 who excreted ECHO-6 virus, had a rise in ECHO-6 N antibody titer from <1:4 on day-4 to 1:128 on day 14.

c. Clinical Response to Immunization: Mild, afebrile upper respiratory disease and/or diarrhea was noted in three immunized and two control volunteers.

Relationship of these illnesses to ADV-21 stool excretion are summarized in Table 14.

Table 14 Experimental ADV-21 Infection in Man: Illness in 5 Volunteers

Volunteer #	Stool Excretion ADV-21 (Study days)	Respiratory Symptoms (Study days)	Diarrhea (Study days)
A. Immunized			
15	none	+7	+9 - +16
10	+6, +8 - +16, +18, +20	+13 - +14	--
1	+6, +8 - +13, +15	--	+16 - +18
B. Control			
4	none	-4 -0	+16
12	none	--	+20

Volunteer #15 who failed to excrete ADV-21 in his stool had intermittent diarrhea from study day +9 to +16. Diarrhea in three other volunteers was observed between day +16 and +20. No viral pathogens or adenovirus Type 21 were isolated from stools collected during these periods. The volunteer who excreted ECHO virus Type 6 in his stool (day -4 to +12) and adenovirus (day +6 to +15) as well as herpesvirus in throat (day -2 to +8) was entirely asymptomatic throughout the study period.

One immunized volunteer (#13) had a 24-hour afebrile illness consisting of mild malaise, headache and backache accompanied only by a mildly injected conjunctivae. One control volunteer (#7) developed a mild papular rash over the supraclavicular region which persisted from day +4 to +13. No other illnesses were detected in any of immunized or placebo volunteers during the duration of this study.

d. Laboratory Response to Immunization. No abnormalities in hematocrit, complete blood count, platelet count, total, direct and indirect bilirubin, SGOT, SGPT, alkaline phosphatase, BUN, or urinalysis was found in a volunteer in the immune or placebo groups during the duration of the study.

e. Discussion: Nine of the 10 volunteers receiving L-AV-21 excreted ADV-21 in the stool; and all of these excretors developed ADV-21 neutralizing antibodies. The infection rate obtained with this lot of vaccine virus (containing $10^{6.5}$ $TCID_{50}$/Tablet) appears to approach 90% and thus be entirely suitable for use in man.

The pattern of stool ADV-21 excretion in immunized volunteers infected with L-AV-21 was found comparable to that of ADV-7 and ADV-4 virus stool excretion as reported by Chanock, et al, JAMA 195: 44, 1966, and Top, et al., J. Inf. Dis. (in press).

ADV-21 was not recovered from the oropharynx of immunized volunteers. No evidence of communicability of the vaccine virus was found in that the five placebo volunteers who also lacked detectable serum ADV-21 N antibody and who were housed together with the immunized group showed no virus excretion and did not develop ADV CF or N antibody rises during the course of the study.

In all immunized volunteers who excreted ADV-21 vaccine virus in stool, serum neutralizing antibody (vs. 10 $TCID_{50}$ of the vaccine strain 35142) was present in the 28-day serum. Titers of ADV-21 serum N antibody in these nine men are comparable to ADV-4 and ADV-7 N antibody titers in volunteers immunized with live, enteric ADV-4 and ADV-7 vaccines respectively (Chanock, et al., and Top, et al., references cited above).

B. Development of Systemic and Secretory Antibody Following Immunization with a Live, Oral Enteric Adenovirus Type 21 Vaccine.

1. Purpose

It has been noted that the immunological responses to live enteric-coated Adenovirus vaccines differ from those infections naturally occurring in the respiratory tract. One difference is the lack of development of Adenovirus antibody in the nasal secretions of the vaccinees. Another is the lower complement fixation responses following enteric Adenovirus infection. This study was designed in conjunction with the previous study to investigate the development of Adenovirus Type 21 (ADV-21) antibody activity in both the serum and in the secretions of immunized volunteers.

2. Methods

a. Collection of specimens: The collection of specimens utilized in this phase of the study was described in Part III A.

b. Preparation

(1) Stools: Samples of selected stool specimens were suspended in twice their weight of distilled water and vortexed with glass beads. The course particulate matter was pelleted by centrifugation at 2,500 rpm for 20 minutes. The supernatant was removed and recentrifuged at 10,000 rpm's for one-half hour. The resulting, clarified solution was dialyzed overnight in distilled water. Following determination of the volume, the specimens were lyophilized and reconstituted to the appropriate concentration with distilled water.

(2) Nasal secretions: Nasal washes were collected as described above. Selected samples were vortexed thoroughly with glass beads or sea sand and centrifuged at 10,000 rpm's for 20 minutes. Supernate was decanted and dialyzed against 200 volumes of distilled water. Protein concentrations were determined on the dialyzed samples using the Aminco-Bowman spectrophotofluorimeter. The solutions were then lyophilized and reconstituted with distilled water to contain 250 mg% protein.

(3) Serum: Sera was collected as described above. Several sera were extracted with DEAE Sephadex A-50 to isolate IgG. See Annual Report, 1968-1969, for method.)

c. Quantitation of immunoglobulins

The immunoglobulins contained in sera, nasal wash, and stools were quantitated using the standard and low level radial immune diffusion kits prepared by Hyland Laboratories.

d. Radio Autography Studies

(1) Antigen preparation - Carbon 14-labeled Adenovirus Type 21 antigen was prepared in monolayer cultures of human embryonic kidney cells. Briefly, Blake bottles containing monolayers of human embryonic kidney cell layers were infected with Adenovirus vaccine virus (Strain W-270) using a multiplicity of infection of approximately 10 to 1. A 90-minute period of incubation was allowed following which maintenance media (Media 199 containing one-tenth the normal Amino Acids and 1% fetal bovine serum) with the addition of 0.2 ml of a ^{14}C Amino Acid mixture containing 0.168 mg of mixed L-Amino acids and 0.1 mc of ^{14}C/ml (New England Nuclear Corp.) was added and the cultures were incubated at 37°. When the cytopathic effect involved 75 to 100 per cent of the cell sheet the media was decanted and the cell sheets from the Blake bottles were harvested into 10 ml of Hink's balanced salt solution. The cells were disrupted by sonication, centrifuged and the supernatant fluid was treated twice with acetone. The aqueous layer was recovered and dialyzed repeatedly against balanced salt solution until the radioactivity of the dialysate was reduced to background level. The preparation used for radioactive binding studies contained 2.7×10^6 counts per minute per ml and 10^8 TCID50 per ml Adenovirus Type 21. Dilutions of 1:10 to 1:100 of this antigen were used. Since all components of the virus were labeled, this antigen was broadly cross reactive.

(2) Radioimmune electrophoresis - Electrophoresis of serum specimens were carried out in 0.9% Ion Agar prepared in 0.05 M barbital buffer, pH 8.6. Slides were prepared and electrophoresed for two hours using 12 volts across the agar. Antisera against whole human serum prepared in rabbits, or antisera against specific immunoglobulins prepared in goats (Hyland Laboratories) were placed in the troughs and the precipitin lines were allowed to develop for 48 hours. Following washing, radioactive antigen was added to the trough and 24 hours later the slides were again washed with saline followed by distilled water, dried and placed in contact with Kodak X-ray film for one to two weeks. The slides were removed from the film, stained for protein with amido black and the X-ray film was developed.

(3) Radioimmune diffusion - Reprisol Micro-Ouchterlony plates were prepared using one per cent agarose in 0.2 M Tris, 0.005 M EDTA buffer, pH 8.0. The peripheral wells were filled with serial liquid dilutions of the specimens to be tested. A specific anti-human immunoglobulin was then placed in the center well of each plate. Forty-two hours incubation at room temperature was allowed for the precipitin lines to develop and the preparation was then washed extensively for 24 hours with normal saline. Following this, the [illegible] with a 1:10 dilution of the radioactive [illegible]. This was also allowed for 24 hours and [illegible]. The plates were extensively washed, dried, [illegible]. Radio

autography was performed as before. The nature of radioactive binding by the immunoglobulin in a specimen was expressed as the reciprocal of the highest dilution showing a detectable line on the radioautograph.

3. Results

a. Neutralizing antibody responses in sera: Nine of the 10 immunized volunteers developed neutralizing antibodies by the twenty-first day following immunization. As was stated previously the responses were variable with four out of nine showing a titer of less than or equal to 1:8. The remainder showing titers of 1:32 or 1:64. Geometric titers are shown in Figure 3.

b. Radioimmune Electrophoresis: Experiments to determine the specificity of the antigen were carried out using Radioimmune Electrophoresis. Adenovirus Type 21 ^{14}C labeled antigen, dilutions containing 10^7 $TCID_{50}$ and 2.7×10^5 Cpm, were found to bind with the immunoglobulin precipitin lines from serum showing complement fixing antibody and/or neutralizing antibody against Adenoviruses. In individuals with no detectable Adenovirus antibody activity, no specific binding was found to occur.

c. Radioimmune diffusion studies on sera: The chronologic development of antibody activity of the three major immunoglobulins was determined. Figure 4 shows the geometric mean titer determined by this method of the specific immunoglobulins. As will be noted, the IgM response began early, developing within seven days after immunization in most of the immunized volunteers. The IgA rose later to higher levels. With IgM and IgA in certain individuals there was a reaction with the antigen in the initial sera indicating probably previous infection with Adenoviruses. IgG from the same sera determined by this method showed a high initial titer in all of the volunteers, immunized and controls. This also was presumably due to high levels of group reacting IgG secondary to previous infection with other Adenoviruses. The IgG titers remain essentially constant in seven of the nine responsive volunteers. In only two of the nine was there a significant four-fold rise in radioimmune diffusion titers.

d. IgG neutralizing activity in sera: Because of the difficulty in interpretation of the IgG responses as monitored by the radioimmune diffusion technique, IgG was extracted from the sera of several individuals using the DEAE Sephadex A-50 method. The extracts were found by immunoelectrophoresis to contain only IgG. This was quantitated with Hyland Radial Immune diffusion plates. Neutralization titers on the whole sera and the IgG showed a parallel rise in neutralizing activity. See Figure 5.

SERUM NEUTRALIZING ANTIBODY RESPONSE FOLLOWING
ADMINISTRATION OF LIVE ENTERIC-COATED ADENOVIRUS TYPE 21 VACCINE

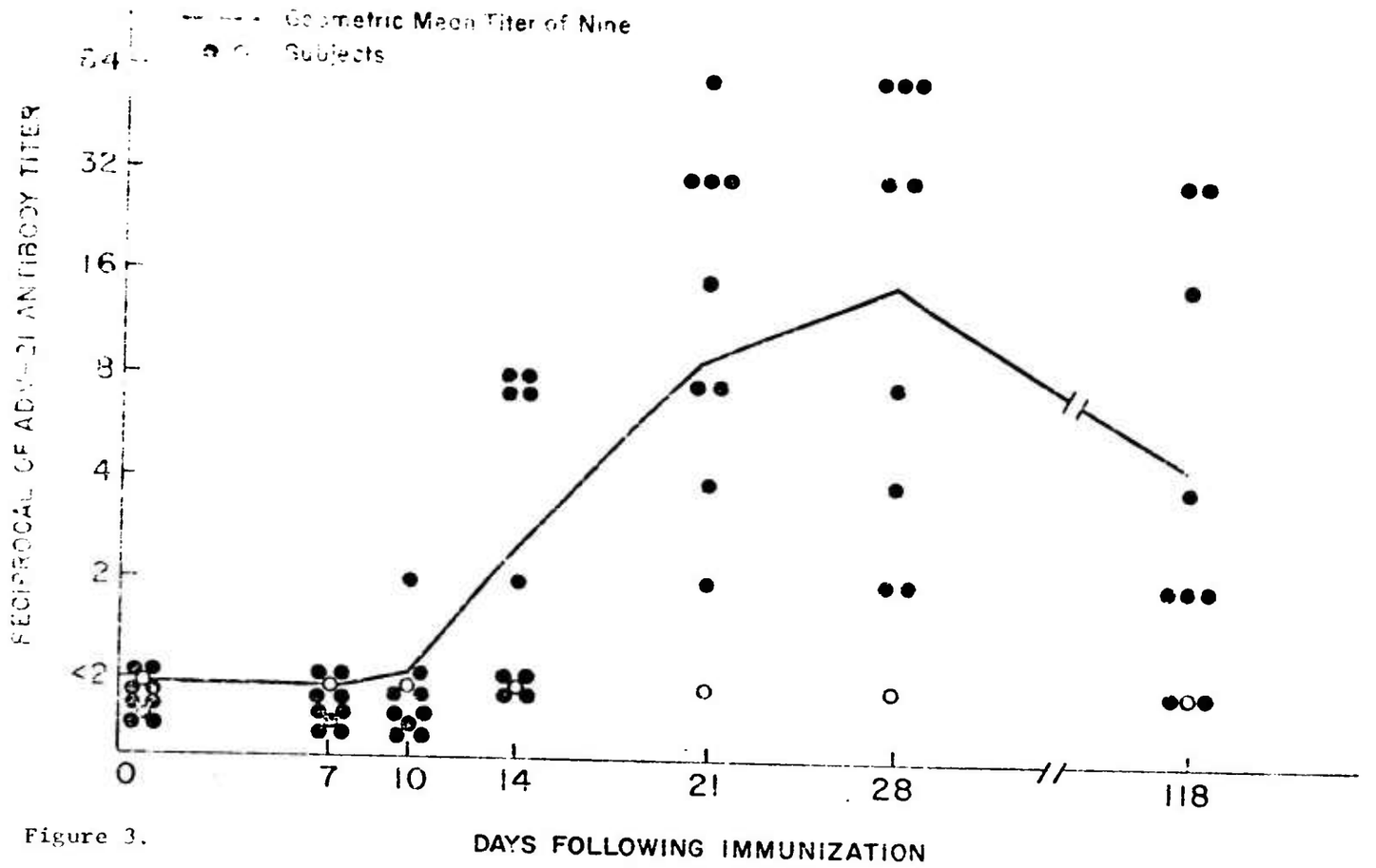


Figure 3.

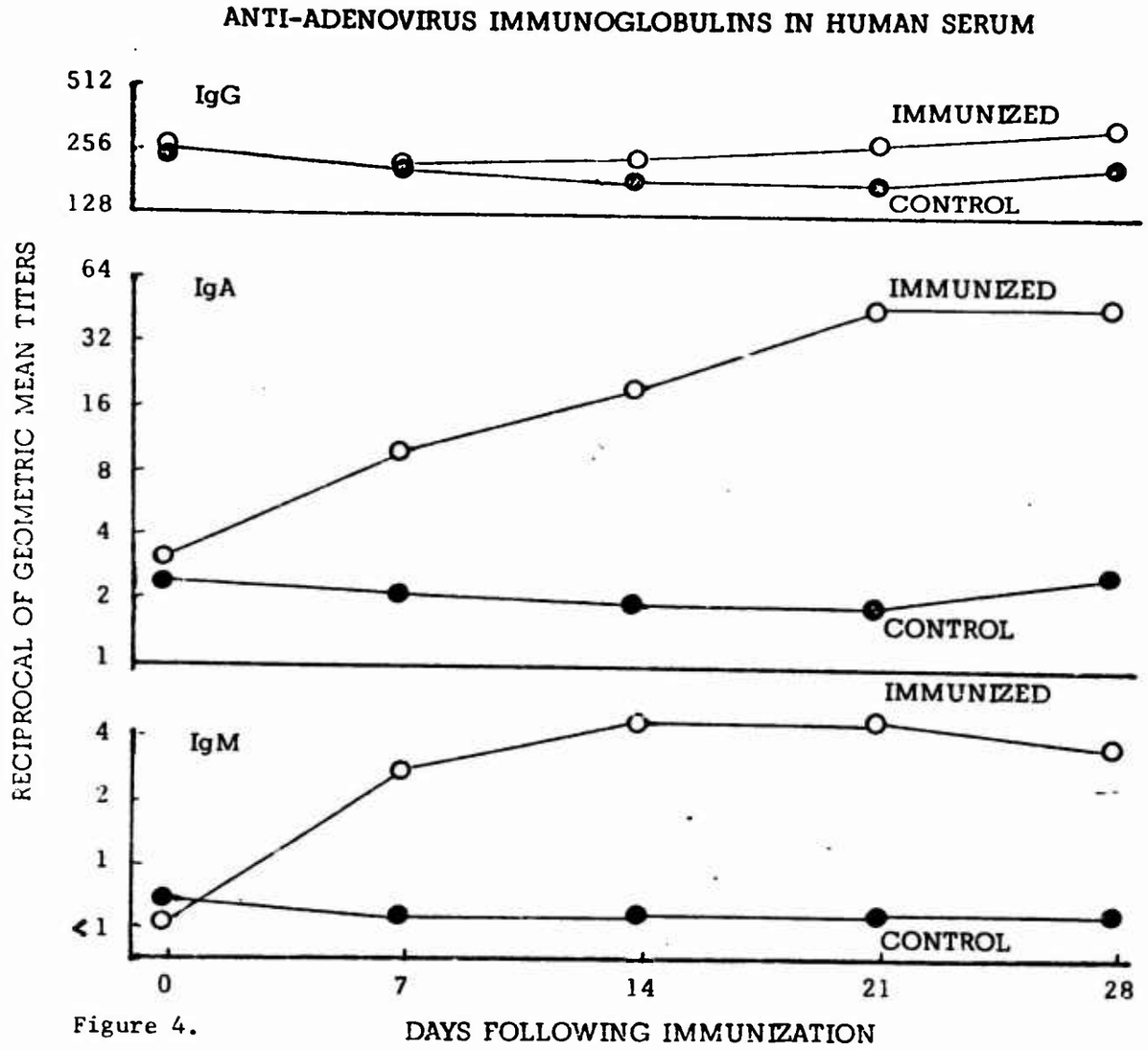

ANTI-ADENOVIRUS IMMUNOGLOBULINS IN HUMAN SERUM
IgG
IMMUNIZED
CONTROL
512
256
128
IgA
IMMUNIZED
CONTROL
64
32
16
8
4
2
1
IgM
IMMUNIZED
CONTROL
4
2
1
<1
RECIPROCAL OF GEOMETRIC MEAN TITERS
0
7
14
21
28
DAYS FOLLOWING IMMUNIZATION

Figure 4.

SERUM ANTIBODY RESPONSE IN ONE VOLUNTEER FOLLOWING ADMINISTRATION OF LIVE ENTERIC-COATED ADENOVIRUS TYPE 21 VACCINE

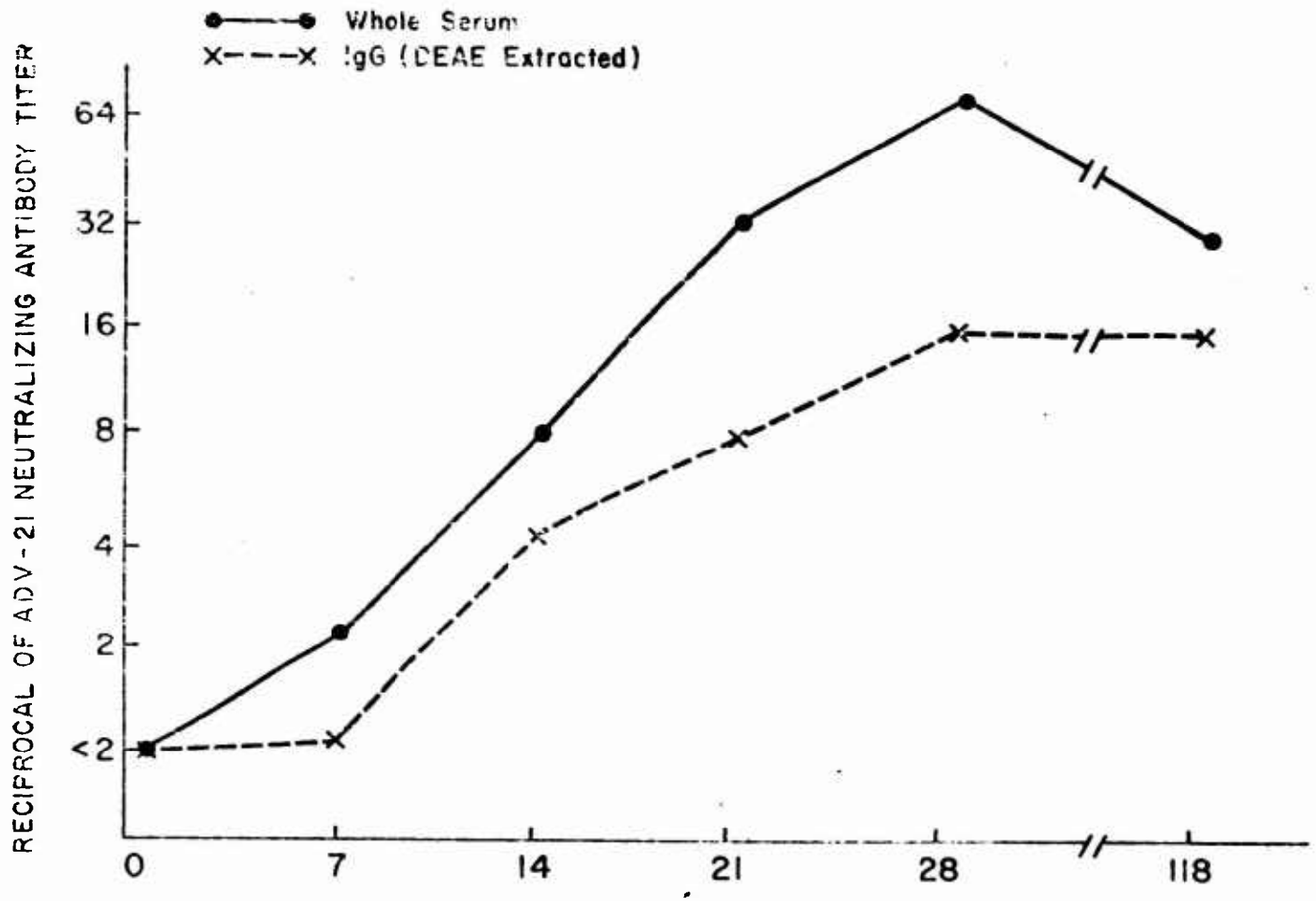


Figure 5.

e. Antibody responses of nasal secretions: Nasal washes were studied for the presence of Adenovirus antibody. The relative amount of IgA, IgM and IgG present in each concentrated specimen was estimated using low level immuno plates and serum standards. Table 15 shows the relative amount of IgA in the nasal washes was approximately the same on day 0 and day 28. The radioimmune diffusion data showed either no uptake of the C^{14}-labeled antigen in the secretory IgA or the same amount of uptake in the day 0 and the day 28 samples. Volunteer No. 10 is an exception. However, it was noted that there was approximately twice as much IgA in the 28 day specimen as was present in the day 0 specimen. It is perhaps significant that this volunteer developed a mild, afebrile upper respiratory illness during the course of this study. The rise in IgA may be attributable to this.

Table 15. RADIOIMMUNE BINDING OF ADENOVIRUS ANTIGEN BY NASAL SECRETORY IgA

SUBJECT	NASAL SECRETIONS*			
	IgA**		RID***	
Immunized ADV-21 Excretors	Day 0	Day 28	Day 0	Day 28
1	38	31	2	< 1
2	37	18	< 1	< 1
3	18	22	< 1	< 1
5	30	29	1	< 1
6	31	45	< 1	< 1
8	16	19	4	4
10	15	33	2	4
11	34	32	1	1
13	39	42	< 1	< 1
CONTROL				
4	--	--	< 1	< 1
7	18	20	2	2
9	31	22	2	1
12	30	28	< 1	< 1
14	33	33	1	1

* Nasal secretions adjusted to 250 mg% protein.
** IgA mg% nasal washes - versus serum standards.
*** Reciprocal of radioimmune diffusion titer.

The volunteers who received placebo had similar relative IgA levels and radioimmune diffusion titers on the early and late nasal washes.

Quantitative IgG levels obtained on nasal secretions ranged between less than 4 mg per cent to 15 mg per cent. Radioimmune diffusion studies on nasal IgG showed low but sustained Adenovirus 21 antibody activity. No IgM was detected in any of the nasal secretions. Neutralization tests were performed on day 0 and day 28. Nasal washes on individuals with Adenovirus antibody activity demonstrated by radioimmune diffusion technique. These were found to have no neutralizing activity against Adenovirus type 21.

f. Antibody responses in the intestinal tract: Vaccine virus replication occurred in the gastrointestinal tract. IgA was the only immunoglobulin found in stool samples. Radioimmune diffusion assay of Adenovirus antibody was carried out on these stool specimens. By this method specific antibodies were found in seven of the nine immunized volunteers who had Adenovirus in their stools. Antibody appeared as earsly as the 14th day in four individuals. Two subjects, one in the control and one in the immunized group, were found to have pre-existing Adenovirus antibody activity in the stool. In one individual, the antibody activity tests were inconclusive due to insufficient amounts of IgA. Beside these exceptions no Adenovirus binding was found in stools from any of the four remaining control volunteers. Four-fold concentrations of day 0 and day 28 stool samples from one of the immunized individuals were tested for neutralizing activity and this was demonstrated only in the 28-day sample. See Table 16.

Table 16. RADIOIMMUNE BINDING OF ADENOVIRUS ANTIGEN BY STOOL SECRETORY IgA

SUBJECT	Stool IgA	
Immunized ADV-21 Excretors	Day 0	Day 28
1	-	+
2	-	+
3	?	?
5	-	+
6	-	+
8	-	+
10	+	+
11	-	+
13	-	+
CONTROLS		
4	-	-
7	-	-
9	+	+
12	-	-
14	-	-

4. Discussion:

These data suggest that enteric infection with Adenovirus results in the development of local secretory antibody only in the intestinal tract. This is in contrast to Adenovirus infections naturally occurring in the upper respiratory tract, which are uniformly accompanied by the development of nasal secretory antibody. That this difference occurs, substantiates observations by others that local stimulation is necessary for the production of local antibody. Either the presence of the virus itself or more likely the replication of viral antigens is necessary.

Adenovirus specific IgG, assayed for the ability to bind radio-labeled antigen, showed less than a four-fold increase in seven out of nine of the immunized individuals. Also Type 21 immunization altered complement fixation titers very little. Similar lack of change in complement fixing antibody titers was seen in studies of Adenovirus Types 4 and 7 vaccines. These serologic responses to immunization differ from those seen in natural infection where significant increases in both complement fixing and IgG activity have been documented by similar methods. This suggests that cross-reactive IgG and complement-fixing antibody are not stimulated by enteric infection. That a low level type specific response does occur was shown by the increases in specific neutralizing activity demonstrated in the DEAE extracted IgG.

The efficacy of the ADV-21 vaccine has not been tested. However, in this initial study, it has lead to similar virologic and immunologic responses in volunteers to those seen following Type 4 and Type 7 vaccines. The similar biologic properties of the Type 21 vaccine indicate that the vaccine should prove efficacious if it should be called into use.

The fact that enteric Adenovirus infections are protective without the development of nasal secretory antibody, suggests that a different protective mechanism exists than that which has been postulated for other upper respiratory virus infections such as Rhinovirus, respiratory syncytial virus or para-influenza. In the latter infections, local respiratory IgA antibody is apparently required for protection against respiratory disease.

Following natural infection with Type 4 Adenovirus reinfection with Type 4 Adenovirus rarely occurs. With enteric immunization, on the other hand, reinfection of the respiratory tract may occur with viral shedding and the development of local nasal antibody. Clinically, this reinfection goes unrecognized or shows only mild upper respiratory symptoms. Disease as defined by temperature elevation and systemic infection does not occur. Invasiveness beyond the mucosa, therefore, appears to be important in the pathogenesis of febrile Adenovirus disease. Demonstration that viremia and viruria occur in individuals

hospitalized with febrile Adenovirus disease, coupled with the observations that parentally administered Adenovirus vaccines cause febrile disease often accompanied by upper respiratory symptoms. Further suggests that viremia or extra-respiratory replication may occur. Thus, it seems possible that the typical disease associated with natural Adenovirus infection may be prevented by the presence of serum neutralizing antibody. Secretory antibody per se is important in the prevention of local infection.

IV. The Relationship of Hepatitis-Associated Antigen (Australia Antigen to Viral Hepatitis.

Studies of Hepatitis-associated antigen (HAA) were directed toward determining (1) if antigen complexes and subtypes exist in patient sera, and (2) the most useful means of detecting HAA in military blood banks. Some related clinical studies were carried out as well.

A. Characterization of HAA

Although antibody to HAA (anti-HAA) is found in persons who have multiple exposures to HAA, e.g., hemophiliacs, it is rarely detectable by complement fixation or gel precipitation in patients who recover from HAA positive hepatitis. This may reflect the presence of an antibody which does not fix complement and is too dilute to precipitate in agar. If this is true, much more sensitive tests will be needed to detect specific antibody. An alternative explanation is that HAA itself is an antigen-antibody complex which must be administered intact to a host to produce anti-HAA. Support for this theory depends on separation of two components of HAA, demonstrating one has antibody characteristics and, if possible, recombining them to restore the original antigenic characteristics.

Antiserum produced in animals from partially purified HAA (Annual Report, 1970) have sometimes contained low titers of anti-normal human IgG. Preliminary experiments were conducted to see if IgG could be separated from HAA prepared by other techniques.

1. A 3.0 ml sample of HAA positive plasma (CK DI387) was passed through a Sephadex G-200 column (2.5 x 90 cm) in 0.02 M sodium phosphate buffered saline (PBS), pH 7.5. A recording was made of the 280 mμ light transmission for each fraction eluted. Protein was detected in 35 fractions. The first eight fractions of the first protein peak contained HAA. Low concentrations of IgG were found in the same fractions by radial immunodiffusion (RID). The presence of IgG in the early peak suggested antibody may be intimately associated with or a part of HAA. A pool was made of 14 fractions including the descending portion of the first peak and mid-portion of the second peak, and an aliquot passed through Sephadex G-200 in 0.2 M Glycine-HCl Buffer, pH 3.0. A single protein peak was recorded which preceded most of the IgG. In CF tests, this peak (a) had a low concentration of HAA; (b) gave a partial reaction with a convalescent serum from a patient who previously had antigen and, (c) gave a weak reaction with fractions containing IgG eluted after the peak. This was further suggestive evidence that the original plasma contained antibody(s) and IgG which were associated with HAA, could be separated by column chromatography and recombined in the presence of complement.

In a follow-up experiment using a freshly prepared Sephadex G-200 column (1.5 x 90 cm) and the same PBS buffer, an HAA positive serum and another aliquot of CK plasma were found to have low concentrations of IgG in fractions containing HAA. Two antigen-negative, "normal" serums had no significant IgG in corresponding elution fractions.

2. An attempt was made to remove all IgG from a sample of CK plasma as a preliminary step in HAA purification. The sample, 18.9 ml, was brought to 37% saturation with $(NH_4)_2SO_4$ for three hours, then centrifuged at 6000 rpm for 60 minutes. Excess $(NH_4)_2SO_4$ was removed from the supernate and sediment by prolonged dialysis with 0.03 M PBS. The dialyzed solutions were tested for HAA by CF. Whereas the original plasma had an HAA titer of 1:32, the supernatant titered 1:48 and the sediment was anticomplimentary.

A 10.0 ml sample of the supernate was placed on a DEAE Sephadex A-50 anion exchange column. Elution of proteins was accomplished with stepwise increases of PBS concentration from 0.003 to 1.0 M. IgG was recovered with the 0.015-0.12 M buffers. The 0.003-0.015 M eluates reacted by CF with three HAA positive sera; the greatest reactivity residing in the 0.003 M portion. They did not react with a normal serum control. HAA was not detected in any of the buffer concentrations.

This indicates that the high salt concentrations used did not precipitate all IgG from HAA positive serum. Furthermore, the IgG which reacts with hepatitis sera may have different charge characteristics than most of the remaining IgG.

3. The effects of 37% $(NH_4)_2SO_4$ treatment on four normal and 10 HAA positive sera were compared. Following a three-hour incubation at 0°C, the treated samples were centrifuged at 2800 rpm for 60 minutes. Supernates and sediments were dialyzed against 0.003 M PBS, pH 6.5.

It was found that HAA activity was partially precipitated by the salt. More importantly, some supernates would react as antigens with homologous or heterologous sediments in immunoelectroosmophoresis (IEOP) tests (Table 17). This ability to recombine was partially lost as the sediments became visibly cloudy when stored at 4°C in 0.003 M PBS for seven days.

It is apparent from these studies that HAA positive sera contain at least two components, which can be separated and recombined. One of these components appears to have IgG characteristics. The exact relationship of these components to HAA is yet to be determined.

TABLE [illegible]

[illegible] OF [illegible] FROM [illegible] WITH
37 $(NH_4)_2SO_4$ AND THEIR HOMOLOGOUS
PRECIPITIN REACTIONS BY IEOP

[illegible]	[illegible] SERUM	$(NH_4)_2SO_4$ SUPERNATE	$(NH_4)_2SO_4$ PRECIPITATE	SUPERNATE AND PRECIPITATE
		HAA	HAA [illegible]	
DT 17	1:256	+	+	-	-
DT 176	[illegible]	+	+	-	-
DT 184	[illegible]	+	+	-	-
DT 389	[illegible]	-	+	+	+
DT 165	[illegible]	-	-	-	+
DM [illegible]	[illegible]	-	+	[illegible]	+
DM 39	[illegible]	-	-	+	-
DM 256	[illegible]	-	+	-	-
DQ 17	1:256	+	+	-	-
DR 161	1:512	+	+	-	+

* CF titers.
** Supernate used as antigen, precipitate as antibody.
\+ Signifies positive IEOP reaction.
\- Signifies negative IEOP reaction.

B. Methods of Detecting HAA and anti-HAA.

1. Immunoelectroosmophoresis (IEOP) was applied to the detection of HAA and anti-HAA (Prince, 1970). In this precipitin test, migration of antigen toward antibody is facilitated by the presence of an electrophoretic field. Under the conditions of the test, negatively-charged HAA migrates toward the anode and the positively-charged specific IgG antibody migrates toward the cathode. The test was performed in standard electrophoresis equipment using glass lantern slides (4 x 3½ inches) coated with 10.0 ml each of 1.0% Agarose in 0.05 M Barbital Buffer, pH 8.6. Antigen was placed in 3 mm wells, antiserum in 2 mm wells and the center-to-center well distance was 5 mm. Electrophoresis was carried out using sufficient constant voltage input to give a 12 volt drop in potential across the agarose slides. Although precipitin reactions were often visible in one hour, routine total running time was two hours. Interpretations were made at one, one and one-half, and two hours.

2. The National Research Council sponsored a cooperative study of the relative sensitivity of different HAA detection methods. This laboratory was one of 20 which received 120 coded test sera from DBS-NCDC for comparative testing. Using four different antisera, each serum was tested for HAA by AGD, IEOP, and CF. In this study, a WRAIR rabbit antiserum was superior to a human and two guinea pig antisera in precipitin tests (Table 19). The IEOP test was shown to be more sensitive than AGD, but less than CF. It was concluded that the rapidity and simplicity of the IEOP make it the most practical test for use in military blood collection centers at this time.

The standard AGD test (Annual Report, 1970) was found to be rather insensitive (Table 19). Sensitivity was improved by preliminary concentration of test sera with Lyphogel (polyacrylamide gel, Gelman Instrument Co.) granules (Peters, 1970). Two to three granules of Lyphogel were placed in 0.3 ml of serum for two hours before loading the wells of an agarose slide. Twice as many sera with CF titers of 1:8 or less were recognized as containing HAA after concentration (Table 20). Preliminary serum concentration is now a routine step in diagnostic AGD tests.

3. Comparative testing of commercially available IEOP equipment was carried out to aid in the selection of equipment for use in military blood donor centers. Comparative tests were limited by the availability of each type of equipment and antiserum. Testing was carried out as follows.

Twenty-four sera were selected from the DBS-NCDC hepatitis panel to include 14 with HAA CF titers of 1:2 to 1:2048 (positives) and 10 with no detectable antigen. One set of sera were repeatedly

Table 18

NCR COOPERATIVE STUDY
THE RELATIVE SENSITIVITY OF THREE METHODS
FOR DETECTING HAA IN 59 HEPATITIS SERA*

WRAIR RESULTS	AGD		IEOP		CF	
Antiserum	No.	%	No.	%	No.	%
WRAIR R227	17	28.8	47	79.7	53	89.8
WRAIR GP6	6	10.2	32	54.2	50	84.7
NCDC GP Pool	9	15.3	38	64.4	58	98.3
NCDC Human	16	27.1	42	71.2		
SUMMARY						
WRAIR	48/236	20.3	159/236	67.4	161/177	91.0
Cooperative Study (All 20 Labs)	2180/4482	48.6	2822/3903	72.3	1617/1941	83.3

* NRC selected 59 sera for comparative analysis which were reported to contain HAA by at least 6% and not more than 92% of the 20 participating laboratories.

Table 19

THE EFFECT OF PRELIMINARY SERUM CONCENTRATION WITH LYPHOGEL* ON THE SENSITIVITY OF AGD

HAA CF Titer	No. Sera Tested	No. Positive by AGD	
		Unconcentrated	Concentrated
> 1:16	61	57	59
1:8	14	5	10
1:4	10	1	1
1:2	3	0	1
Total Positive	88	63	71
% Positive	100.0	71.6	80.7

* Polyacrylamide gel granules.

[illegible]	[illegible]	Power	[illegible]	Running Time (Minutes)	Maximum Test Per Run	Reliability, Simplicity and Safety	Other Features
[illegible]	[illegible]	60 C.V.	2000 ml	120	6+	Cannot judge on a prototype	1. Terry Cloth Wicks. 2. Two plate sizes.
[illegible]	[illegible]	100 C.V.	500 ml	45	24	Good	1. Adjustable timer. 2. Sponge wicks
[illegible]	Ortho	175 C.V.	[illegible] Liquid buffer	60	12	Good	Electrodes embedded in plate.
4.	Pfizer	90 C.V.	200 ml	35 Preceded by 15 min wait	32		1. Rectangular wells. 2. Flexible plates.
5.	Savant	300-400 [illegible] C.V.	400 ml	120	96	Very good	Cooling plate required
6.	Shandon	- Choice of User -			96		Versatile equipment
7.	Spectra	170 C.V.	300 ml	120	30	Good	1. Versatile plates. 2. Built-in viewing light.

tested in each piece of equipment with as many commercial antisera as possible. This permitted the evaluation of the performance of each type of equipment and antiserum independently. In each run, sera were arranged randomly. Precipitin reactions were interpreted by multiple readers, including a company representative whenever possible. Only the observations of one reader were used for comparing results.

Each type of equipment used a barbital buffer. Buffer pH ranged from 8.2 to 8.6 as specified by the company. Table 21 demonstrates the major differences between types of equipment. In comparing the performances of equipment and antisera, both false negative and false positive reactions were considered (Tables 22, 23). A per cent total error was determined by adding the number of false negative and false positive reactions and dividing by the total number of tests. Usually, each type of equipment had fewer false negatives when using its own antiserum (Table 24). Precipitation reactions are dependent on obtaining an optimum concentration of antigen versus an optimum concentration of antibody. "False negative" reactions are frequently due to high concentration of antibody (in the undiluted antiserum) which inhibits precipitation due to antibody excess.

This principle of the precipitation reaction limits the use of a precipitating antigen-antibody system as a method of antigen detection over a wide range of antigen concentrations.

An evaluation of each type of equipment was forwarded to the Surgeon General's Office.

Table 21

Performance of IEOP Equipment*

Equipment	No. Tested	No. Ab. Pos.	No. False Neg.	No. False Pos.	False Neg.	False Pos.	Total Error
WRAIR	94	55	17	0	30.9	0.0	17.9
Hyland	119	69	29	1	42.0	2.0	25.2
Pfizer	48	28	5	2	17.9	10.6	14.6
Ortho	23	13	2	1	15.4	10.0	13.0
Abbott	61	32	8	0	25.0	0.0	13.1
Spectra	94	52	16	2	30.8	2.5	18.5
	(419)	(236)	(70)	(4)	(30.0%)	(2.2%)	(17.8%)

* Based on repeated tests of 24 sera.

Table 22

Performance of Antisera*

Antiserum	No. Test Sera	No. Au Pos.	No. False Neg.	No. False Pos.	False Neg.	False Pos.	Total Error
WRAIR R230	110	61	17	0	27.9	0.0	15.5
Hyland Horse	94	54	19	0	35.2	0.0	20.2
Ortho Rabbitt Pool	94	54	14	3	25.9	7.5	18.1
Abbott GP Pool 2200910	46	26	11	0	42.3	0.0	23.9
Spectra Human Pool Lot 05	46	26	9	2	34.7	16.0	23.9

*Based on repeated tests of 24 sera.

Table 3

False Negative Reactions
in Repeated IEOI
Tests of Some Allergen Extracts

Equipment	WRAIR Rabbit 23+	Hyland Horse	Abbott GP Pool	Spectra Human Pool	Ortho Rabbit 1:1	Total	% False Neg.
WRAIR	4/14	6/14	4/13	4/13	3/13	21/67	34.3
Hyland	6/14	7/14	8/13	7/13	4/14	32/67	47.1
Abbott	1/6	2/13	5/13	2/13		10/45	22.2
Spectra	4/13	4/13	6/13	2/13		16/52	30.8
Ortho					2/13	2/13	15.4
Pfizer	2/14				3/14	5/28	17.9
Total	17/61	19/54	23/52	15/52	14/54		
% False Negative	27.9	35.2	44.2	28.8	25.9		

C. Clinical Studies

1. The immediate family members of an infant with giant cell hepatitis and HAA were followed over a 12-month period (Bancroft, 1971). Although the mother and two sisters were asymptomatic HAA carriers without histories of liver disease, percutaneous liver biopsies showed pathologic changes in each (Table 24). These findings support former suggestions that hepatitis virus may cause different pathologic manifestations in different people depending upon their age, length of exposure, and immune responsiveness (Aterman, 1963).

2. In order to estimate the HAA carrier rate in military blood donors, pilot tubes from 1112 units of blood collected from 9 June to 10 July 1970 were tested by CF. Suspicious and positive sera were retested by AGD. Three units (0.26%) were HAA positive and 55 (4.0%) were anticomplementary. The carrier rate in this sample was 2.6/1000 with this detection method.

3. Because of the increased risk of transfusion hepatitis in hemodialysis units, a program was initiated of routine monthly testing of all patients and employees on Ward 38, Walter Reed General Hospital. Between 1 April 1970 and 31 March 1971, 23 ward personnel and 53 patients were tested with an average of 5.7 and 2.2 sera each, respectively. Two ward personnel (8.7%) and two patients (3.8%) were found to have HAA. In each case, antigen was found in the first serum tested. The one carrier who subsequently became HAA negative did not have detectable anti-HAA by CF. It is possible that more patients would have been found HAA positive if more sera had been received from them.

Table 24

Comparative Laboratory Findings in Family Members with Hepatitis

Patient	HAA CF	AGD	SGOT Maximum Values	T. Bilirubin Maximum Values	Liver Biopsy
Infant Male (22 wks)	1:2	0	4300 u/ml	7.3 mg/	Giant cell hepatitis; portal fibrosis
Mother (32 yr)	1:64-256	+	30	0.8	Resolving acute hepatitis
Sister (11 yr)	1:512-1024	+	33	0.3	Resolving acute hepatitis
Sister (14 yr)	1:16-32	+	45	1.4	Chronic hepatitis; portal fibrosis

V Antigenic Analysis of Dengue Viruses.

A. Comparison of Denue-2 and Dengue-3 Strains by Neutralization Tests.

The occurrence of two major dengue epidemics in the Caribbean region within the past decade has raised important questions concerning the existence of possible geographic variants and the geographic origin of the epidemic strains. The first dengue viruses recovered in the Caribbean region were Dengue-2 (DEN-2) strains found associated with sporadic disease in Trinidad in 1954. More recently, DEN-2 viruses were recovered on multiple occasion from patients during an outbreak in Jamaica in 1969 and during the 1969 Puerto Rico epidemic. Dengue-3 (DEN-3) strains from the Caribbean were first isolated during the dengue epidemic in Puerto Rico in 1963 and DEN-3 virus was again found associated with the small outbreak in Jamaica in 1968. The low mouse virulence and consequent difficulties in isolating the DEN-3 strains responsible for the 1963-1964 Caribbean epidemic provided the first observation which suggested a difference between Caribbean and Old World strains.

In 1964 epidemic dengue occurred in Tahiti and was found to be caused by a dengue virus with very low mouse virulence (Rosen, 1967). Subsequent identification of the Tahiti agent as a DEN-3 strain raised the question of its antigenic relationship to the Caribbean DEN-3 strain and to contemporary southeast Asian strains. The recent recovery of dengue viruses in Africa (Carey, 1971) again raised the question of possible strain variation related to geograp'ic origins. In the hope that subtypes of epidemiologic significance could be distinguished, we compared several DEN-2 and DEN-3 strains from the Caribbean with prototype viruses and with strains Southeast Asia, Africa, and Tahiti.

The strains included in this study are described in Table 25. The TR-1751 and the H-11234 strain of DEN-2 were obtained from Dr. Jordi Casals. The Pr-6 strain, a mouse-adapted DEN-3 strain from the 1963 Puerto Rico epidemic, was obtained from Dr. Charles Wisseman. The J-1007 strain was re-isolated in this laboratory from serum provided by Dr. Arnoldo Ventura. The Tahiti-4 strain was supplied by Dr. Leon Rosen.

Hyperimmune mouse ascitic fluids were prepared against the mouse-adapted strains by a modification of the method of Brandt. Ascites was induced in the immunized mice by the use of sarcoma 180 cells. Human convalescent sera were obtained in the course of epidemiologic studies in Puerto Rico and Tahiti. For these studies sera were selected from patients who were thought to have had a primary type antibody response to the dengue infection. Selection was made on the

Table 25.

Virus Strains Tested

Designation	Original Isolation Year	Original Isolation Location	Passage Level*	Serotype
New Guinea C	1944	New Guinea	sm-27	DEN-2
TR-1751	1954	Trinidad	sm-55	DEN-2
PR-109	1969	Puerto Rico	sm-5	DEN-2
H-11234	1966	Nigeria	sm-27	DEN-2
H-87	1956	Philippines	sm-26	DEN-3
21153	1965	Thailand	sm-10	DEN-3
Tahiti-4	1964	Tahiti	tc-5	DEN-3
PR-38	1963	Puerto Rico	tc-6	DEN-3
PR-6	1963	Puerto Rico	sm-14	DEN-3
J-1007	1968	Jamaica	tc-6	DEN-3

* sm - suckling mouse passage.

tc - tissue culture passage.

basis of absence of detectable HI antibody in the acute phase specimen and a rise to low or moderate levels (1:80-1:320) in the convalescent specimen obtained two to four weeks after onset of illness. The convalescent sera from Tahitian patients was supplied by Dr. Leon Rosen.

Plaque reduction neutralization tests were carried out in LLC-MK_2 Cell cultures. Tests were carried out in 30 ml plastic flasks (Falcon Plastics, Oxnard California). Two-fold dilutions of immune ascitic fluid were mixed with virus and incubated at 25° C for 30 minutes prior to adsorportion. Adsorption was carried out at 37° C for one hour. Control plaque counts were between 30 and 100 pfu/flask. Fifty per cent plaque reduction end points were estimated by the probit method.

The results of cross neutralization tests with the Dengue-2 strains are shown in Table 26. The neutralization titers in each case failed to show any marked difference between the Caribbean and prototype strains. The slightly lower (approximately two-fold) titers of the TR-1751 ascitic fluid and Puerto Rican human serum against the African H-11234 strain are within the variation expected between tests.

DEN-3 strains. The neutralizing antibody titers of the hyperimmune ascitic fluids shown in Table 27 indicate that the PR-6 ascitic fluid, a 1963 Caribbean strain, neutralized the Southeast Asian strains very poorly. Titers against H-87 and 21153 viruses were five-fold or more lower than the titers against other Caribbean and the Tahitian strains. With the H-87 and 21153 ascitic fluids, apparent differences between the Asian and the Caribbean and Tahitian strains are much less marked. The homologous and heterologous titers of the 21153 ascitic fluid vary by less than two-fold. The H-87 ascitic fluid has somewhat lower titers (two to three-fold) against the Caribbean strains.

Results with human convalescent sera as shown in Table 28 confirm the results obtained with the mouse ascitic fluids. A significant difference is again apparent; the Puerto Rican sera neutralize the Southeast Asian strains to a much lower titer than the titers against Caribbean and Tahitian strains. The Tahitian human convalescent sera, while neutralizing homologous strains and Caribbean strains, fail to neutralize Southeast Asian strains.

Table 26.

Neutralizing Antibody Titers of Hyperimmune Mouse Ascitic Fluids and Human Convalescent Serum Against Dengue-2 Strains

	Mouse Ascitic Fluids			Human Convalescent
Virus	NG C	TR-1751	PR-109	Puerto Rico 1969
NG C	3000*	1700	2000	300
TR-1751	1300	1300	1200	200
PR-109	2800	1600	1400	400
H-11234	1900	600	1200	110

* Reciprocal of 50% plaque reduction titer.

Table 27.

Neutralizing Antibody Titers of Hyperimmune Mouse Ascitic Fluids Against Dengue-3 Strains

	Ascitic Fluids		
Virus	H-87	21153	PR-6
H-87	180*	240	70
21153	200	420	130
PR-6	50	250	>640
PR-38	80	280	>640
J-1007	90	230	1100
Tahiti-4	100	250	>640

*Reciprocal of 50% plaque reduction titer.

Table 28.

Neutralizing Activity of Human Convalescent Sera Against Several Strains of Dengue 3

	Tahitian Serum - 1964			Puerto Rican Serum - 1963	
Virus	4364	4365	4366	YB-6	YB-4
H-87	<20*	<20	<20	30	<20
2115	<20	<20	<20	40	25
PR-6	60	60	60	190	50
PR-38	50	50	30	220	115
J-1007	95	140	70	180	75
Tahiti-4	70	150	50	200	100

* Reciprocal of 50% plaque reduction titer.

Discussion.

The above neutralization tests indicate that the DEN-2 strains from Southeast Asia, Africa, and the Caribbean form a relatively homogenous antigenic group. This is consistent with other observations that all DEN-2 strains are readily adapted to suckling mice and grow readily in several cell culture systems. Southeast Asian and Caribbean DEN-2 strains are similar in that both exhibit a high degree of plaque size variation when freshly isolated strains are tested in LLC-MK_2 cell cultures. It, therefore, appears impossible to determine by these methods whether the 1969 epidemic of DEN-2 in the Caribbean was caused by an endemic strain which persisted since 1954 or by a strain of DEN-2 introduced from another region.

The observations on the DEN-3 strains are quite different from those seen with DEN-2 and clearly indicate that the Caribbean and Tahitian strains of DEN-3 form a distinct antigenic subtype. Continued classification of these strains as Dengue-3 appears justified since the neutralization of Caribbean and Tahitian strain by antisera to the Southeast Asian strains is so close to homologous titers. Of considerable interest is the fact that differentiation of this subtype by neutralization tests appears to correlate with the biologic marker of low virulence for suckling mice.

The recovery of the Caribbean subtype in Jamaica in 1968 suggests that this subtype has remained endemic in the Caribbean region since at least 1963. The origin of the Tahiti strain remains obscure but it appears unlikely that it was introduced from Southeast Asia. The very low neutralizing antibody titers of the Tahitian and Puerto Rican patients to the Southeast Asian strains raises the question of their susceptibility to infection with strains similar to the H-87 prototype. It appears possible that the Tahitians may remain susceptible to infection with the Southeast Asian DEN-3 even though immune to the Caribbean-Tahitian subtype.

B. Separation of Dengue Strains on the Basis of a Nonstructural Antigen.

Accurate characterization of intratypic strain variations within the dengue virus serotypes is critical to our understanding of the pattern of epidemics of dengue and dengue hemorrhagic fever. The ability to positively identify subtypes could provide clues as to the origin of newly introduced epidemic strains or possibly indicate changes in the nature of endemic strains. Intratypic strains have been extensively studied using serologic techniques, primarily, complement-fixation. The significance of these tests has been obscured by the use of antigen preparations which contained a mixture of structural and nonstructural antigens in both natural and degraded states, as shown in previous annual reports.

Attempts to differentiate strains by plaque reduction neutralization have provided results which have led to differences of opinion as to whether these strains could or could not be separated. These differences of opinion are based upon the interpretation of observed data and may be due to the inherent variability in such tests when unpurified reagents are used.

Antigenic analysis of purified antigens offers many theoretical and practical advantages over the standard systems. The four dengue serotypes have been separated on the basis of a purified soluble complement-fixing (SCF) antigen by Ouchterlony immunodiffusion methods. Subsequently, the SCF antigens of the four dengue serotypes were separated biophysically by disc gel electrophoresis. In the following experiments, this combination of biophysical and serological analysis was applied to the problem of intratypic strain variation. Two strains of dengue-1 (TH-Sman and Hawaii) were chosen to illustrate the usefulness of this approach.

Materials and Methods

Viruses. Mouse adapted prototype strains of Dengue-1 (Hawaii and TH-Sman) and Dengue 2 (New Guinea C and TH-36) were obtained from Dr. William McD. Hammon, University of Pittsburgh; these strains had undergone purification by terminal dilution in suckling mice.

Immune ascitic fluids. The antibody source for each strain was hyperimmune ascitic fluids prepared in adult female mice as previously described. The immunogens in each case were 20% suspensions of infected suckling mouse brain.

Purification of SCF Antigens. The SCF antigens were prepared from each strain of dengue virus by methods in previous annual reports.

Briefly, suckling mice were inoculated intracerebrally and virus-infected brains were harvested when the mice became moribund. Their brains were homogenized (20 w/v) in 0.02 M Tris-HCl buffer at pH 7.2. These suspensions were clarified by precipitation of excess brain tissue with 2 mg/ml protamine sulfate and by centrifugation at 9000 X G for 30 minutes; recentrifugation of the supernatant at 78,000 X G for three hours removed all detectable hemagglutinating viral antigens. The SCF antigen was then precipitated from the resulting ultracentrifuge supernatant with 60% ammonium sulfate. The SCF antigen was resuspended in Tris-HCl buffer and applied to a 5 X 80 cm Sephadex G-100 column. Filtration was carried out in 0.02M phosphate-buffered saline at pH 7.2. Fractions containing the SCF antigens were pooled and concentrated by pressure dialysis.

Complement-fixation (CF) tests. CF tests were carried out by a microtiter modification of methods described by Kent and Fife, Department of Serology, WRAIR. In block CF tests, master dilution sets of antigens and antibodies were prepared by pipette in tubes before they were added to the microtiter plates.

Immunodiffusion tests. Ouchterlony plates were prepared by using 1.0% agarose in 0.02 M Tris-buffered saline, pH 8.2, in plastic petri dishes as described in previous annual reports. Before use in immunodiffusion tests, antigen and antibody preparation were diluted to a uniform CF titer of 1:64.

Disc gel electrophoresis. Acrylamide monomer and N,N'-methylene-bis-acrylamide were recrystallized from acetone. The desired concentration of polyacrylamide was prepared by dilution of a 30% acrylamide monomer and 1 bis-acrylamide stock. Stacking took place at pH 7.3 and separation at pH 8.9 (Hedrick and Smith, 1968). Individual SCF antigens or mixed pairs were electrophoresed at 5 ma/tube. At the end of each run the gels were sliced transversely on razor blades with 1mm spacers, eluted in saline and tested for CF activity and compared by their mobilities relative to the dye front.

Results.

Immunodiffusion. As expected, the Dengue-1 strains could be differentiated from the Dengue-2 strains in Ouchterlony plates by spur formation (Annual Report, 1970). The strains within each serotype, however, could not be differentiated from each other by immunodiffusion. As specifically illustrated, the two Dengue-1 strains (TH-Sman and Hawaii) formed spurs when adjacent to Dengue-2 (New Guinea C) in the presence of Dengue-1 antibody, but, when adjacent to each other, their precipitin lines fused without spurring (Fig. 6). Similar relationships existed when the Dengue-2 strains were compared with the Dengue-1 strains in the presence of Dengue-2 antibody (Fig. 6).

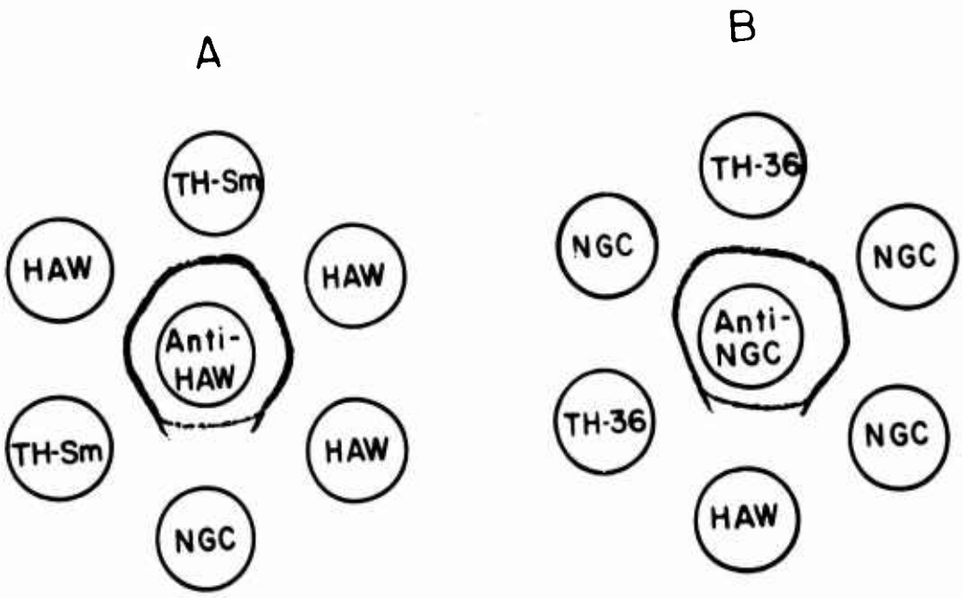


Figure 6 ...gram of immunoprecipitation by strains of dengue-1 (Hawaii ...H-Sman) and dengue-2 (New Guinea C and TH-36). The SCF antigens are arranged in the peripheral wells to demonstrate homologous and heterologous reactions.

A. Hawaii (HAW) and TH-Sman (TH-Sm) SCF antigens compared with each other and with the New Guinea C (NGC) strain of dengue-2 when they are reacted with anti-Hawaii in the center well.

B. New Guinea C and TH-36 SCF antigens compared with each other and with the Hawaii strain of dengue-1 when they are reacted with anti-New Guinea C in the center well.

Complement-fixation. Comparisons of strains within dengue serotypes 1 and 2 were carried out by CF tests on block titrations of antibodies and antigens. When type 1 strains were compared, the homologous strain block patterns were identical (Fig. 7). The block pattern of TH-Sman antigen and anti-Hawaii differed only slightly from the homologous reactions in shape but not in total size. A significant difference in size and shape was observed only when the Hawaii SCF antigen was tested in the presence of anti-TH-Sman. The block comparisons of the Dengue-2 strains (New Guinea C and TH-36) were essentially identical in all combinations (Fig. 7).

Disc gel electrophoresis. Preliminary experiments were carried out by using 70 mm long separating gels, previously shown to separate SCF antigens of the four major dengue serotypes (Annual Report, 1970). Under these conditions, however, there was no difference in mobility between New Guinea C and TH-36 (Dengue-2 strains) or between Hawaii and TH-Sman (Dengue-1 strain) SCF antigens. In order to maximize the separation of molecules with very closely related molecular sizes and/or charges, a separating gel of 10% polyacrylamide 150 mm in length was employed. Under these conditions, Hawaii and TH-Sman had different relative mobilities when they were electrophoresed either separately or together (Fig. 8). In contrast, the SCF antigens of New Guinea C and TH-36 could not be separated when they were electrophoresed under the same conditions (Fig. 9).

Discussion.

In 1945 Sabin reported cross challenge experiments in man using dengue viruses from Hawaii and New Guinea which resulted in the designation of the first two dengue serotypes. In 1956 two more viruses were isolated in the Philippines which were antigenically related to Types 1 and 2 but clearly distinguishable from them by both complement-fixation and neutralization (Hammon, et al, 1960). These two isolates were designated dengue Type 3 and Type 4. The 1958 dengue hemorrhagic fever epidemic in Bangkok produced another pair of isolates, TH-Sman and TH-36, which were proposed as separate serotypes based upon small but reproducible differences observed by complement-fixation utilizing selected human antisera and mouse brain derived antigens (Hammon and Sather, 1964). Subsequently, evidence of strain variation was presented based on plaque reduction neutralization and immunoprecipitation on the same mouse brain passage virus (Ibrahim and Hammon, Ibrahim, et al, 1968). Cross neutralization in other laboratories using mouse and monkey antisera, however, failed to clearly differentiate between TH-Sman and Hawaii (Type 1) or between TH-36 and New Guinea C (Type 2) (Russell and Nisalak, 1967). Further, cross challenge in mice failed to reveal intratypic variation (W. McD. Hammon, personal communication). These discrepancies between the various studies illustrate the problems of serotyping dengue strains before purified antigens became available for antigenic and biophysical analysis.

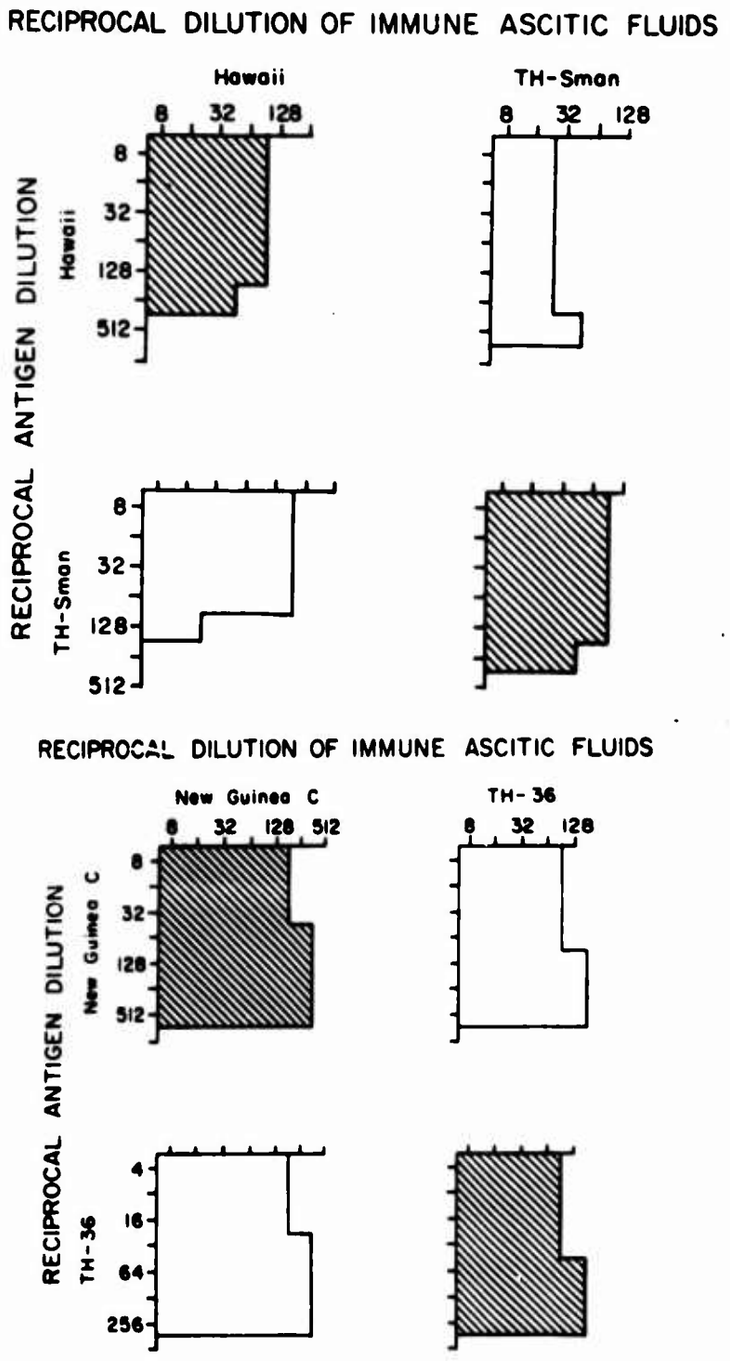
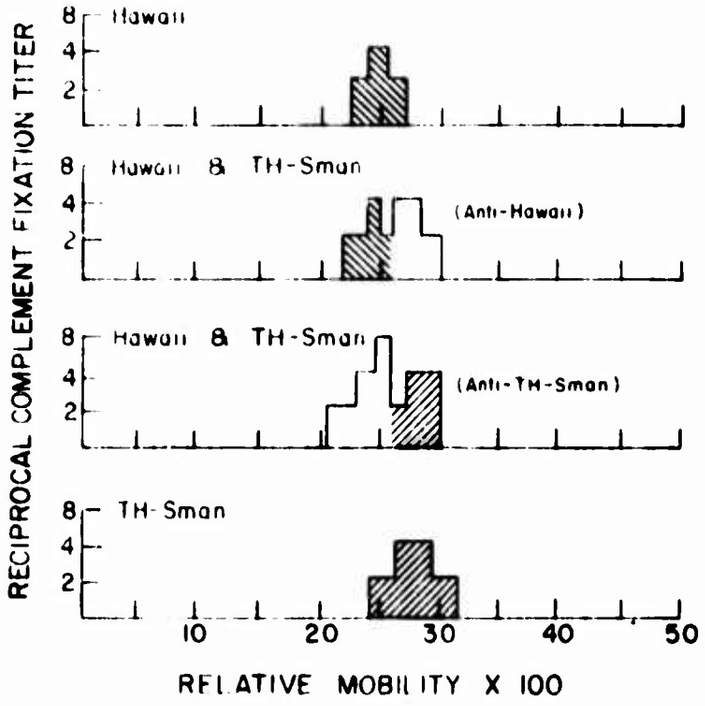


Figure 7. Cross complement-fixation tests on A. dengue-1 strains (Hawaii and TH-Sman) and dengue-2 strains (New Guinea C and TH-36). Homologous strain reactions are shaded.



[illegible] antigens (dengue-1 strains) [illegible] electrophoresis when electrophoresed separately (top and bottom panels) or together (middle panels). Homologous reactions are open, while heterologous reactions are open.

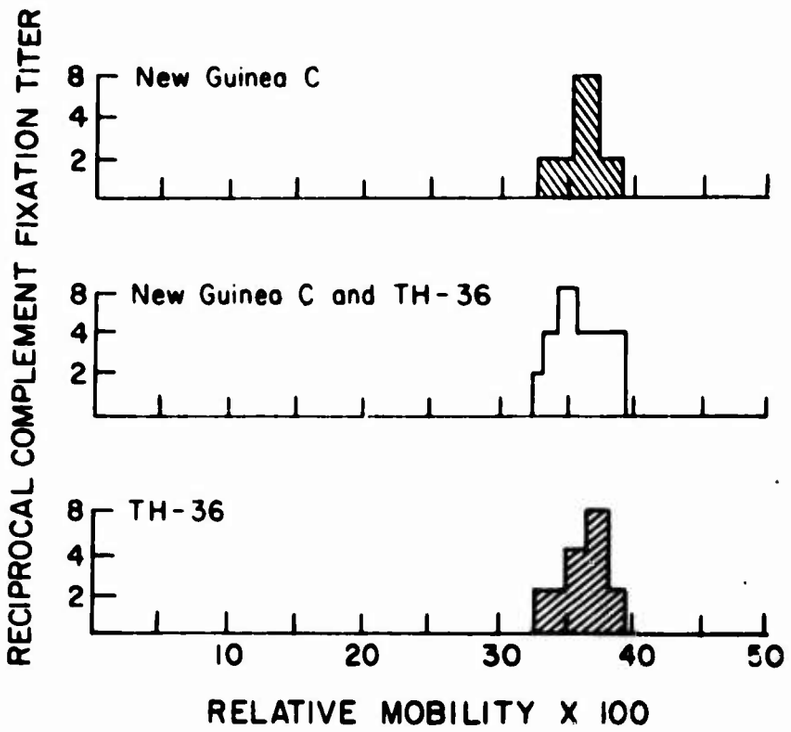


Figure 9. Motility of New Guinea C and TH-36 SCF antigens (dengue-2 strains) by disc gel electrophoresis when electrophoresed separately (top and bottom panels) or together (middle panel). In this example the fractions were reacted with anti-New Guinea C.

Immunological and biophysical analysis of a partially purified viral antigen provides an opportunity to investigate intratypic strain variation at a molecular level. The SCF antigen used in the above experiments has been found to be a major CF antigen in dengue infected cells (Annual Report, 1969). The SCF antigen of each serotype has both a type and group specific antigenic determinate and can be separated from the SCF antigens of other serotypes by immunodiffusion and disc gel electrophoresis (Annual Report, 1970). Antibodies against SCF do not bind to or neutralize the virion and purified SCF does not block antibodies which neutralize the virus. This and other evidence indicates that SCF is a type specific nonstructural antigen (i.e., SCF is not in or on the virion) (Annual Report, 1970). It has an estimated molecular weight of 39,000 daltons and assuming that it is in-toto a polypeptide, SCF theoretically accounts for 10-15% of the dengue genome. The functional significance of the antigen is not known.

The SCF antigens of TH-36 and New Guinea (Dengue-2 strains) could not be separated by any of the three methods utilized in these experiments. This indicates that either the molecules are identical or the difference is below the resolution of the techniques used. While no separation of SCF antigens was achieved, other portions of the genome may well contain differences which are responsible for the serological separations reported by Hammon. The differentiation, however, is probably not based upon the SCF antigens of the two strains.

When the purified SCF antigens of Hawaii and TH-Sman (Dengue-1 strains were compared by immunodiffusion, a single line (identity) was observed. The CF block patterns were virtually identical when anti-Hawaii was reacted in the presence of TH-Sman and Hawaii SCF antigens. Small, but probably significant CF block differences between TH-Sman and Hawaii were observed only when anti-Sman was used as the antibody. This serological differentiation suggests that both the type specific and group specific determinates of the two strains are so closely related that they can be distinguished only by tests which indicate a difference in relative antibody avidity.

The above interpretation is reinforced by the biophysical separation of the antigens by disc gel electrophoresis. When electrophoresed independently, TH-Sman and Hawaii migrated as single peaks with slightly different mobilities. When they were co-electrophoresed in the same gel, a bimodal peak of CF activity was observed, confirming that the two antigens have different relative mobilities. These data are consistent with the CF results and indicate that the SCF antigens of TH-Sman and Hawaii contain minor differences in amino acid sequence and/or conformation resulting in differences in the size, shape and/or charge of their respective molecules. Since SCF is a major CF antigen in dengue infected mouse brain, these subtle differences in the SCF

molecules of TH-Sman and Hawaii may be sufficient to account for the original separation of the two Dengue-1 strains. Molecular variation in SCF, however, would not necessarily be associated with differences in the coat antigens of the virions and thus, could account for the difficulties experienced in separating the two dengue-1 strains on the basis of neutralization tests.

The nonvirion SCF antigen of dengue viruses does not elicit the formation of neutralizing antibodies and would presumably have no protective effect against secondary infection. Variation in the SCF molecule, therefore, should have no effect on the natural selection of dengue strains through immunization of the population at risk. However, a nonstructural component, such as an enzyme, could provide a biologic advantage within primate or insect cells. Variations in the SCF molecule could, therefore, still have selective value within certain types of host cells, but this theoretical advantage must await elucidation of the function of SCF. The concept of classification of viruses solely on the basis of the properties of their virions appears to be inadequate for considering intratypic variation in Dengue-1. At least one pair of Dengue-1 strains (TH-Sman and Hawaii) can be separated on the basis of a nonstructural antigen. Nonstructural components are clearly taxonomically important in this case and must be considered in any classification based on serological characteristics.

VI. Structure and Morphogenesis of Arboviruses.

A. The Proteins of Japanese Encephalitis Virus.

Knowledge of the molecular structure of Japanese encephalitis virus (JEV) and related group B arboviruses is limited, with very little known concerning the events leading to the formation of mature virions. Particulate antigens produced during JEV infection are heterogeneous and the relationship of subviral particles, such as the slowly sedimenting hemagglutinin (SHA), to the morphogenesis of the virion remains unexplained. Studies of the polypeptides of the related viruses, dengue (Stollar, 1969), St. Louis encephalitis (SLE) (Trent, et al., 1969), and Kunjin (Westaway and Reedman, 1969), have resulted in unresolved differences on such matters as the composition of viral cores and the number of virion polypeptides. A comprehensive study of at least one group B virus system will be required to fully understand the biology of the group.

An initial step toward providing a complete explanation of the morphogenesis and antigenic composition is the identification and comparison of polypeptides present in the virion, in subviral particles and in virus infected cells. In this paper we report the polypeptide composition of the JEV virion, core, slowly sedimenting hemagglutinin and the intracellular virus specified proteins.

Preparation of Japanese encephalitis virus (JEV).

The virus used was JEV strain M1/311, mouse passage 27, originally isolated from Culex tritaeniorhynchus in Japan. The seed virus consisted of a 20% infected suckling mouse brain suspension in saline containing 4% bovine plasma albumin. Monolayers of LLC-MK_2 cells in 32 oz. bottles were grown in medium 199 containing 20% heat-inactivated fetal bovine serum (FBS), penicillin (100 u/ml) and streptomycin (100 ug/ml) (complete 199). Cell cultures were infected with 3 ml of stock virus diluted in complete 199 containing 0.15% sodium bicarbonate at a multiplicity of infection (MOI) greater than 10. After adsorption for 60 to 90 minutes, 27 ml of medium 199 lacking amino acids and serum (minimal 199) was added and the bottles incubated at 36°C. To prepare radioactive virus either: 1. ^{3}H-amino acids; 2. ^{3}H-uridine; or 3. ^{14}C-amino acids; or 4. 2 + 3 (all generally labeled and from New England Nuclear) was added to final activities ranging from 6 to 12 microcuries per ml. The pH was adjusted to approximately 7.3 with bicarbonate when necessary. Culture fluids were harvested 40 hours after infection and clarified at 2000 rpm for 10 minutes, then at 10,000 rpm for 50 minutes. The virus was then pelleted by centrifugation at 25,000 rpm for three hours, and usually resuspended in 0.02 M Tris (hydroxymethyl) aminomethane (Tris), 0.15 M NaCl, 0.001 M EDTA, pH 8.7 (called TNE, pH 8.7). The virus suspension was sonicated by two one-minute cycles in a 10-kc Raytheon Sonic Oscillator and then sedimented through a sucrose gradient (see below). When virus was grown in chick cells, the same procedures were used.

Preparation of JEV-infected cell extracts.

1. Cycloheximide pulsed cells. At various times after infection, chick cells were pulsed-inhibited with cycloheximide in the presence of actinomycin D, shown schematically in the following columns, where x is defined as the time (hours after infection) at which cycloheximide was added:

Time after infection (hours)	Operation
0	infected
x - 9	actinomycin added
x	cycloheximide added
x + 0.5	cycloheximide removed; actinomycin D re-added
x + 1	isotope added
x + 5	cells dissolved in SLS

When actinomycin D addition, at x - 9 hours, occurred prior to infection, the drug was removed throughout adsorption and re-added after adsorption.

Specifically, chick embryo cells were prepared from 9 to 11 day-old embryos and grown in 30 cm^2 plastic Falcon tissue culture flasks in 0.5% lactalbumin hydrolysate medium supplemented with 5% FBS and 0.075% sodium bicarbonate. The cells were washed once with Hank's balanced salt solution (HBSS), and infected with 0.2 ml virus diluted in complete 199 + bicarbonate (MOI greater than 50). Following adsorption for 60 to 90 minutes at 36°C, the cell monolayers were washed with 5 ml of HBSS and then 0.5 ml complete 199 + 4.5 ml minimal 199 was added. At the appropriate time (x - 9 hours) 0.1 ml of actinomycin D (50 micrograms per ml) was added to the cells which were then protected from light. At the indicated time (x hours) in Figure 12A, 0.3 ml of cycloheximide (5 mgm/ml) was added. The pulse was terminated after 30 minutes (x + 0.5 hr) by washing the cells four times with 5 ml of minimal 199 and then adding 0.5 ml complete 199, 4.5 ml of minimal 199 and actinomycin D. Thirty minutes later (x + 1 hr) 0.1 ml of ^{3}H-amino acid mixture (100 microcuries/ml) was added. After four hours (x + 5 hr) the cell monolayers were washed three times with 5 ml saline; 1 ml of 0.01 M phosphate, pH 7.3, containing 1% sodium lauryl sulfate (SLS) was then added. Aliquots of 0.1 ml were taken for determination of protein concentration (Lowry, et al., 1951 and acid-insoluble radioactivity. One-tenth volume of 10% 2-mercaptoethanol (2-ME) was added and the viscous extracts were heated at 95°C for 10 minutes, after which they were not notably viscous. Unless otherwise indicated, the samples were dialyzed against at least 200 volumes of 0.01 M phosphate, 0.1% SLS, and 0.02% sodium azide at room temperature for 15 to 36 hours prior to polyacrylamide gel electrophoresis (PAGE) described below. Extracts of uninfected cells were prepared in an identical manner except that no virus was present at the time of mock-infection.

2. Noncycloheximide-treated extracts.

Chick cells were infected as described above. Actinomycin D (final concentration 1 ug/ml) was added nine hours after infection and ^{3}H-amino acids were added 19 hours after infection. At 23 hours the cells were washed three times with saline and then 1 ml of 2% NP 40 in RSB (0.01 M NaCl, 0.0015 M $MgCl_2$, 0.01 M Tris, pH 7.4) was added. The cells were allowed to swell for 10 minutes at room temperature with gentle rocking of the flasks, scraped into the buffer with a pasteur pipette, and disrupted with 20 strokes by the tight fitting pestle in a small Dounce homogenizer. The material was clarified at 2000 rpm for two minutes in an IEC PR-6 centrifuge, dialyzed overnight against 250 volumes of 0.02 M Tris, 0.15 M NaCl, pH 8.1 at 4°C and then frozen at -20°C. It was further clarified in a Beckman 152 Microfuge at 15,000 rpm for five minutes just before use in radioimmune precipitation (RIP). To the appropriate fractions obtained after RIP 1/10 volume of 10% SLS and 1/10 volume of 10% 2-mercaptoethanol were added and then the material was heated and dialyzed as above. Extracts of uninfected cells were treated identically except for mock-infection.

Preparation of radioactive Sindbis virus.

The procedures used were similar to those employed for JEV. Confluent monolayers of chick embryo cells in 32 oz. bottles were prepared and infected with AR339 strain of Sindbis virus at 36°C at an MOI greater than 10. After adsorption for one hour, medium and isotope were added. The culture fluids were harvested 12 hours after infection, clarified and pelleted as described for JEV. The virus was purified on a linear 5-40% sucrose gradient in TNE buffer, pH 7.6, at 25,000 rpm for 2.5 hours at 4°C in the Spinco 25.1 rotor. To the appropriate fractions 1/10 volume of 10% 2-mercaptoethanol and 10% SLS were added and then the material was treated as described above.

Plaque assay of JEV.

Confluent monolayers of chick embryo cells were infected with JEV. After adsorption, they were overlaid with 7 ml of media containing 0.8% agarose, Hank's balanced salt solution, 0.5% lactalbumin hydrolysate, 0.1 Yeastolate, 2 mM glutamine, 5% FBS, 0.3% $NaHCO_3$, and antibiotics. On the third day of incubation at 36°C, 5 ml of a second overlay medium consisting of 4% of 1:300 neutral red solution (Gibco), Hank's balanced salt solution and 0.8% agarose were added. After incubation at 36°C for two hours the cells were left at room temperature overnight; plaques were counted the next day.

Sucrose gradients.

All sucrose gradients were linear, preformed with a mixing chamber and prepared with ribonuclease-free sucrose. For large volumes, 2.5-3 ml samples were applied to 27 ml gradients and the tubes were centrifuged at 25,000 rpm in the Spinco 25.1 rotor for three hours unless otherwise indicated. For small volumes, 0.25 ml samples were applied to 4.8 ml gradients and the tubes were centrifuged at 50,000 rpm in the Spinco SW-50L rotor for 35 minutes unless otherwise indicated.

For isopycnic runs, 0.5 ml samples were applied to 4.6 ml gradients of 20-70% sucrose in deuterium oxide (D_2O) and centrifuged at 65,000 rpm in the Spinco SW-65 rotor for at least 4.5 hours. In a similar system it is known that a 200S particle of density 1.26, after centrifugation at 47,000 rpm for four hours, was at equilibrium. Density was determined by direct weighing of ice-cold 0.05 ml aliquots of each fraction.

Fractions from all gradients were collected dropwise from the bottom of the tube. One ml of NCS (Amersham Searle) was added to 0.1 ml aliquots of radioactive fractions in scintillation vials, the mixture shaken, and then 10 ml of Liquifluor diluted in toluene as recommended was added. Samples were counted in a Packard Tri-Carb

scintillation counter. The channels were set to exclude ^{3}H from ^{14}C and to allow for 5% spillover from ^{14}C to ^{3}H.

Assay for hemagglutination (HA) and complement-fixation (CF).

HA and CF was measured by microtiter modification of the techique used in the Department of Serology (previous Annual Reports).

Polyacrylamide gel electrophoresis.

Continuous neutral SDS polyacrylamide gel electrophoresis (PAGE) was performed essentially according to Maizel (Maizel, 1969). Acrylamide and N, N'-bismethylene acrylamide were purchased, water washed, from Canalco and recrystallized from acetone. A stock solution of 30% acrylamide and 1% bis was prepared and diluted appropriately; gels were 8% acrylamide unless otherwise indicated. The 1M phosphate buffer stock was composed of (per liter) 107 gm Na_2HPO_4, 38.6 gm NaH_2PO_4 H_2O and 0.2 gm NaN_3. A Buchler Polyanalyst apparatus (Buchler Instruments) was employed. The gels (7 x 0.5 cm) were polymerized and electrophoresed while immersed in water at room temperature. Glycerol (0.05 ml) and saturated bromphenyl blue solution (0.01 ml) were added to 0.25 ml of the sample (usually prepared as described for preparation of infected cell extracts), 0.05 ml of the mixture was usually applied. Electrophoresis was performed at 40 volts for the first five minutes, then at 60 volts until the dye front migrated about 6 cm; this usually required two hours. The gels were then removed, placed in a metal trough, frozen on dry ice, and pressed against a transverse slicer containing razor blades at 1 mm intervals. The slices were placed in scintillation vials with 0.075 ml water and 1 ml of NCS, heated at 65°C overnight and then mixed with Liquifluor diluted in toluene.

Molecular weight estimation.

We determined that with 8% gels containing a 30:1 ratio of acrylamide to bis, a linear relationship between relative migration and log molecular weight was valid for a molecular weight range of at least 12,000 to 67,000 (standards were bovine plasma albumin, rennin, B-lactoglobulin and cytochrome C. To determine molecular weights, ^{14}C-amino acid labeled Sindbis was added to ^{3}H-amino acid labeled samples and the mixture co-electrophoresed. Molecular weights of 53,000 and 30,000 were obtained from the literature for the Sindbis proteins.

Radioimmune precipitation (RIP).

The RIP procedure was described in detail in the previous annual report. Essentially, 0.05 ml of diluted radioactive antigen was added to 0.05 ml of TBS (0.1% bovine plasma albumin in 0.15 M NaCl, 0.1 Tris-HCL pH 7.4) in a 0.5 ml polyethylene micro test tube; 0.05 ml of diluted hyperimmune ascitic fluid was added, stirred, and incubated at

37°C for one hour. Then, 0.05 ml of a 1:10 dilution of rabbit anti-mouse serum was added, the tube mixed, incubated at 37°C for one hour, and placed in the refrigerator 2-24 hours. The tubes were centrifuged in a Beckman 152 microfuge at 15,000 rpm for 5 min; 0.1 ml was removed and designated the "supernatant." The tube was mixed, and the remaining 0.1 ml was removed and designated the "pellet." Per cent precipitation was calculated in the following manner:

$$\frac{\text{CPM "pellet"} - \text{CPM "supernatant"}}{\text{CPM "pellet"} + \text{CPM "supernatant"} - 2 \times \text{background CPM}} \times 100 = \% \text{ RIP}$$

For preparative purposes, larger volumes were used but the micro-tubes were still employed and filled with about 0.5 ml. Centrifugation was increased to 10 minutes. The supernatant was removed except for about 0.02 ml. The tubes were mixed, the "pellets" pooled and diluted with an equal volume of TBS, incubated at 4°C for two hours and again centrifuged at 15,000 rpm for 10 min. The supernatant was removed except for about 0.02 ml, the tubes were mixed and the pellet fractions were pooled, diluted to the desired volume with 0.01 M phosphate buffer, dissociated with SLS and 2-ME, dialyzed, and mixed with glycerol and bromphenyl blue prior to PAGE as previously described.

Determination of acid-insoluble radioactivity.

A 0.1 ml aliquot of extract was added to 2 ml of 5% trichloroacetic acid (TCA), heated at 90°C for 15 min, and then cooled in ice-water for at least two hours. The material was pured through 0.45 mu membrane filters (Millipore Corp.) and the filters rinsed witn 3-2 ml aliquots of cold 5% TCA. They were placed into scintillation vials, dried in an oven at 65°C, and then Liquifluor diluted with Toluene was added.

Results

Growth of radioactive JEV in LLC-MK$_2$ and chick embryo cells.

The growth curves of JEV in LLC-MK$_2$ and chick embryo cells are presented in Fig. 10A. Virus appeared in the medium by 11 hours after infection and reached maximal titers by 40 hours in both cell lines. Therefore, when preparing radioactive virus, culture medium was harvested at 40 hours. When examining stable intracellular proteins of infected cells, long radioactive pulses beginning at least 12 hours after infection were used.

When radioactive JEV was prepared from LLC-cells as described above and sedimented through a sucrose gradient, one major coincident peak of radioactivity, hemagglutination and complement-fixation was present (Fig. 10B), this peak has been shown to be infectious virus. A more slowly sedimenting peak of HA (SHA) was also present and will be discussed below.

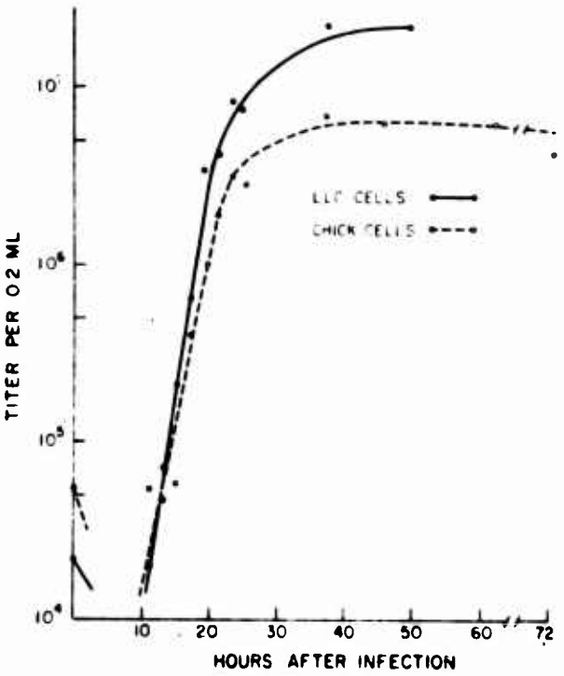


Figure 10A. Growth curve of Japanese encephalitis virus in monkey kidney (LLC-MK_2) and chick embryo cell cultures.

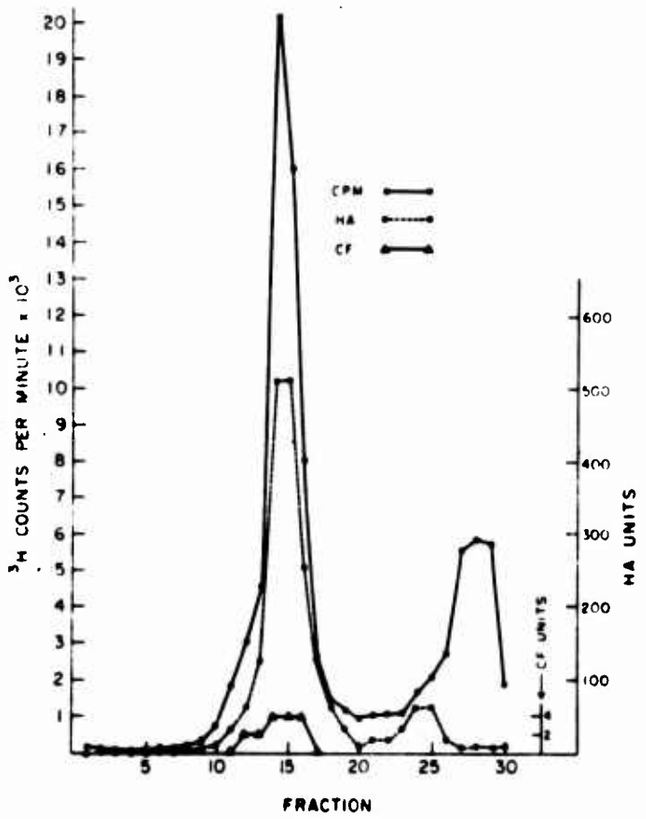


Figure 10 B. Radioactive JEV centrifuged in 5-45% sucrose gradients at 25,000 rpm for 2.5 hours.

Polyacrylamide gel electrophoresis (PAGE of ^{3}H-amino acid labeled JEV.

When ^{3}H-JEV was dissociated with sodium lauryl sulfate (SLS) and 2-mercaptoethanol (2-ME), dialyzed, and electrophoresed on varying concentrations of polyacrylamide gels, three virion polypeptides were always detected (V-1, V-2, and V-3) (Fig. 11). The fastest migrating polypeptide (V-1) co-migrated with the dye front in 8% acrylamide gels. However, electrophoresis through 10% and 12% gels clearly separated V-1 from the dye front, indicating that this radioactive peak actually did correspond to a polypeptide. ^{14}C-Sindbis proteins were used as reference markers as described below. Occasionally, a very minor radioactive peak migrated slower than V-3 (Fig. 13A); however, this was not reproducible and therefore not considered to be a virion polypeptide.

Polypeptides in JEV-infected cells.

Protein synthesis in chick cells was inhibited 25% by JEV infection; actinomycin D treatment of chick cells suppressed protein synthesis by 54% (Table 29). Despite these levels of inhibition, we were unable to identify virus specific polypeptides in JEV-infected cells in the presence or absence of actinomycin D; residual host protein synthesis effectively masked the presence of virus-specified proteins.

Table 29. Protein Synthesis in Chick Cells[1]

Treatment	Acid Insoluble cpm
None (Mock-infected)	40,000
JEV infection	30,000
Actinomycin D addition (alone)[2]	18,200
Actinomycin D plus cycloheximide pulse[3]	5,200
Cycloheximide addition (alone, continuous)[4]	
a. Mock-infected	725
b. Infected	673

1 The results presented here are the controls for the experiment in Fig. 13A; the general procedures are described in Materials and Methods or in the legend to Fig. 12A. Isotopes were added from 1½ hrs to 5½ hrs after infection in all samples. The relative results were the same when expressed as cpm or calculated as specific activity.

2 Cells pretreated with 1 ug/ml of actinomycin D for 9 hrs prior to mock-infection; actinomycin D readded 1½ hrs after mock-infection and isotope added from 1½ to 5½ hrs after mock-infection.

3 From the "uninfected curve," 1½ hr point in Fig. 13A.

4 Mock-infected or JEV-infected cells; cycloheximide (300 ug/ml) added 1 hr after infection; isotope and cycloheximide present from 1½ to 5½ hr. after infection.

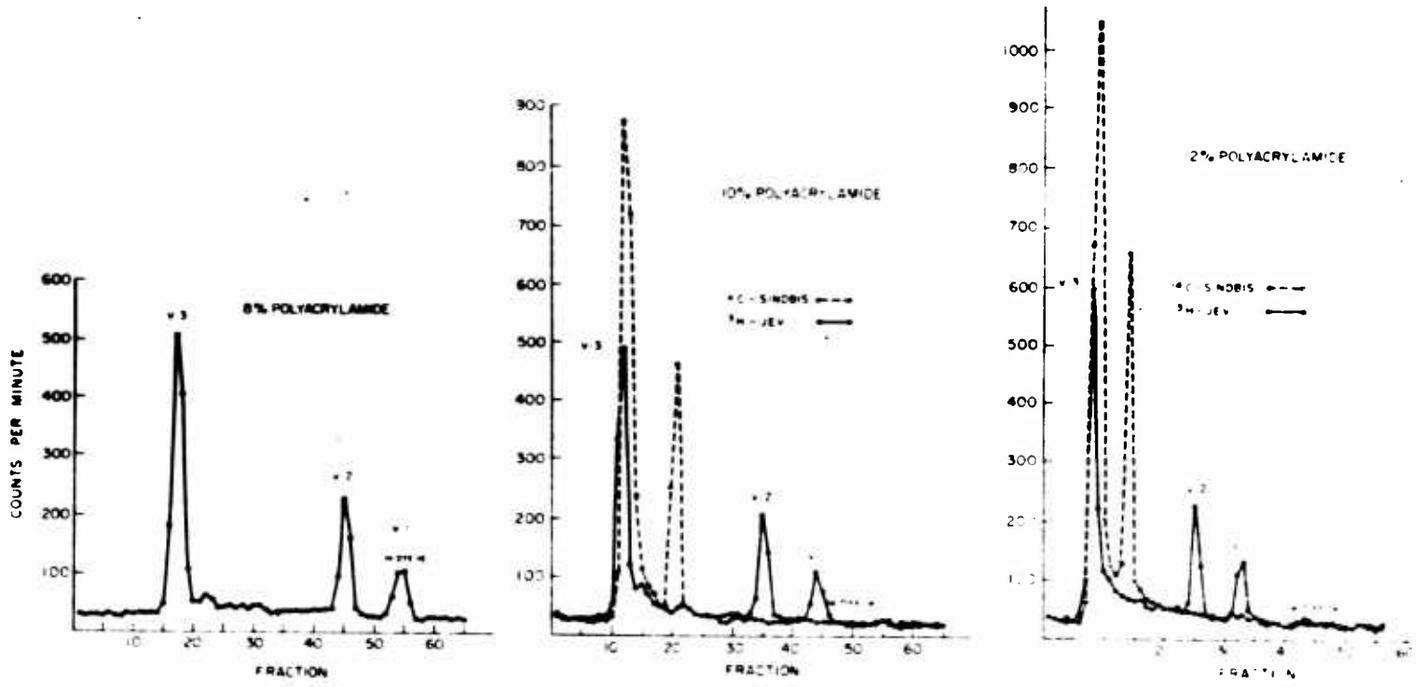


Figure 11. Polyacrylamide gel electrophoresis of ^{3}H-amino acid labelled Japanese encephalitis virus (JEV). The 10% and 12% gels contained 0.01ml ^{14}C-sindbis virus in addition to the 0.05 ml JEV sample

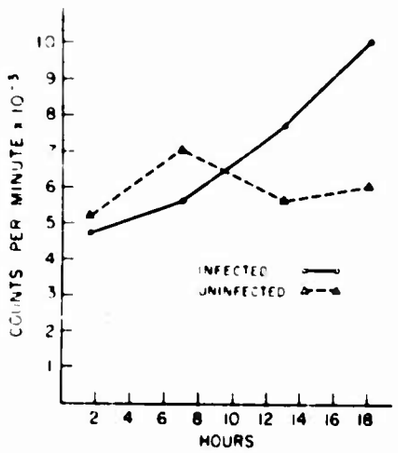


Figure 12A. Protein synthesis in Japanese encephalitis virus infected, actinomycin D-treated, cycloheximide pulse-inhibited chick cells.

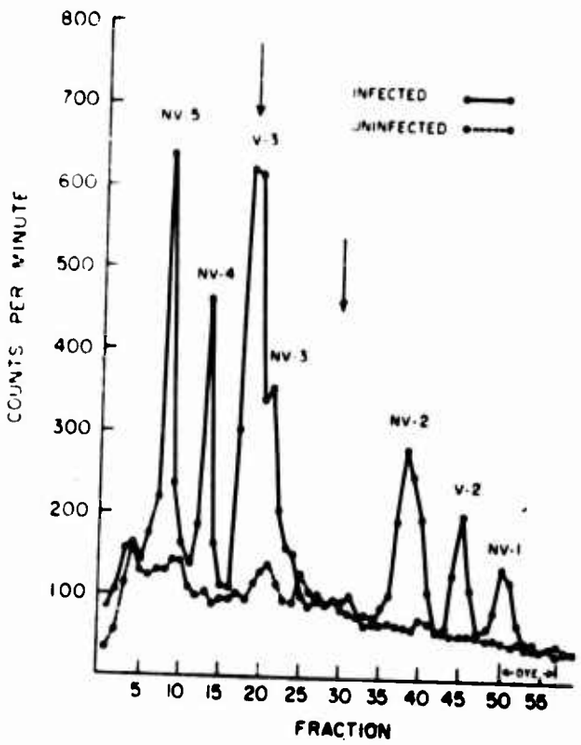


Figure 12B. Polyacrylamide gel electrophoresis of extracts of JEV-infected, actinomycin D treated, cycloheximide pulsed, chick cells. Arrows represent position of ^{14}C-Sindbis markers.

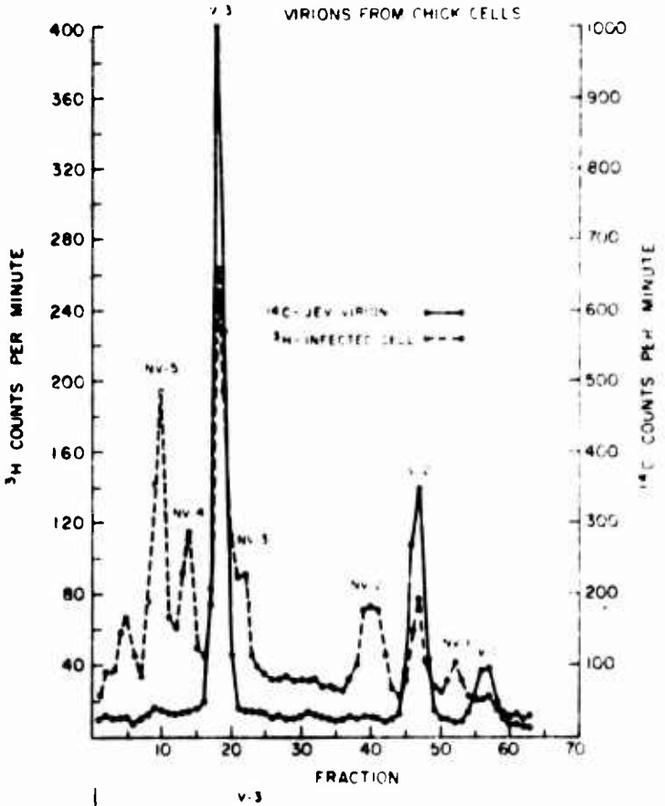


Figure 13A. Polyacrylamide electrophoresis of JEV virions and intracellular proteins from chick cells.

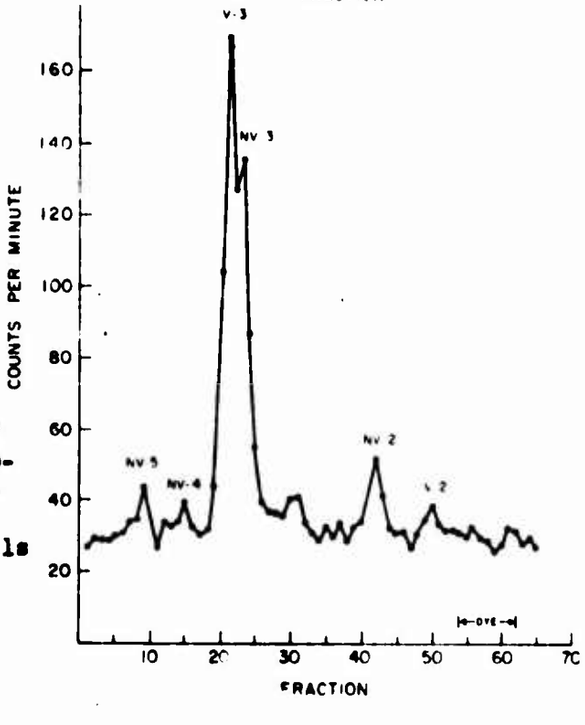


Figure 13B. Polyacrylamide electrophoresis of radioimmune precipitate from actinomycin D treated, infected chick cells

In order to specifically and more severely suppress host protein synthesis, it was necessary to utilize Trent's procedure in which a brief pulse of cycloheximide is administered to cells pretreated with actinomycin D. When chick cells were treated with cycloheximide continuously, protein synthesis in both infected and uninfected cells was abolished (Table 29). When normal chick cells were treated with a pulse of cycloheximide, there was only a transient inhibition of protein synthesis. This procedure was, therefore, not useful for the initial identification of virus-specified polypeptides. However, when normal chick cells were treated with a pulse of cycloheximide after pretreatment with actinomycin D, protein synthesis was inhibited by 87% (Table 29). When this same procedure was applied to JEV-infected cells at various times after infection, an increase of protein synthesis, relative to uninfected cells, was observed when the cycloheximide was added 12 hours or more after infection (Fig. 12A).

The observed increase in protein synthesis in JEV-infected cells treated with actinomycin D and cycloheximide was shown to be due to the selective synthesis of viral specific proteins. When extracts of infected and mock-infected chick cells which were pretreated with actinomycin D and pulse-inhibited with cycloheximide 22 hours after infection were analyzed by PAGE, seven polypeptides were detected in the infected cell extract (Fig. 12B). By contrast, in the uninfected cell extract, only a low, generally flat background was present (Fig. 12B). Two of the polypeptides corresponded to virion polypeptides V-2 and V-3; the five additional, nonvirion polypeptides were designated NV-1 through NV-5. NV-3 was usually a distinct peak, but occasionally its presence was indicated by a shoulder on the right side of V-3. The same polypeptides were identified in infected cells pulse-inhibited with cycloheximide in the absence of actinomycin D. This result indicated that pre-treatment with the latter drug was not essential for severe inhibition of host protein synthesis in the infected cell, although it was necessary in the uninfected cell.

In order to estimate the molecular weights of the JE proteins, ^{14}C-amino acid-labeled Sindbis was added to ^{3}H-amino acid-labeled samples, and the mixture co-electrophoresed. From runs similar to those illustrated in Figs. 11 and 12B (Sindbis proteins indicated by arrows), molecular weights of 8,700, 13,500 and 53,000 were calculated for V-1, V-2, and V-3, respectively, and values of 10,500, 19,000, 45,000, 71,000, and 93,000 for NV-1 through NV-5, respectively.

There was no convincing evidence for the presence in infected chick cells of a polypeptide of 8,700 daltons corresponding to V-1, the smallest virion polypeptide of LLC-MK_2 derived virus.

One possible explanation for this would be that virions derived from chick cells, in contrast to virions from LLC-MK_2 cells, lack V-1 and contain as a structural component one of the "non'virion"

polypeptides. However, co-electrophoresis of chick cell-derived virus with infected chick cell extracts indicated that the virus from chick cells also contained V-1 and appeared similar to virus from LLC-MK_2 cells (Fig. 13A).

Radioimmune precipitation (RIP) of JEV proteins.

We felt that it would be desirable to determine virus-specified intracellular proteins by another, independent method. We chose to use radioimmune precipitation, which relied on their presumed antigenicity. Hyperimmune ascitic fluid prepared in mice immunized with JEV-infected mouse brain was able to specifically precipitate greater than 85% of radioactive JEV virions. When a cytoplasmic extract prepared from JEV-infected, actinomycin-D treated, chick cells that had not been pulsed with cycloheximide was treated with this antisera, approximately 20% of the radioactivity was precipitated. No radioactivity was precipitated from identically prepared extracts of uninfected cells in parallel experiments; other controls were also negative (substitution of normal ascitic fluid for hyperimmune ascitic fluid or omission of rabbit anti-mouse serum). When material precipitated in this manner was analyzed by PAGE, it appeared that six of the seven previously described polypeptides were identifiable although there was insufficient radioactivity to be certain of the presence of all of them (Fig. 13B).

Isolation and PAGE of JEV "core."

When virions which were doubly labeled with ^{3}H-uridine and ^{14}C-amino acids were centrifuged through a sucrose-D_2O gradient they appeared to have a density of 1.23 (Fig. 14A). When an aliquot of the original preparation of virions was treated with NP-40, a structure was obtained which was denser than the virion and which contained proportionately more ^{3}H-RNA (relative to protein) than the virion (Fig. 14B). Co-electrophoresis of this presumed "core" structure with ^{3}H-amino acid labeled virions indicated that the "core" contained principally one polypeptide, V-2 (Fig. 14C).

Characterization of the slowly sedimenting hemagglutinin (SHA).

When cell culture fluid was harvested at 64 hours after infection, a slowly sedimenting hemagglutinin (SHA) was present and shown to be RNA-poor (Fig. 15). When this material was centrifuged to equilibrium, most of the protein banded as a discrete peak with density of 1.23 g/cm^3 (similar to the density of the virion) (Fig. 16A). Most of the small amount of RNA in the preparation had a greater density and was, therefore, not associated with the SHA particle.

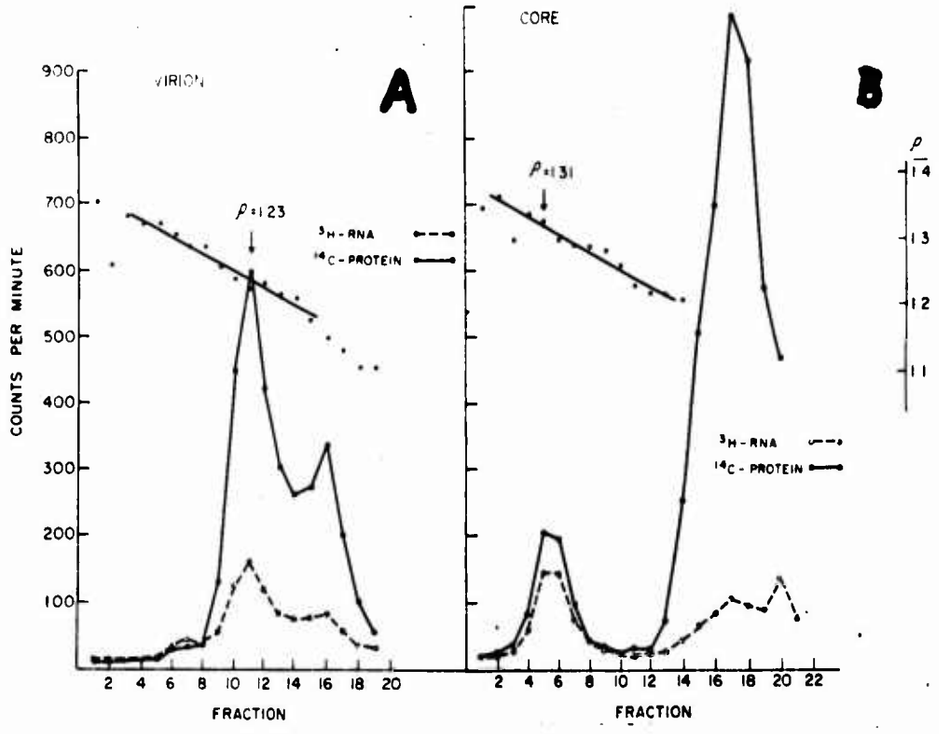


Figure 14. Isopycnic centrifugation of JEV virion (A) and NP-40-derived core (B) on 20-70% sucrose-D_2O gradients (65,000 rpm for 4.5 hours).

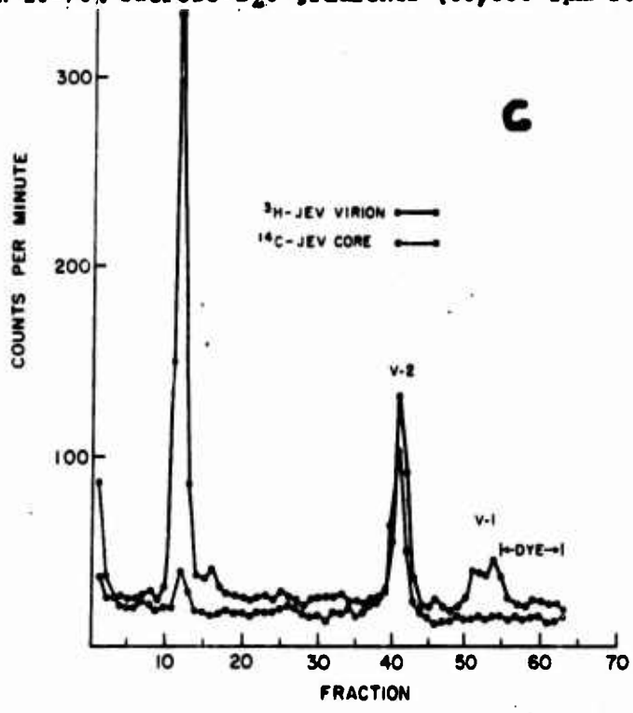


Figure 14C. Co-electrophoresis of JEV virions and core proteins.

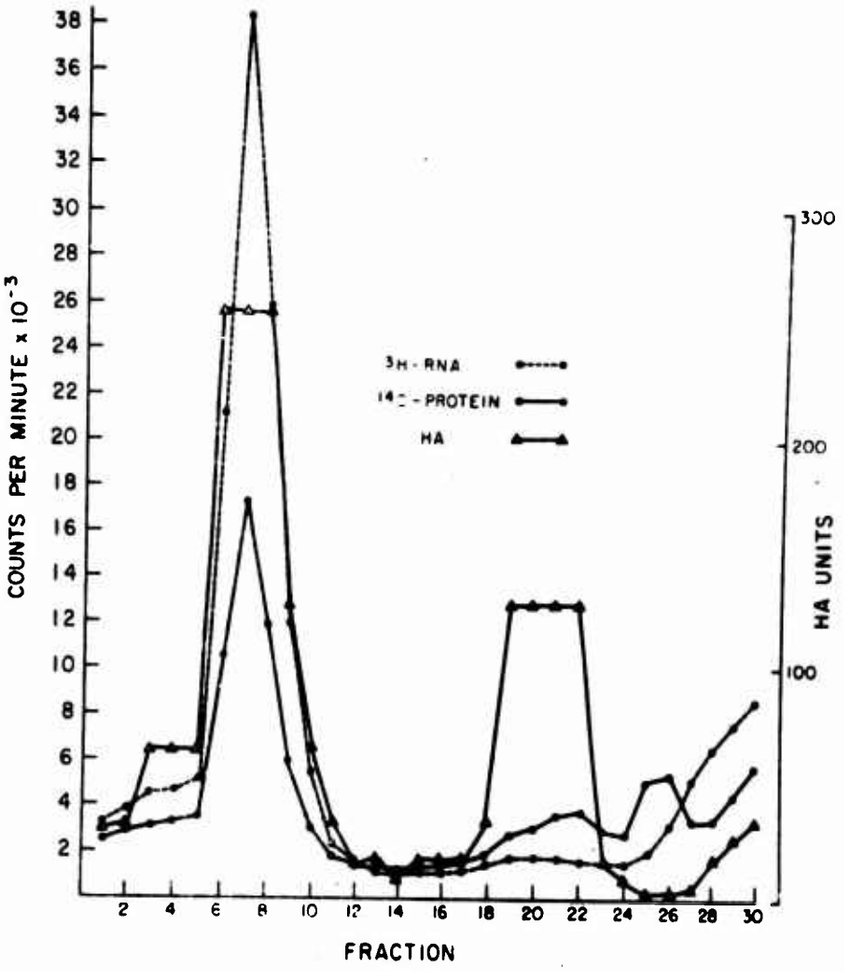


Figure 15. Radioactive JEV harvested 64 hours post infection and centrifuged in 5-35% sucrose gradients at 25,000 rpm for 3.5 hours. Slowly sedimenting hemagglutinin found in fractions 19-22. LLC-MK_2 cells were supplied with radioactive amino acids and uridine.

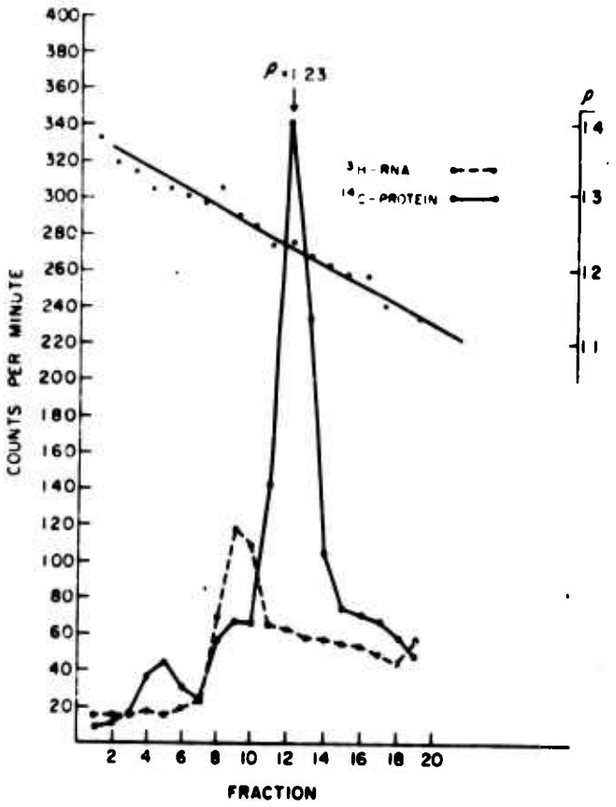


Figure 16A. Isopycnic centrifugation of JEV slowly sedimenting hemagglutinin shown in the previous figure (fractions 19-22).

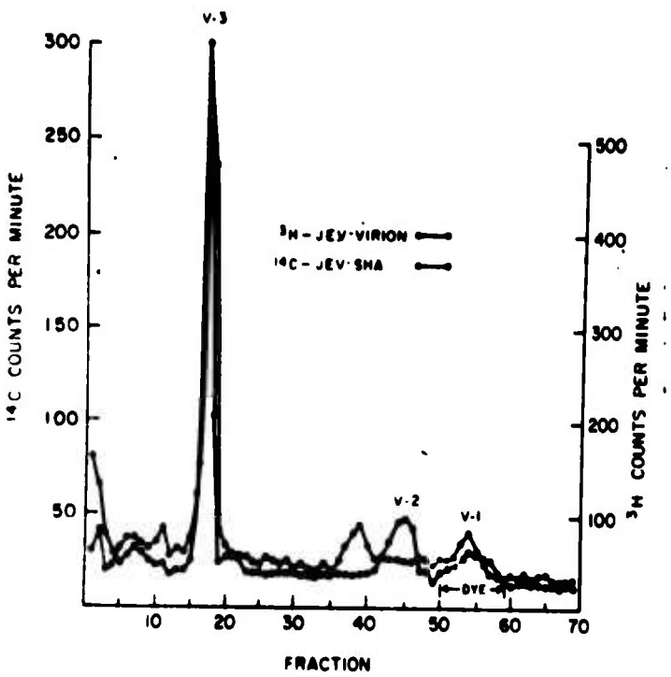


Figure 16B. Polyacrylamide coelectrophoresis of JEV virions and slowly sedimenting hemagglutinin (SHA)

When labeled SHA was taken directly from the gradient in Fig. 15 and co-electrophoresed with ^{3}H-amino acid virions three major polypeptides were shown to be present in the SHA (Fig. 16B). Two corresponded to V-1 and V-3 and the third had an estimated molecular weight of 18,000, corresponding to NV-2.

However, when analogous material was taken from a gradient of virus harvested at 40 hours rather than 64 hours, only V-1 and V-3 were present.

Discussion

We have analyzed the polypeptides of a group B arbovirus "system" consisting of the virion, slowly sedimenting hemagglutinin (SHA), "core" and intracellular proteins. The observation that moderately radioactively labeled virion is composed of only three major polypeptides is in agreement with results for dengue (Stollar, 1969) and St. Louis encephalitis (Trent, et al, 1969) but differs from results reported for Kunjin (Westaway and Reedman, 1969). A highly-radioactively-labeled preparation of Kunjin was reported to contain four polypeptides. Since Kunjin is very closely related to JEV this difference is unexpected. It is possible that the fourth minor radioactive peak represents an artifact due to aggregation similar to what was observed with the "core" protein of Semliki Forest virus (Acheson and Tamm, 1970). We have occasionally observed a minor inconstant peak in acrylamide analyses of virions which migrated with NV-5 in co-runs. However, in most newly prepared samples, including one containing a 4000 cpm V-3 peak, similar to Kunjin, the fourth peak was absent. Unambiguous demonstration of minor structural polypeptides will require functional tests.

We were able to detect seven polypeptides in infected cells. One of the polypeptides, NV-3, was not always separable from V-3, and in fact, resembled an intramolecularly-rearranged form of V-3 (Strauss, et al, 1969). However, we felt that NV-3 was a discrete polypeptide for the following reasons: 1. Sindbis proteins, which are known to be subject to this kind of artifact, always behaved as two homogenous narrow peaks in co-electrophoresis runs; 2. V-3 derived from the virion also always behaved as a single, narrow peak; 3. Co-electrophoresis of virion and intracellular polypeptides indicated the presence of NV-3 only in the intracellular material; and 4. Isolation of the intracellular proteins by an immunological procedure revealed a markedly increased ratio of NV-3 to V-3, suggesting, but not proving, independent behavior of the two proteins.

We have shown that a JEV "core" obtained by NP-40 treatment of the virion contained one polypeptide. It was claimed that treatment of Kunjin virions with deoxycholate resulted in a "core"

containing two polypeptides. Two core polypeptides were also reported for St. Louis encephalitis virus treated with deoxycholate (Trent, 1969). However, one core polypeptide was reported for dengue virions treated with NP-40. These differences may arise because of the different detergents used. It appears likely that the use of NP-40 produced a completely "stripped-down" core derived from JEV and dengue virions and that the single polypeptide in this particle interacted most strongly with RNA and is therefore the "core" protein. It is not unreasonable to expect that less "stripped-down" cores would have additional proteins bound primarily to the "core" protein rather than to the RNA. Until a naturally-occurring core is isolated from infected cells and analyzed (Acheson and Tamm, 1970) we must conclude that group B arbovirus "cores" contain one polypeptide.

The polypeptides found in the infected cell constitute 300,000 daltons of protein, which is approximately the coding capacity of the viral genome assuming that JEV-RNA has the same molecular weight (3.3×10^6) as dengue RNA (Stollar, et al, 1966). Furthermore, they were found after long labelling times in cells beginning at about 12 hours after infection. Therefore, they probably constitute the major species of stable virus-specified polypeptides. Because the polypeptides are not present in equimolar amounts, they are unlikely to result from unvarying translation of a single giant m-RNA molecule. Whether they arise as separate monocistronic translations of m-RNA, or as post-translational cleavage products (Jacobson and Baltimore, 1968; Burrell, et al, 1970; Cooper, et al, 1970; Hosada and One, 1970; Katz and Moss, 1970), and whether they are differentially synthesized in an expanding intracellular pool in a manner similar to what is observed for group A arboviruses (Scheele and Pfefferkorn, 1969) remain to be determined.

The intracellular deficit of V-1 may be relevant here. Since group B arboviruses appear to penetrate through membranes into intracellular vacuoles (Murphy, et al, 1969; Ota, 1965), one would expect to find at least traces of all virion polypeptides within the infected cell merely because of the vacuolar entrapment of fully mature virions. The deficit of intracellular V-1 is therefore unexpected and may result from the following:

a. V-1 may merely be synthesized in small amounts too low to be detected here. If true, this would raise the possibility that the amount of V-1 available limits virus assembly or release.

b. V-1 may be rapidly turned over with respect to V-2 and V-3, or else may be preferentially synthesized relatively early in infection.

c. Actinomycin D-cycloheximide pulse-inhibition may irreversibly inhibit V-1 synthesis.

d. V-1 may be derived by cleavage from one of the non-virion polypeptides during virus assembly or release. This suggests the possible existence of a precursor particle similar to the polio procapsid (Jacobson and Baltimore, 1968).

Related to the last possibility is the existence of a subviral particle (SHA) which contains at least one polypeptide not present in the virion and lacks V-2. However, instead of representing a normal precursor, it may result from aberrant morphogenesis perhaps occasioned by cellular degeneration. This situation is complex since, with varying conditions of infection, we have observed several varieties of subviral particles sedimenting similar to SHA. We are currently investigating their relationship to each other and their role in virus maturation.

B. Characteristics and Synthesis of Proteins in Japanese Encephalitis Virus Infected Chick Embryo Fibroblasts.

Information obtained on thepolypeptide composition of JEV as well as the viral-specified proteins within infected host cells (see above) provided the means to begin investigating the site(s) of synthesis of viral antigens in the infected cell. It has been shown that the major coat protein of the JE virion is a glycoprotein, and it may be true that the major viral antigens are glycoproteins. For this reason, the nature and site of antigen synthesis within infected cells was first approached by determining which of the viral specified, but nonvirion proteins are glycoproteins. Next, the membranes of infected cells were fractionated by centrifugation methods that required extensive development and examined for their relationship with viral specified proteins.

1. Glycoproteins of JEV-infected Chick Cells.

Chick cells were treated with actinomycin D after infection with JEV and then labeled with ^{3}H-amino acids and ^{14}C glucosamine from 12 to 22 hours after infection. The cells were dissolved in 1% NP-40 in RSB, Dounced 25 times and dialyzed against RSB. JEV hyperimmune mouse ascitic fluid was able to precipitate about 20 per cent of the radioactivity whereas normal ascitic fluid did not precipitate any radioactivity. When this material was dissolved in SLS and 2-ME and analyzed by polyacrylamide gel electrophoresis (PAGE), three major peaks were present: V-3, NV-3, and NV-2; all were glycoproteins (Figure 17A). Therefore, we conclude that there are three JEV-specified glycoproteins and that all three comprise the principle antigens of infected cells. The peak labeled "A" is not generally observed.

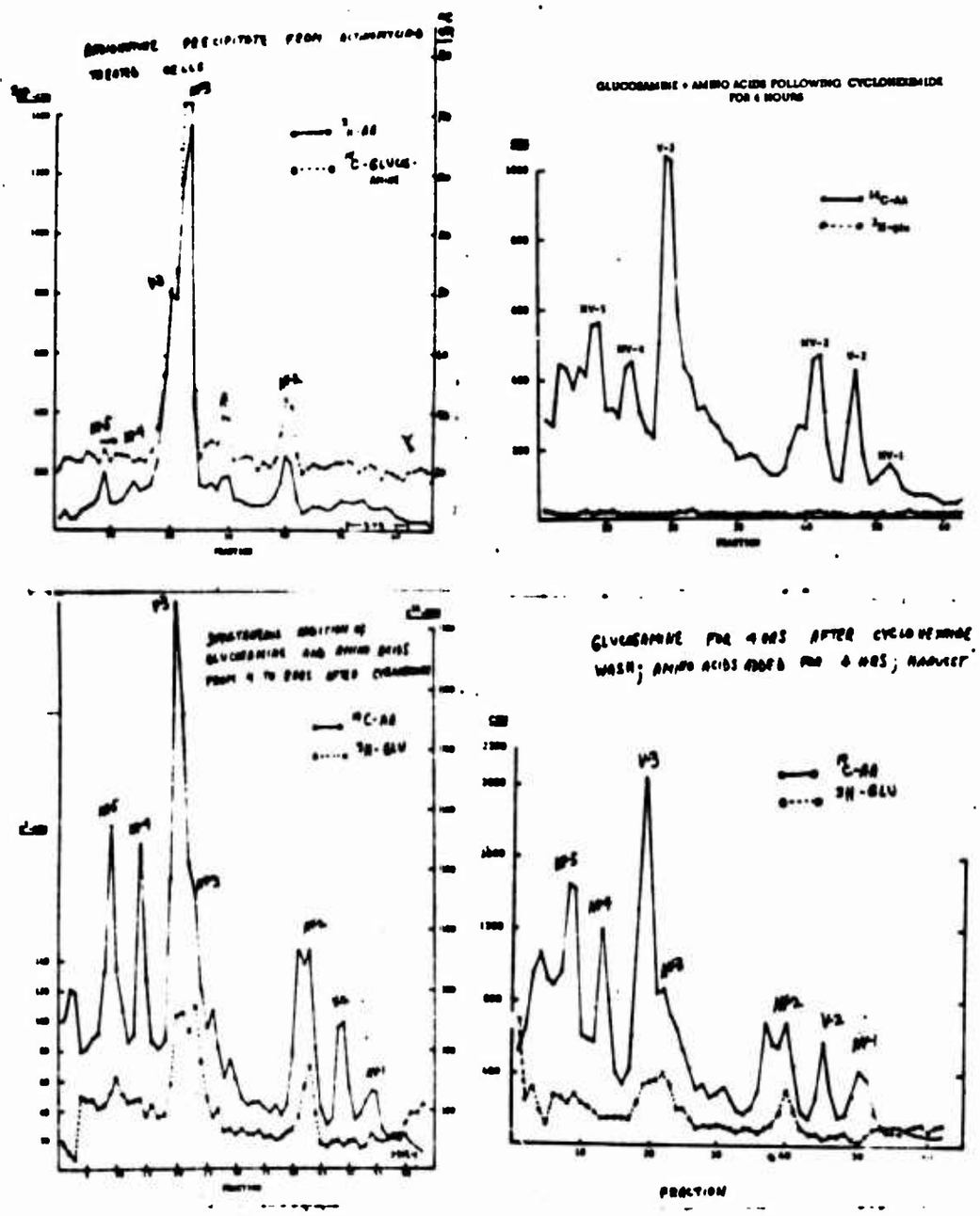


Figure 17. Polyacrylamide gel electrophoresis of radioactive extracts of chick embryo cells infected with Japanese encephalitis virus and treated as indicated in each graph.

When JE infected cells were treated with actinomycin D and pulse-inhibited with cycloheximide, glycosylation was found to be more persistently suppressed than protein synthesis. In fact, when glycosamine and amino acids were added right after pulse-inhibition, no carbohydrate was incorporated into glycoproteins (Figure 17B). By contrast, after a delay of four hours, incorporation of carbohydrate did occur (Figure 17C). To demonstrate that glucosamine was able to enter the cell and presumably the nucleotide sugar pool during the period of time when glycosylation was inhibited, the glucosamine was added for four hours then removed, after which amino acids were added. As can be seen by PAGE of the cell extract, glycosylation did occur after this treatment (Figure 17D). The explanation for these unexpected findings is not clear.

2. Membranes of JEV-infected Chick Cells.

We have adapted procedures for isolating membranes of chick embryo cells; the scheme is shown in Figure 18A. Briefly, monolayers of cells from 1 to 3 32-oz prescription bottles containing 2 to 10 x 10^8 cells are scraped off into 0.25 M sucrose in RSB and then dounced 25 times in a 7 ml homogenizer with a tightly fitting pestle. The material is centrifuged at 2000 rpm for 2 min and the pellet is resuspended and recentrifuged at 2000 rpm for two min; the resulting pellet is called the "2P" fraction, corresponding to a nuclear-enriched fraction. The supernatant, called "2S", is then centrifuged through 0.3M sucrose onto a 2.6 M sucrose cushion at 10,000 x g for 30 min. The pelleted material is called "10P". The supernatant is called "10S" and centrifuged through 0.3M sucrose onto a 2.6M sucrose cushion at 308,000 g (65,000 rpm) for 45 min. The pellet is called "65-P" and the supernatant fluid is called "65-S". The three fractions are then centrifuged on the discontinuous sucrose gradient illustrated in Figure 18B. For convenience, the method of Caliguiri is included for comparison. The gradients are collected in 0.1 ml fractions by bottom puncture. Eight bands of membranous material can be distinguished in regions corresponding to the various sucrose interfaces. In general, electron micrographs indicate that smooth membranes are found in bands I, II, and III, whereas rough (and smooth) membranes are present in bands IV through VII. Figures 19 A, B, C, illustrate the results of membrane fractionations when a pulse-inhibited cell is labeled with 3H-amino acids for four hours. The 2P fraction contains essentially only band VI. The 4P fraction contains three prominent bands: VI, V_B, V_A, and III. The 65-P fraction contains predominantly III. On occasion, the majority of V_A can be found in the 65P fraction rather than the 10P fraction. The distribution of radioactivity is given in Table 30; 60% of total cell radioactivity is present in 2P, 30% is found in 10P, and 5% in 65. Only 4% is found in 65S, indicating that essentially every JEV-specified protein is membrane bound. There is a 5 to 10-fold enrichment of radioactivity in bands IV - VII as compared to bands I - III.

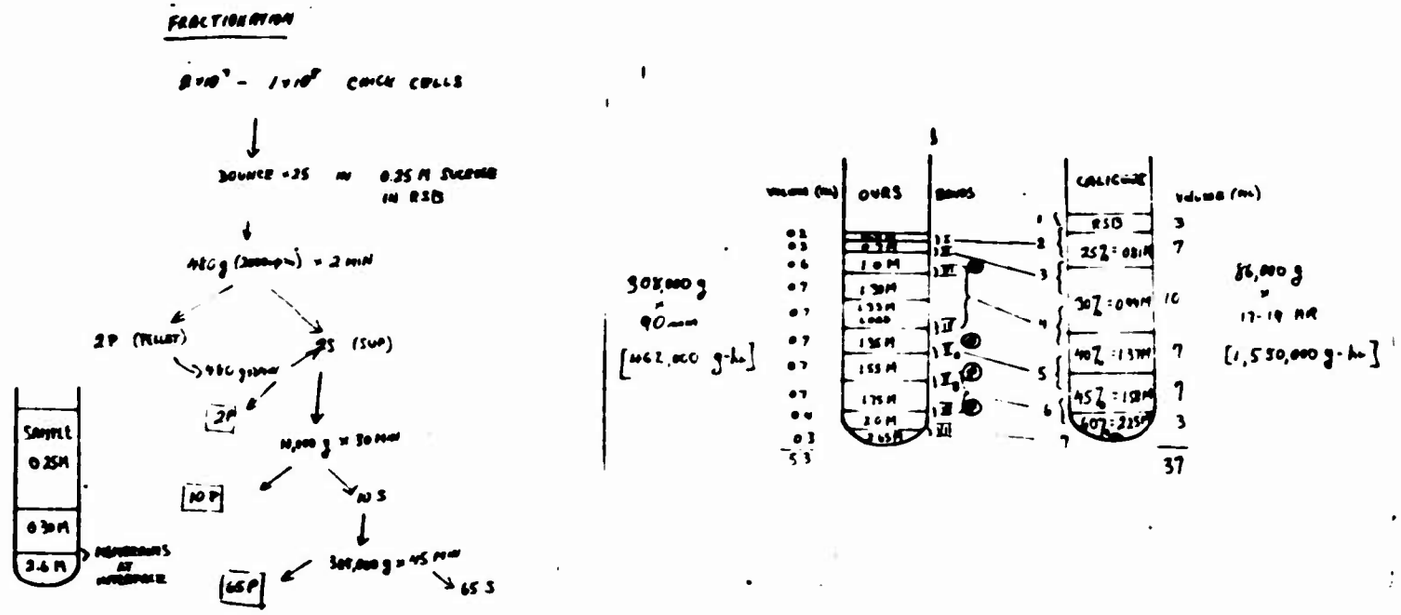


Figure 18. A. Crude fractionation scheme used to isolate membranes from chick embryo fibroblasts.

B. Discontinuous sucrose gradient used to separate the different types of membranes obtained in the 2,000 rpm pellet, 10,000 rpm pellet, and 65,000 rpm pellet fractions shown in "A". Samples are loaded into the 1.33 molar sucrose about the center of the gradient. The method of Caliguiri is shown for comparison.

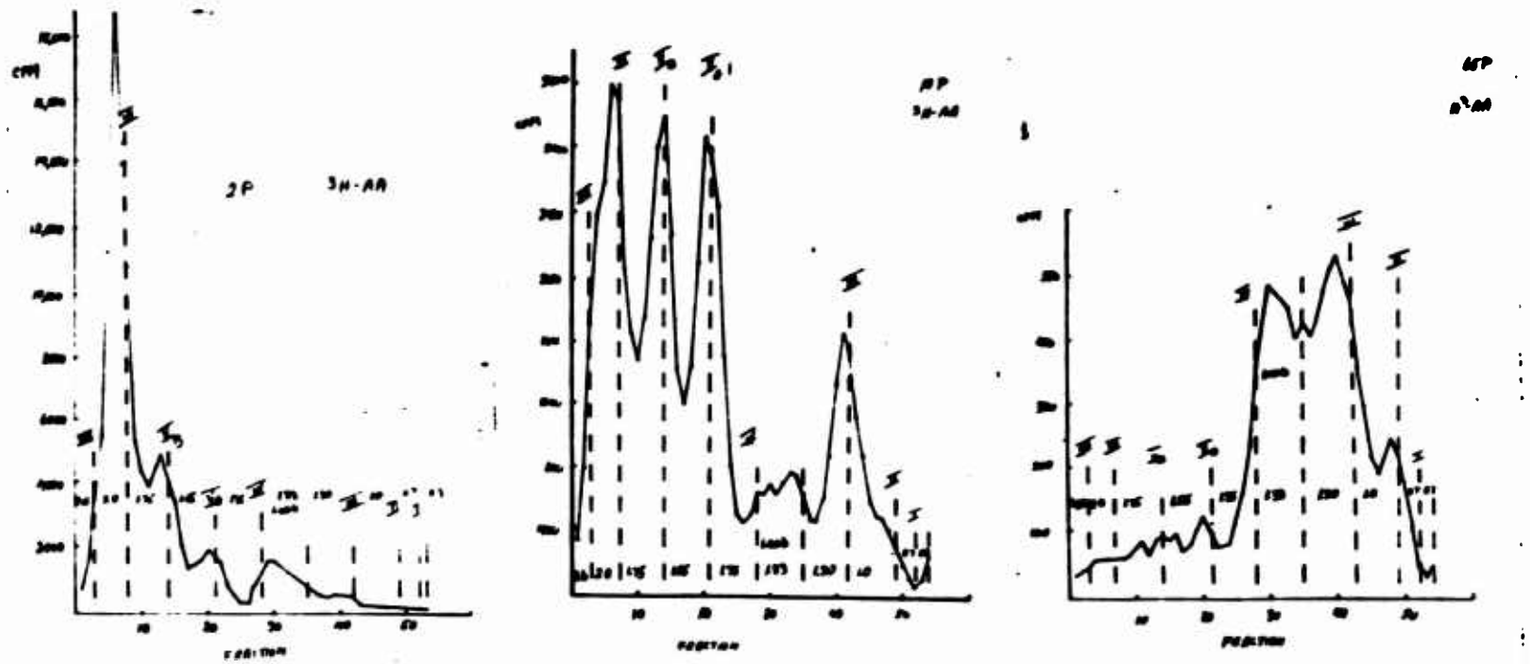


Figure 19. Radioactivity in fractions from discontinuous sucrose gradients illustrated in Fig. 18B. Visible bands were marked by Roman numerals and fractions were collected in 0.1 ml aliquots from the bottom of the centrifuge tube and assayed for radioactivity. The gradients of the 2,000 rpm pellet, 10,000 rpm pellet, and 65,000 rpm pellet are shown in A, B, and C, respectively.

When the membrane fractions were analyzed by PAGE it appeared that dense membranes had similar polypeptide compositions (Figure 20, A-D). Light membranes were strikingly different (Figure 21, A-C); they obtained a predominance of NV-5 and lacked NV-4. When the soluble cytoplasmic proteins (65 supernate) were analyzed, there was a large proportion of NV-5 (Figure 21D). Neither one of the three prominent peaks A, B, or C co-ran with marker V-3. Furthermore, a high background of radioactivity was present throughout the run. This suggests that proteolysis is partly responsible for "soluble" proteins. There was also no enrichment for glycoproteins. These results do not support the hypothesis that smooth membranes are the sites of glycosylation. To look at this more closely, infected cells were treated with actinomycin D and labeled with ^{3}H-galactose and ^{14}C-glucosamine for eight hours. The membrane gradients are presented in Figure 22 A, B, C. There is virtually a constant ratio of galactose/glucosamine in all membrane fractions. Furthermore, there is a two to four-fold enrichment of radioactivity in bands IV - VI as compared to I - III which, although less than the five to ten-fold enrichment found in the case of amino-acid labeled cells, does not implicate smooth membranes (or at least not light smooth membranes) as the site of glycosylation.

Table 30.

Distribution of Radioactivity through Membrane Fractions

	Percentage of Total Radioactivity		
Fraction	^{3}H Amino Acids	^{3}H Galactose	^{14}C Glucosamine
2P	59	39	42
10P	30	50	44
65P	5	6	5
65S	4	4	5
Bands IV-VII of 2P, 10P, and 65P	84	74	73
Bands I-III of 2P, 10P and 65P	8	26	20
Bands IV-VII of 10P and 65P only	31	40	35
Bands I-III of 10P and 65P only	6	21	18

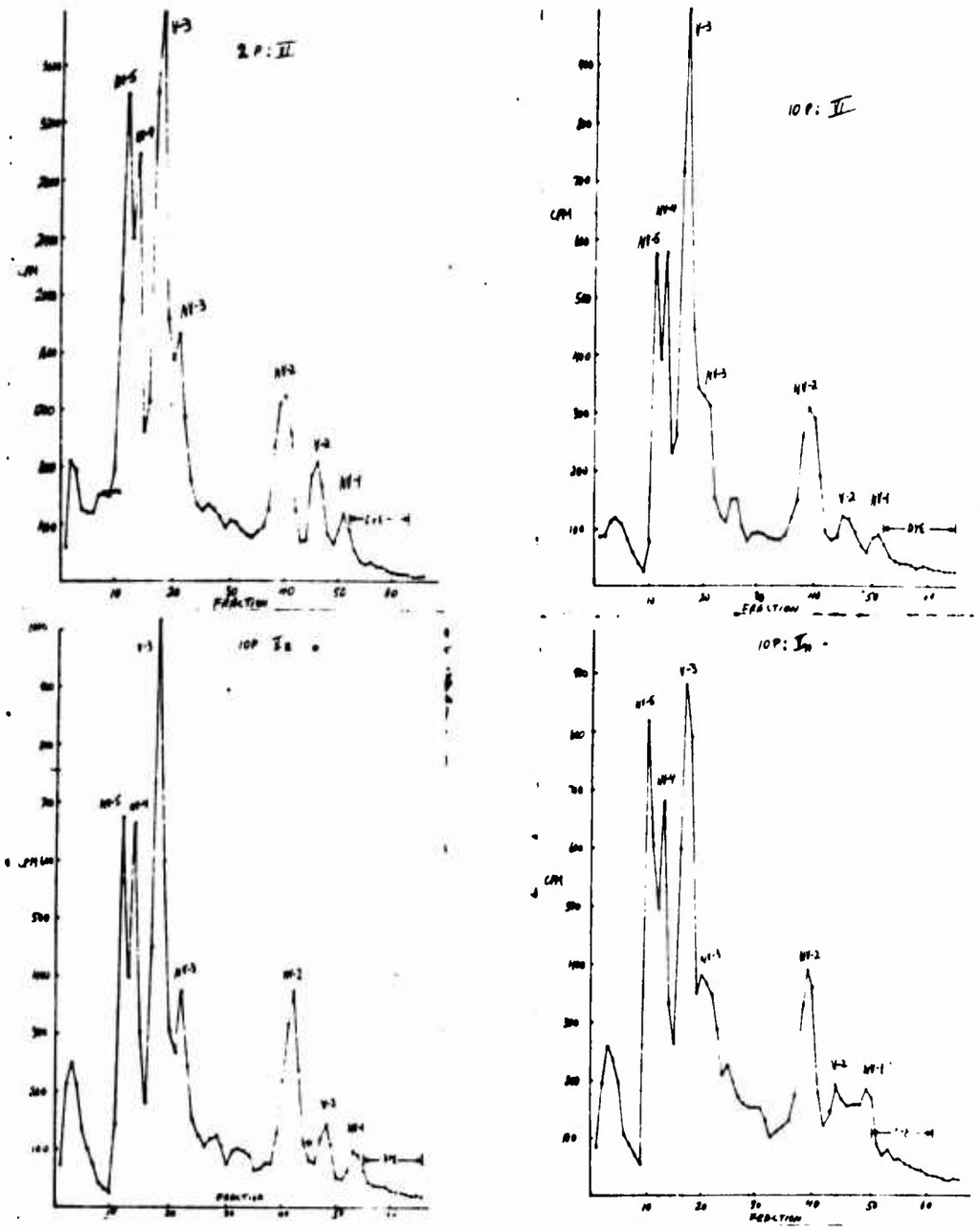


Figure 20. Polyacrylamide gel electrophoresis of dense membranes in the indicated bands (Roman numerals) isolated as shown in Figure 19.

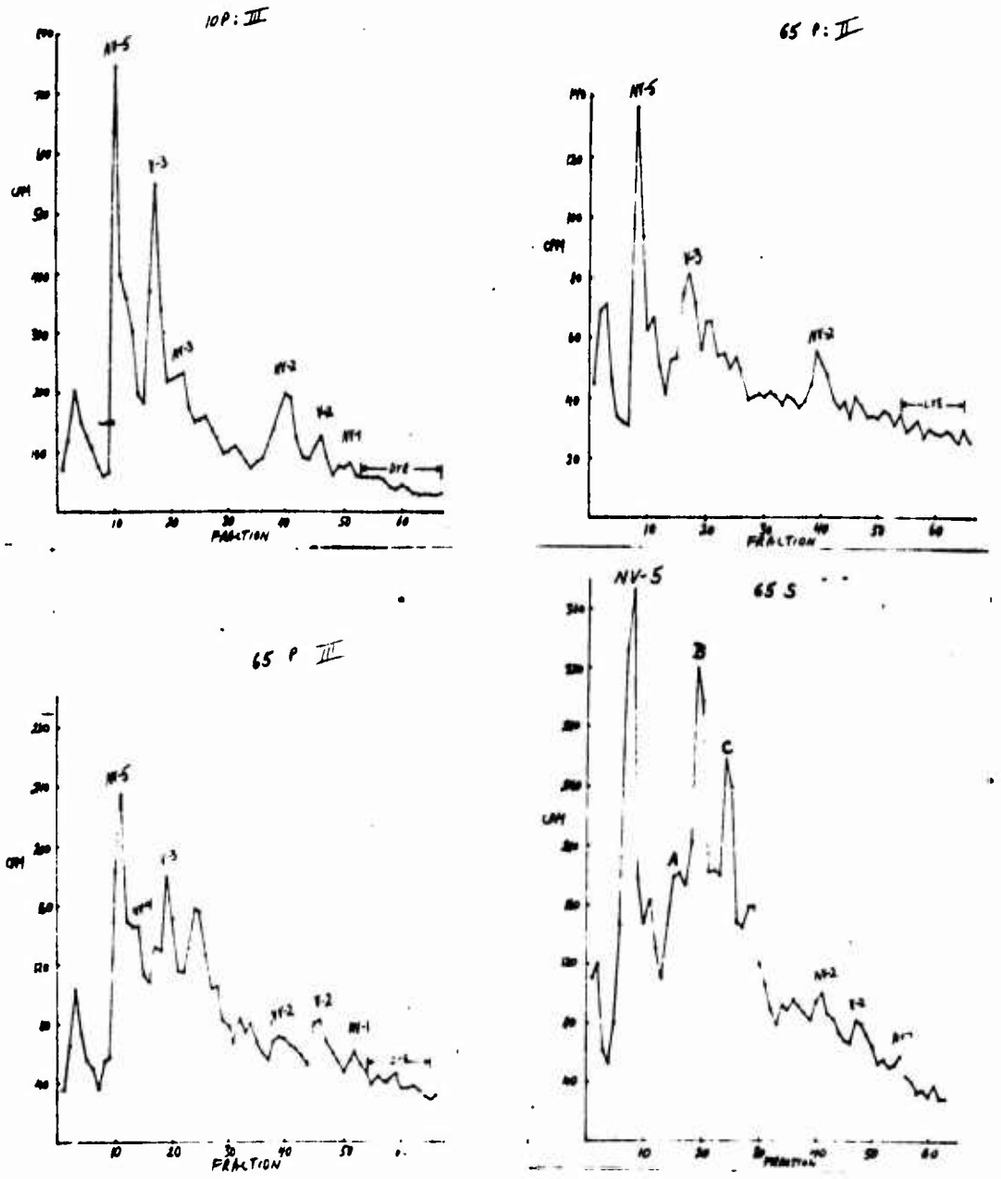


Figure 21. Polyacrylamide gel electrophoresis of light membranes (A, B, C) in the indicated bands (Roman numerals) isolated as shown in Fig. 21D. Analysis of soluble cytoplasmic proteins in the 65,000 rpm supernate.

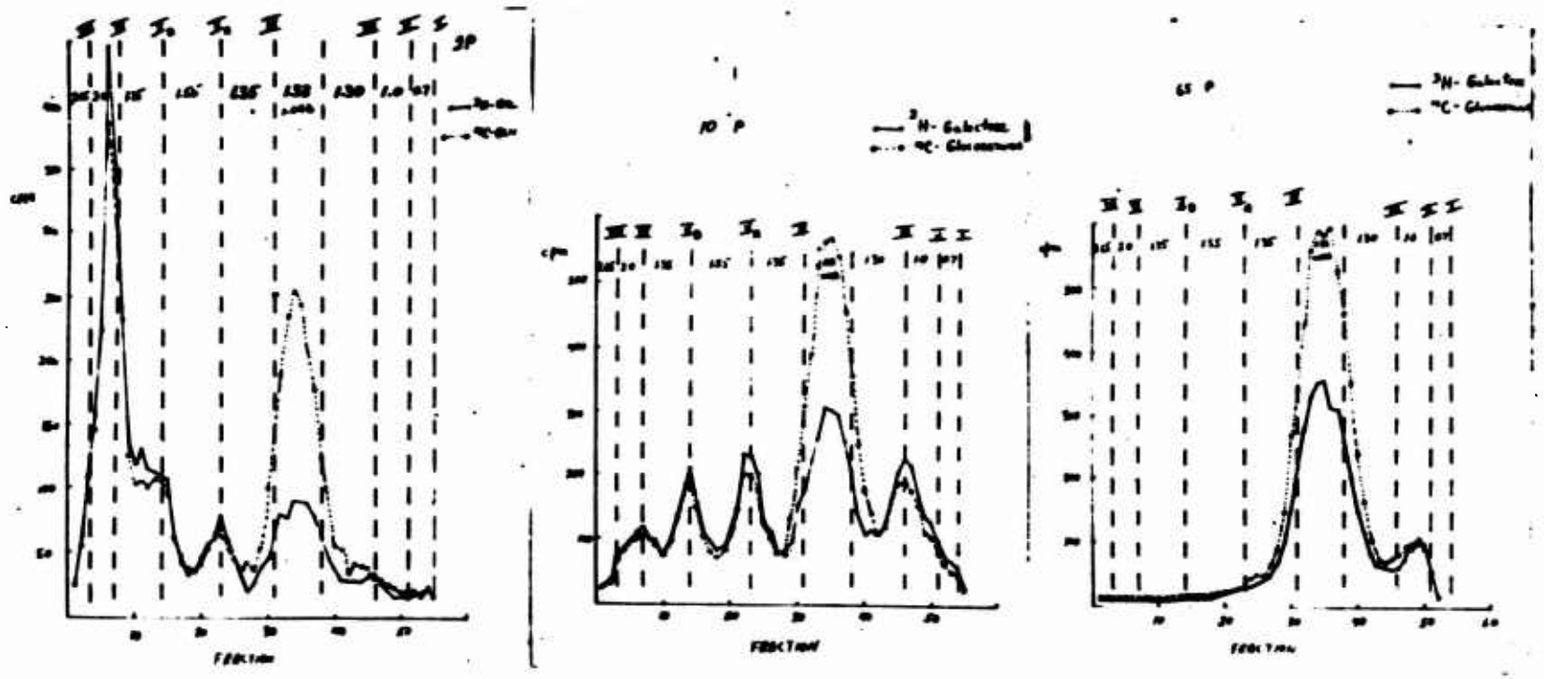


Figure 22. Radioactivity of membranes labeled with galactose and glucosamine and separated on discontinuous sucrose gradients. Graphs A, B, and C show the distribution in the 2000 rpm pellet, 10,000 rpm pellet, and 65,000 rpm pellet, respectively.

C. Fluorescent Antibody and Electron Microscopic Studies of group B Arbovirus-infected Cell Cultures.

There are no published descriptions of dengue virus morphogenesis. Electron microscopic studies of cells infected with other group B arboviruses have developed the concept that group B arboviruses somehow mature within the cell in association with the endoplasmic reticulum (Ota, Z., 1967 and Murphy et al, 1968).

Cytological studies using fluorescent antibodies (FA) have shown dengue antigens restricted to the perinuclear zone (Atchison et al, 1966; Bhamarpravati, N., et al, 1964). These studies were performed before the current data concerning dengue antigens became available at WRAIR but they do implicate the golgi zone and the endoplasmic reticulum as a prime area of viral morphogenesis.

We have recently used antibodies against soluble complement fixing antigen (anti-SCF), the virion or rapidly sedimenting hemagglutinin (anti-RHA) and crude dengue-infected brain extract (anti-Den-2) to establish by FA the relative sensitivity of the antigens to fixatives, their cytological distribution and their time of appearance.

Dengue-2 infected cells were then examined under the electron microscope to determine the morphology of these cells in relationship to the FA findings.

Materials and Methods.

Cells and media: LLC-MK2 cells were grown in plastic petri dishes containing 10 x 22 mm glass coverslips using medium 199 in Hank's balanced salt solution with tricine buffer, 3% fetal calf serum and antibiotics added (Robb and Martin, 1970).

Virus Infection. At appropriate times LLC-MK2 cells were overlaid with 20% dengue-2 infected mouse brain suspensions. The mouse brain suspensions were diluted with medium 199 to an appropriate MOI and allowed to adsorb 2-4 hours before removal. Following the adsorption period, the cells were washed with buffer and a replacement volume of medium was placed on the culture.

Antisera. Mouse hyperimmune ascitic fluid produced as described in previous reports was used as the source of anti-dengue antibodies. Fluorescein labeled anti-mouse globulin was purchased from Antibodies, Inc., Davis, California, and passed through a sephadex G25 column before use.

Fixation. Media was removed from cells at appropriate times and rinsed 2 times with PBS and fixed 5 minutes with cold 2.5% poraformal dehyde in isomolar sucrose buffered at pm 7.2 with phosphate. The cells were then rinsed twice with PBS and stored at -70°C until use.

Fluorescent Staining. The coverslips were thawed and fixed with one of several lipid solvents cooled at -70°C for 15 minutes. The solvent was removed and the cells rinsed with PBS. 200 lambda of MHAF or norma ascitic fluid control was then placed on the cells and incubated at 37°C for one hour with constant agitation. The antibody was removed and the cells were washed with three changes of PBS for one hour. Flourescein conjugated antiglobulin was then put on the coverslips and incubation took place with constant agitation for 30 minutes at 37°C. The conjugate was removed and rinsed as above. The coverslips were mounted with glycerol and viewed and photographed with a Leety UV microscope with an automatic orthomat camera.

EM Studies. Cells for EM were fixed in the petri dishes with 2.5% Glutaraldehyde containing 2.5% sucrose in phosphate buffer pH 7.2 24 hours post adsorption. The cells were scraped with a rubber policeman pelleted by centrifugation at 600g and embedded in 7% noble agar. Following embedding in agar the cells were fixed in 1% OsO_4 for one hour, dehydrated in a graded series of alcohols and embedded in epon. Thin sections were cut on an LKB Ultratome II using a diamond knife, caught on copper grids, stained with uranyl acetate and lead citrate and viewed under a RCA-EMV 4B.

Results

Effects of Fixation on FA. The results of fixation experiments are summarized in Table 31. Several points are important: (1) Aldehyde fixation alone led to diminished FA. (2) This diminution could be readily overcome using cold lipid solvents. (3) The SCF antigen survived all fixatives. (4) The HA antigens were destroyed by methanol.

Distribution of FA with different antibodies. The three MHAF preparations provided different patterns of fluorescene in the dengue-2 infected cells. Anti-SCF was strictly limited to the perinuclear region of the cell. The fluorescene with anti-RHA was also perinuclear but tended to be more granular and the fluorescent granules spread throughout the cytoplasm. The fluorescence observed using anti-DEN-2 is a combination of the two previous with both perinuclear and cytoplasmic fluorescence depending on the fixatives used. Selected photomicrographs appear at the end of this section.

Time of Appearance of SCF. A series of Kinetic experiments were used to determine the earliest time of appearance of SCF. SCF was first identifiable at 10-12 post adsorption. This correlated with the first observable fluorescene with anti Den-2. Therefore, SCF is one of the early antigens of dengue-2 infected cells.

Table 31.

Effects of Fixatives on Immune Fluorescence in Dengue-2 Infected Cells

Fixative	MHAF anti-DEN-2	anti-SCF	anti-RHA
Paraformaldehyde alone	+1	+1	±
Glutaraldehyde alone	0	0	0
Methanol alone	+4	+4	0
Acetone alone	+3	+3	+3
Paraform + methanol	+4	+4	0
Paraform + acetone	+4	+4	+4
Paraform + ethanol	+4	+4	+4
Glut + any solvent	0	0	0

Table 32. Group B Cross Reactions by FA

MHAF	Cell-Virus-Fix D-2 LLC Acetone	D-2 LLC Methanol	JEV - CEC Acetone	JEV - CEC Methanol
anti D2	+4	+4	0	+3
anti SCF	+2	+4	0	0
anti D4	+3	+4	-	-
anti JEV	0	+4	+4	+4
anti JEV SCF_1	-	-	+2	0
anti JEV SCF_2	-	-	+2	0
anti Langat	0	+2	-	-
anti St. Louis	0	+4	-	-

Correlation with other group B viruses. Similar studies have been carried out with JEV infected cells. Fixation affects the FA distribution of antigens in JEV infected cells in a similar manner. That is, any combination of fixatives with methanol results in a tight perinuclear halo. Fixation with acetone results in a diffuse cytoplasmic fluorescence. Antigens identified by antisera against column chromatographed complement-fixing JEV antigens, however, proved to be destroyed by methanol but not by acetone. Because of the methanol labidity, the column purified antigens apparently are not similar to dengue SCF.

Cross reactions observed. A comparison of dengue antigens and JEV antigens were made using viral specific MHAF and various JEV or dengue infected cells. Both antisera crossreacted under varying fixative schedules but interestingly, anti-SCF did not identify any JEV antigens. (See Table 32)

Further studies were made using dengue-2 infected cells and MHAF against Langat and dengue-4. These are summarized in Table 32. Of particular interest is the fact that methanol-stable cross-reacting antigens in addition to SCF are identified in this system. Acetone fixation tends to reduce this cross reaction to negative levels.

Comparison of FA patterns in group A and group B viruses. The pattern of FA in group B infected cells is characteristic. This is dramatized by the FA pattern in the group A viruses. In general, the group A FA pattern is diffuse, involving both cytoplasm and membranes. The FA is diminished when acetone is the sole fixative used. All other combinations of fixation are equally effective.

EM Correlation. In view of the group B FA pattern it was of interest to examine the perinuclear zone of group B infected cells. Pertinent electron micrographs follow this section.

The most prominent features of the dengue or JEV infected cells were the large accumulations of virus particles in the cisternae of smooth and rough endoplasmic reticulum in the perinuclear zone and the proliferation of 1 micron oval, membrane limited vesicles with reticular electron dense cores (CPV-1).

The virus particles observed towards the periphery of the cell were generally found within smooth membrane vacoules.

Many cells had widely dilated vesicles which contained many electron dense particles which resembled virus particles with aberrent shapes. These dilated cesternae also contained vesicles which appeared to be collapsed CPV-1.

Discussion

Previous studies in this laboratory have indicated that the dengue SCF antigen is a nonstructural antigen. The FA studies demonstrate that SCF and the hemagglutinins also differ in their stability in methanol. SCF apparently appears earlier and is restricted to the perinuclear zone of dengue infected cells. HA on the other hand is found not only in the perinuclear zone but throughout the cytoplasm. This suggests that SCF is an early function protein antigen of dengue-2 and is involved in synthetic functions rather than structural functions.

Electron microscopy of group B infected cells has thus far been concentrated in the perinuclear zone. This zone is rich in all forms of endoplasmic reticulum and contains many virus particles. The zone also contains a strange vesicular structure which has previously been described in group A infected cells and called the cytopathic vacoule Type I (CPV-1) (Grimby, et al, 1968). The CPV-1 of group A infected cells has been implicated in RNA synthesis and its location in the group B cells would be consistent with a synthetic function.

The comparative studies suggest that the group B infected cells all contain a nonstructural methanol stable antigen. This antigen(s) is perinuclear (zone of synthesis) but is apparently not SCF. At least anti-SCF from dengue-2 does not cross react. The lack of cross reaction with acetone treated cells may mean either a low level of cross reactivity, loss of antigen with acetone fixation, or the lack of cross-reacting acetone stable antigens.

From these studies, the membrane studies and the comparison with the group A viruses we can begin to piece together the topography of group B synthesis, assembly and release.

All group B synthesis and assembly occurs within the deep endoplasmic reticulum as is evidenced by the lack of surface FA and the requirement for a lipid solvent for adequate levels of fluorescence. This is in direct contrast to the FA patterns found in the group A viruses. The early appearance of a nonstructural antigen SCF in the perinuclear zone, the lack of change in the distribution of SCF with time and the appearance of CPV-1 in the perinuclear zone all suggest that this area is a zone of viral synthesis.

The presence of virus particles in the perinuclear zone implies that assembly also occurs in these synthetic zones. The data from membrane experiments suggest that the major synthesis occurs on rough endoplasmic reticulum although no clear morphological data has been obtained. The mode of viral assembly is not clear. The presence of lipids in the virus suggests that the virus buds through the internal membrane. There are no electron micrographs which clearly

show the process and there is no clear evidence of nucleoid structures on the cytoplasmic side of the endoplasmic reticulum as is found in the group A viruses. Therefore, the question of the actual mode of assembly remains unanswered.

Finally, the virus is apparently transported from the zone of synthesis and assembly in smooth membrane vacoules and released by "reverse pinocytosis" much as the manner of many normal cell products are transported and released. This is in distinct contrast to the mechanisms of release of group A viruses which can readily be found budding through surface membranes.

Figure 23. Fluorescence obtained with anti-DEN-2 on dengue-2 infected LLC-MK_2 cells. Concentric perinuclear staining extends into surrounding cytoplasm in the form of a granular fluorescence (600X).

Figure 24. Fluorescence obtained with anti-SCF on dengue-2 infected LLC-MK_2 cells 14 hours post infection. Most of the fluorescence is in the perinuclear zone (600X).

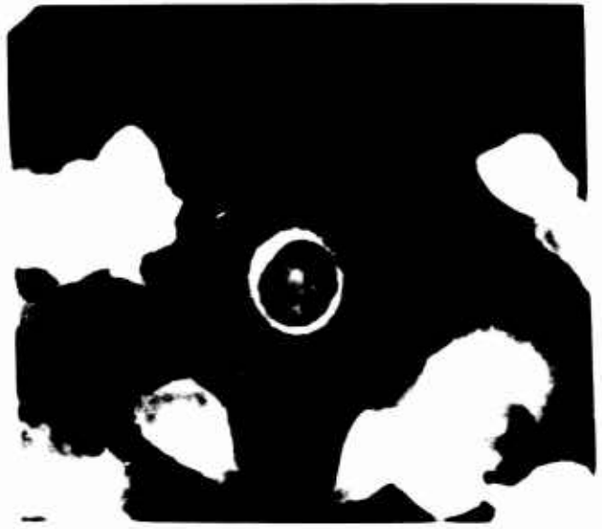


Figure 25. Fluorescence obtained with anti-SCF on dengue-2 infected LLC-MK_2 cells 24 hours post infection and following fixation with paraformaldehyde and ethanol (1000X).

Figure 26. Fluorescence obtained with anti-RHA on dengue-2 infected LLC-MK_2 cells 24 hours post infection and following fixation with paraformaldehyde and ethanol (1000X).

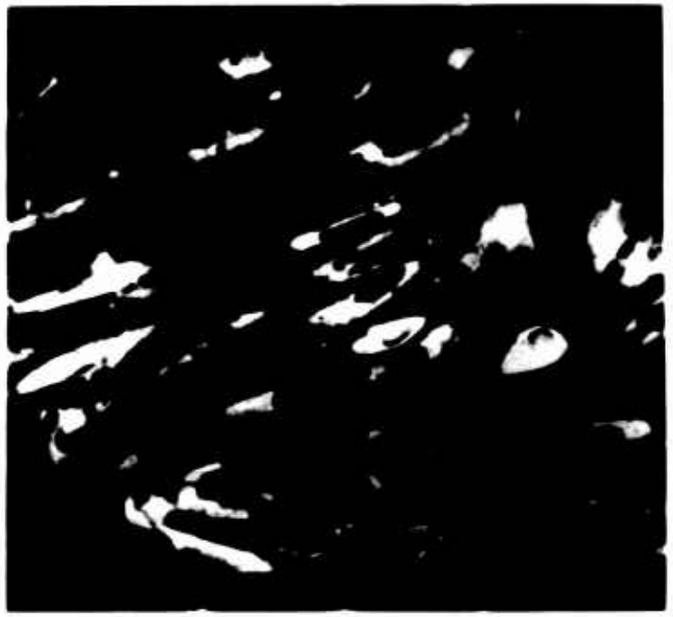


Figure 27. Fluorescence of Japanese encephalitis virus infected chick embryo fibroblasts after methanol fixation; note the perinuclear staining (600X).

Figure 28. Fluorescence of Sindbis infected chick embryo fibroblasts after methanol fixation; compare with methanol fixed Japanese encephalitis infected cells and dengue infected cells in the first photomicrograph (600X).

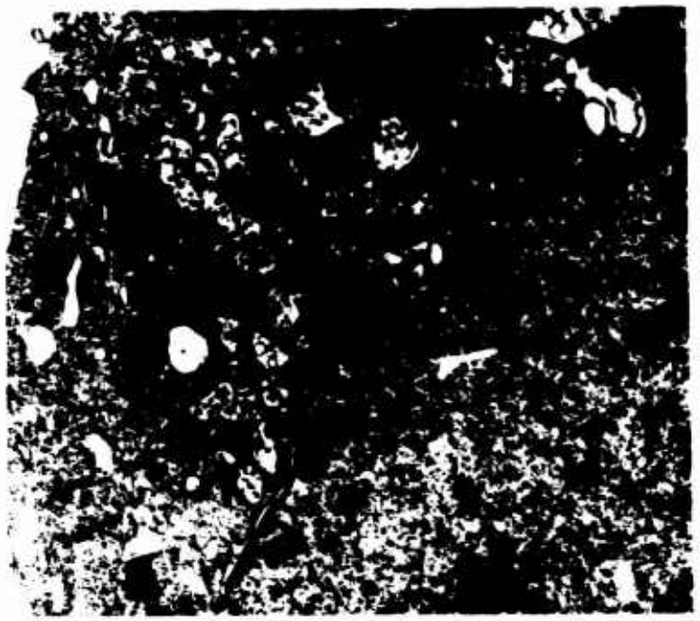


Figure 29. Electron micrograph of LLC-MK_2 cell 24 hours after infection with dengue-2 virus. Dilated endoplasmic reticulum filled with mature virions and cytopathic vacuoles can be observed in the perinuclear zone (PN). Some virions near the cell surface are encased in smooth membrane vacuoles (arrows), (21,000X)

Figure 30. Electron micrograph of perinuclear zone of dengue-2 infected LLC-MK_2 cell. Numerous virus particles (V) can be seen in smooth and rough endoplasmic reticulum. Cytopathic vacuoles (CPV) are observed throughout the endoplasmic reticulum (33,000X).

Figure 31. Electron micrograph of the area adjacent to the nucleus (N) in a dengue-2 infected LLC-MK_2 cell. Dilated endoplasmic reticulum contains numerous virus particles (V) and cytopathic vacuoles (CPV), (33,000X).

Figure 32. Electron micrograph of zone adjacent to nucleus (N) in a dengue-2 infected LLC-MK_2 cell. Numerous virions (V) and cytopathic vacuoles are found in a dilated membrane system (33,000X).

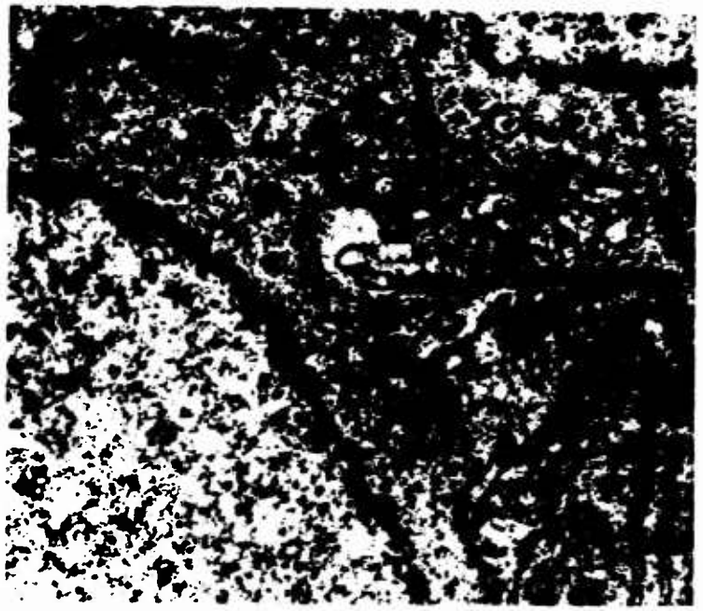


Figure 33. Normal LLC-MK_2 cell showing nucleus (N), golgi (G), rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), and mitochondria (M), (33,000X).

Figure 34. Electron micrograph of perinuclear zone of normal LLC-MK_2 cell showing nucleus (N), rough endoplasmic reticulum (RER), golgi zone (G), and mitochondria (M), (33,000X).

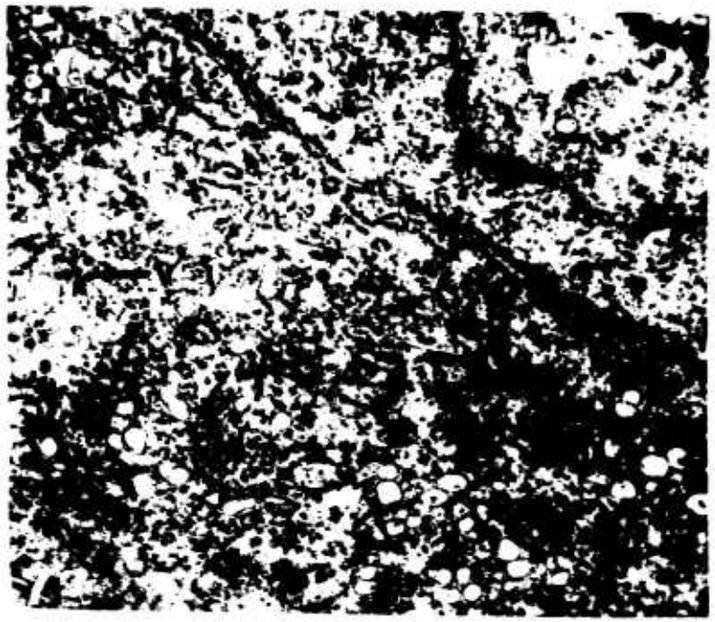


Figure 35. Electron micrograph of the region next to nucleus (N) in a Japanese encephalitis virus infected chick embryo cell. Virus particles (V) and cytopathic vacuoles can be seen scattered throughout the endoplasmic reticulum (33,000X)

Figure 36. Electron micrograph of perinuclear region of Japanese encephalitis virus infected chick embryo cell. Virus particles (V) and cytopathic vacuoles are scattered throughout the endoplasmic reticulum (33,000X).

D. Group B Arbovirus Polymerase and Polymerase RNA Products.

In the previous annual report and departmental publications, it was shown that nonvirion but viral specified antigens, such as the soluble complement-fixing antigen (SCF), were produced in dengue infected cells in substantial quantities; preliminary work indicated a similar antigen probably also occurs in JE virus infected cells as well. In order to selectively control synthesis in group B arbovirus infected cells, it is necessary to know the function, sites of synthesis, and composition of the various particulate and soluble virus specified antigens and proteins. Some approaches to this have been described in the preceding sections. In this section, characterization of dengue and JEV polymerase and polymerase RNA products was carried out to (1) support studies on sites of synthesis on the various infected cell membranes and (2) to determine whether SCF is part of a polymerase molecule by testing the capacity of anti-SCF to inhibit the formation of polymerase RNA products.

Ribonucleic acid polymerase is necessary for the replication of the genome of RNA viruses (Spiegelman, 1967). This enzyme has been described in detail in several RNA bacteriophage and identified in a number of animal RNA viruses by the RNA products generated in a suitable incubation medium. RNA polymerase has been described in several group A arboviruses by Sreevalsan and Yin (1969), Martin and Sonnabend (1967), and Levin and Friedman (1971). In this report we describe the characteristics of the RNA polymerase and polymerase products of two group B arboviruses, Japanese encephalitis and dengue-2.

Materials and Methods. Continuous rhesus monkey kidney cells (LLC-MK_2) were grown in 32 oz glass prescription bottles in medium 199 and chick embryo cells were grown in roller bottles as described above.

Preparation of Enzyme.

Chick embryo fibroblast cultures were infected with Japanese encephalitis virus in 20% mouse brain suspension at an MOI of 10. The inocula was not poured off but merely diluted to volume after two hours. Either 6 or 8 roller bottles were used for each experiment. The JEV infected cells were harvested at 18 to 24 hours post infection.

About 30 - 32 oz bottles of LLC-MK_2 cells were prepared and infected with mouse brain seeds of dengue 2 at an MOI of 2. Following 22 hour adsorption the inoculum was washed off and replaced with growth media. After 48 to 92 hours the cells were harvested. Enzyme preparation was the same for both virus infected cell types. The media was poured off and the cell monolayers were washed twice with 10cc of hypotonic buffer (Sreevalson and Yin, 1969). One cc of hyptonic buffer was then placed in each bottle and incubated at 4°C for 30 min.

The cells were then scraped of the wall with a rubber policeman, pooled and homogenized in a glass pestle Dounce homogenizer with 30-35 strokes.

The homogenate was centrifuged at 2000 g x 5 min and the supernatant was removed and centrifuged at 10,000 g x 20 min. Again, the resulting supernatant was removed and centrifuged at 65,000 g x 180 min. The pellets from each step were resuspended in hypotonic storage buffer (Sreevalsan and Yin, 1969), and aliquots from each supernatant were taken. The samples were frozen and stored at -70°c prior to being monitored for CF activity, protein content (Lowry, et al, 1951), and enzyme activity.

Enzyme Reaction

Most of the enzyme assays were performed in 0.1 ml quantities with the following constituents: 10 to 50 lambda of an enzyme preparation and 0.05M Tris (hydromethyl) aminomethane (pH 8.0); 0.1M $MgCl_2$; 0.01M 2B mercaptoethanol; 0.025 micrograms actinomycin D; 0.01M phosphoenolpyruvate; 4 micrograms pyruvate kinase; 10 micromoles of ATP, UTP, CTP and 3_H guanosine triphosphate (1mc/mM) (Levin and Friedman, 1971).

The reactions were terminated at appropriate times with 5% TCA in saturated solution of sodium pyrophosphate. The samples were filtered on a .45 micron millipore filter with exhaustive washes of 5% TCA. The filters were dried in a 60° oven, placed in scintillation vials containing liquiflor and counted in a Packard liquid scintillation counter.

Batch preparations for product analysis contained the same proportions but in greater quantities.

Variations in the various components of the reaction mixture were used in control experiments.

RNA Extraction.

The RNA extraction was performed in 1% SLS (0.1 or NaCl, 0.01M Tris-HCl pH 7.4; and 0.001M EDTA with cold phenol repeated three times. The RNA was precipitated out of the acquous phase following the addition 1/10 volume of 2.5M NaAc pH 5.2 using cold 95% ethanol. The RNA was pelleted by centrifugation at 18,000 g x 20 min and redissolved in 0.001M NaCl, 0.01M Tris MCl pH 2.2, and 0.001M EDTA. The samples were either used immediately or stored at - 20°c until use.

Sucrose Density Gradients.

Rate zonal centrifugation was performed in preformed sucrose gradients. Unless otherwise noted the gradients were 20-5% sucrose (w/v) in 0.001M EDTA, 0.01M Tris HCl pH 7.2 and 0.1M NaCl. Following

centrifugation at the appropriate g force, the gradients were collected in 5 drop (about 200 lambda) fractions by bottom puncture of tubes.

Some gradients were collected directly into scintillation vials, dissolved in liquiflor containing 1/20 volume of NCS, and counted as total counts.

Acid precipitable counts were determined by the addition of 10 volumes of 5% TCA, and collection of precipitate of 0.45 micron nitrocellulose millipore filters which were then dried in a 60° oven. The dried filters were counted in a Packard liquid scintillation counter following the addition of liquiflor.

Polyacrylamide-agarose electrophoresis of RNA.

Preparation of 2.0% polyacrylamide, 0.5% agarose gels (0.6 cm x 7 cm) was performed by combining 15 ml of melted 1% agarose (w/v) with 15 microliters of N, N, N^1, N^1 tetramethylethylenediamine, and 15 ml of the following mixture: 0.72 ml of water; 5.28 ml of a stock solution containing 15% acrylamide (w/v) and 0.75% N, N^1-methylene bisacrylamide (w/v); and 12 ml of gel buffer (0.04M Tris, 0.06M NaAc; 0.003 MEDTA pH 7.2). Solidification of agarose was prevented by mixing the reagents at 45°c. After air evacuation 0.3 ml of a fresh solution of 10% ammonium persulfate (w/v) was added and the mixture rapidly transferred to warm glass tubes. The gels were stored in electrophoresis buffer to remove excess catalyst. Prior to electrophoresis the uneven tips were sliced and the gels were replaced into glass tubes which had one end capped with dialysis tubing. The sample 50-100 microliters were applied to the gel and run at 5 ma until the dye front reached the end (60 min). The gels were removed, frozen, sliced on a transverse slicer into 1.8 mm sections, placed in scintillation vials containing liquiflor and 1/20 volume of NCS. The gels were allowed to swell overnight and then were counted in a Packard liquid scintillation counter.

Results.

Fractionation of Cells and Polymerase.

The virus infected cells were fractionated into nuclear (2000g), mitochondrial (10,000g) and microsomal (65,000g) fractions. The enzymatic activity and antigen of each fraction was monitored (Table 33 and 34). The major portion of the enzyme was found in the 2,000g and 10,000g fractions with the highest specific activity in the 10,000g fraction. Electron microscopic examination of these fractions revealed that the 2,000g pellet contained nuclei, and cytoplasmic fragments attached to nuclei. The 10,000g contained fewer nuclei but an admixture of smooth and rough endoplasmic reticulin. Further

Table 33.

Distribution of Polymerase Activity and Antigen from a Typical Extraction of JEV-infected Chick Embryo Cells from 8 Roller Bottles. Sample Number Eight Represents a 10,000 x g Pellet of Uninfected Cells.

JEV	Net cpm	Protein (mgm/ml)	Specific Activity (cpm/mgm x 10^3)	CF Titer
(1) Homogenate	143	1.97	7.3	1:64
(2) 600 g Pellet	5296	3.50	151	1:512
(3) 600 g Sup	0	1.83	0	1:16
(4) 10,000 g Pellet	1784	0.625	288	1:512
(5) 10,000 g Sup	10	0.45	2.4	1:8
(6) 65,000 g Pellet	0	0.25	0	1:16
(7) 65,000 g Sup	50	0.64	16	1:2
(8) CEC Cells	1410	4.8	5.8	0

Table 34.

The Distribution of Polymerase Activity and Antigen in a Typical Extraction of dengue-2 Infected LLC-MK_2 Cells from 30 - 32 oz. Bottles.

Fraction	Net cpm	Protein (mg/ml)	Specific Activity (cpm/mgm x 10^3)	CF Titer Anti-D2	CF Titer Anti-SCF
(1) Dounced Cells	507	.47	108	-	-
(2) 600 g Pellet	454	.82	56	1:128	1:8
(3) 600 g Sup	641	.20	320	1:8	1:2
(4) 10,000 g Pellet	1777	.56	317	1:64	1:16
(5) 10,000 g Sup	187	.18	102	1:64	1:16

douncing and recentrifugation of the 2,000 g pellet failed to significantly reduce the amount of cytoplasmic debris or the enzymatic activity.

The enzymatic activity found in these fractions varied directly with the total protein added to the reaction mixture (Figure 37).

Reaction Characteristics.

Both JEV and dengue-2 polymerases required Mg++, Mn++ could not be substituted and all three triphosphates were required (Table 35).

Table 35. The Effect of Incomplete Media on Polymerase Reactions of Japanese Encephalitis and dengue-2 Viruses

Incubation Media	Dengue-2 cpm	JEV cpm
Complete	612	1284
Without Mg+	88	252
Without ATP	139	160
Without CTP	250	236
Without UTP	119	159

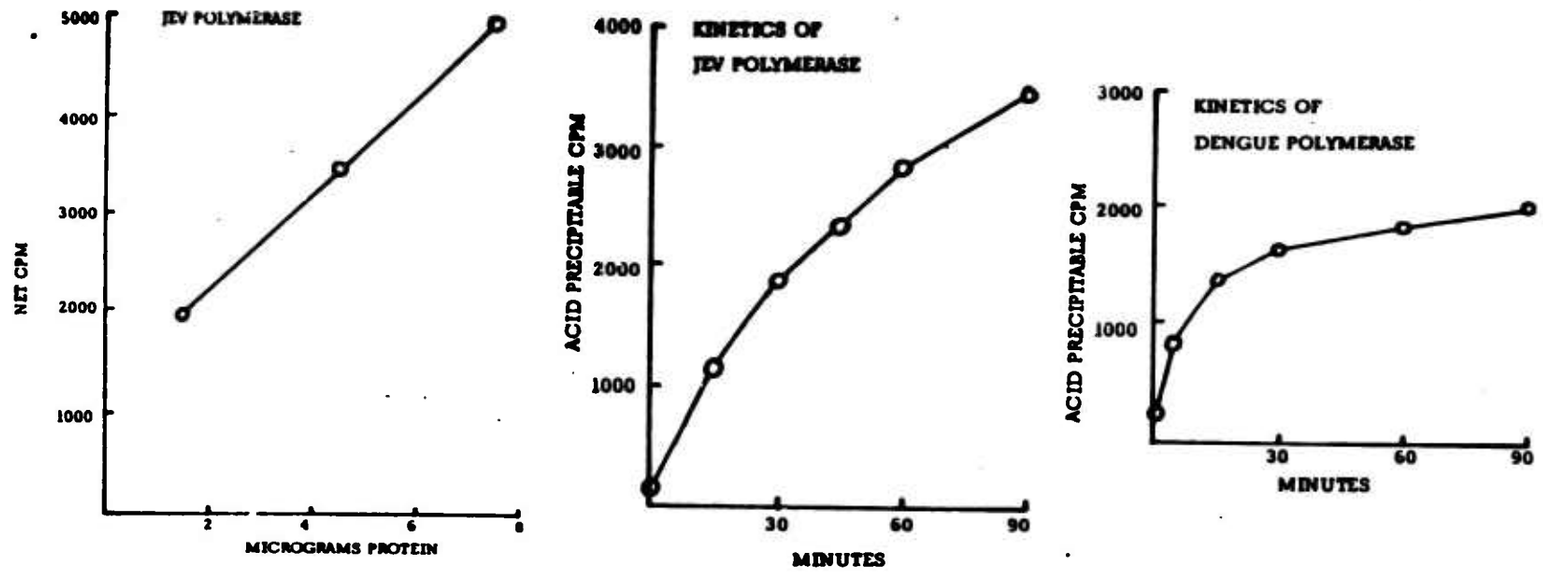


Figure 37. a. Graph showing linear relationship between acid precipitable GTP counts generated and the protein added.

b. Kinetics of incorporation of ^{3}H-GTP into acid precipitable counts by JEV polymerase, and

c. Dengue-2 polymerase.

Points are the mean of triplicate samples.

Kinetics

JEV polymerase reaction proceeds in close to a linear pattern over the first two hours (Figure 37B). After two hours, the reaction rate declines and frequently there is less acid percipitable reaction product at eight hours than at three hours (Figure 38A). This suggests that a significant proportion of the reaction product is single stranded and that the enzyme preparation contains significant levels of nuclease.

In contrast the dengue-2 polymerase reaction proceeds with linear kinetics only over the first 30 - 60 min (Figure 37C). The rate decreases for the next 30 mins and generally lapses into a negative phase thereafter (Figure 38B). This again suggests single stranded RNA and nuclease.

Products

A. Cell Products

A small amount of acid precipitable RNA was observed in the reaction using uninfected LLC-MK2 cells or uninfected chick embryo cells. This material was phenol extracted and precipitated in ethanol and sedimented by rate zonal centrifugation. The products produced by these normal cells did not sediment and did not overlap with the products observed from infected cells (Figure 39 A and B).

B. JEV Polymerase Products

JEV polymerase generated RNA which was either biphasic or was heterodispersed by rate zonal centrifugation. The most interesting aspect of this asymmetrical pattern was that the asymmetry was in front of the migrating major peak. The most characteristic peak of RNA generated sedimented at about 22s (Figure 40A). When a distinct second peak was observed, it was about a 26s peak (Figure 40B). When the individual fractions were treated with RNase after rate zonal centrifugation, RNase resistant RNA (double stranded) was limited to the 22s peak (Figure 40A and B); quite significantly, the pattern of radioactivity in the gradient becomes symmetrical following RNase treatment.

When the polymerase product was treated with RNase prior to rate zonal centrifugation, the double stranded RNA migrated as a 15s peak rather than a 22s peak (Figure 41) .

These results are highly suggestive of a double stranded replicative form (RF) and a heterodispersed partially double stranded replicative intermediate (RI).

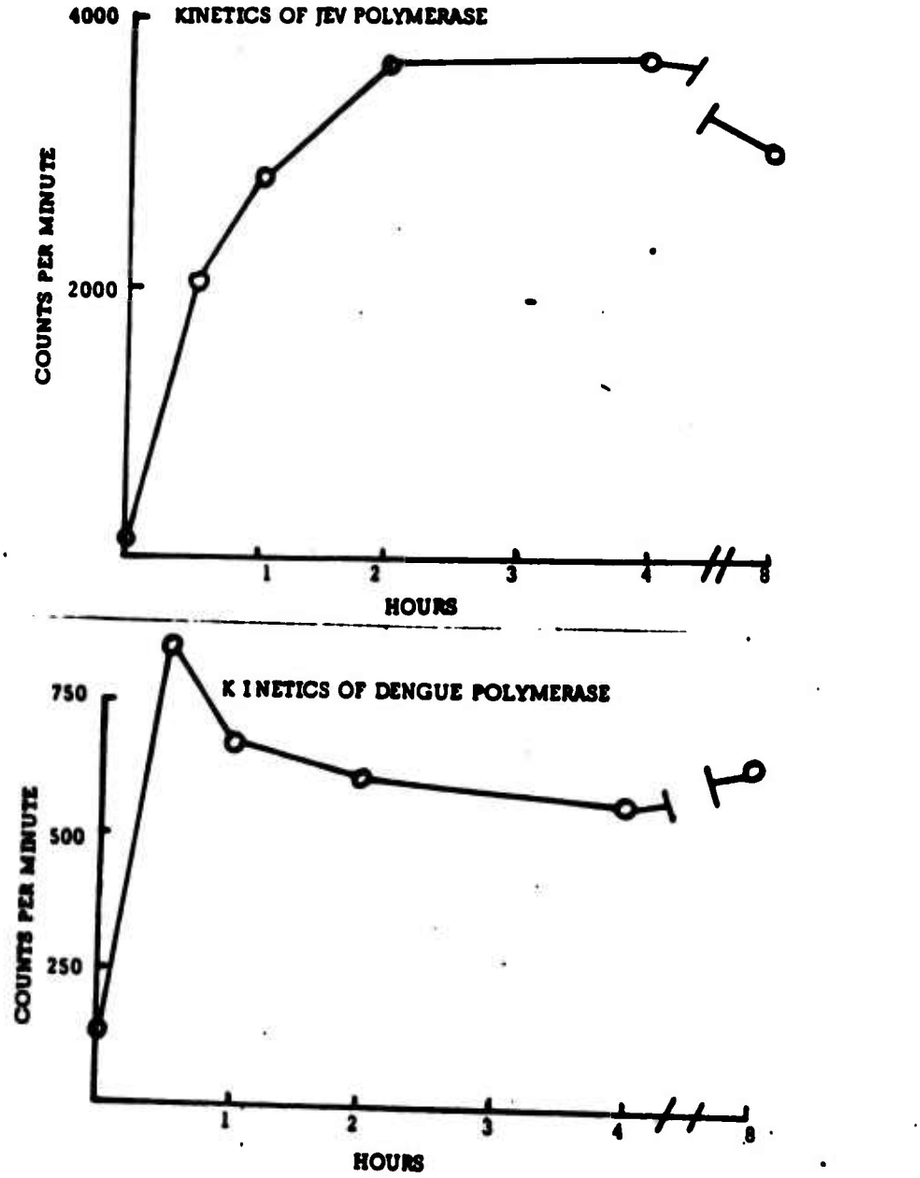


Figure 38. Kinetics of incorporation of ^{3}H-GTP into acid precipitable form by:

a. JEV polymerase, and
b. dengue-2 polymerase.

The points are the average of triplicate samples.

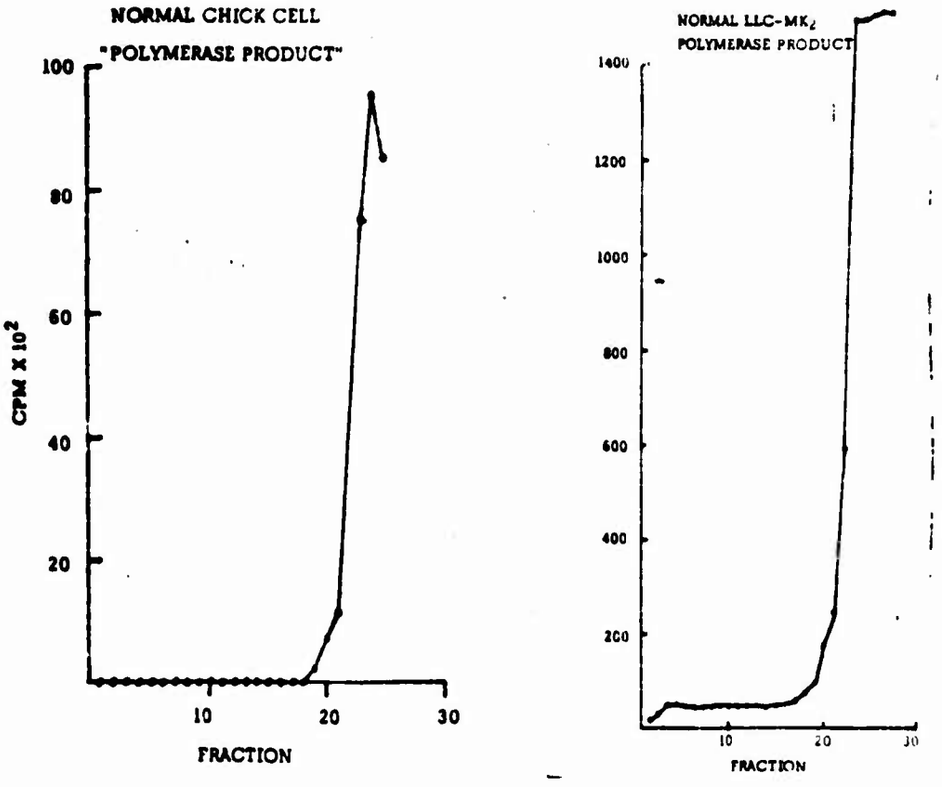


Figure 39. A. Sucrose gradient distribution of polymerase product from normal chick cell extract; 0.48 mgm extract was incubated with enzyme medium for 120 minutes at 37C. Following SLS-phenol extraction and ethanol precipitation, the product was dissolved in 0.1 M NaCl and placed on a 5 ml 20-5% (w/v) sucrose gradient and centrifuged in the SW-65 rotor at 65,000 rpm for 120 minutes.

B. Sucrose gradient distribution of acid precipitable counts from the 10,000 x g pellet of normal LLC-MK2 cells; 0.5 mgm pellet was incubated 60 minutes in complete media. Following SLS-phenol extraction and ethanol precipitation, the resulting sample was placed on a 5 ml linear 20-5% sucrose gradient and centrifuged at 65,000 rpm for 120 minutes in an SW-65 rotor.

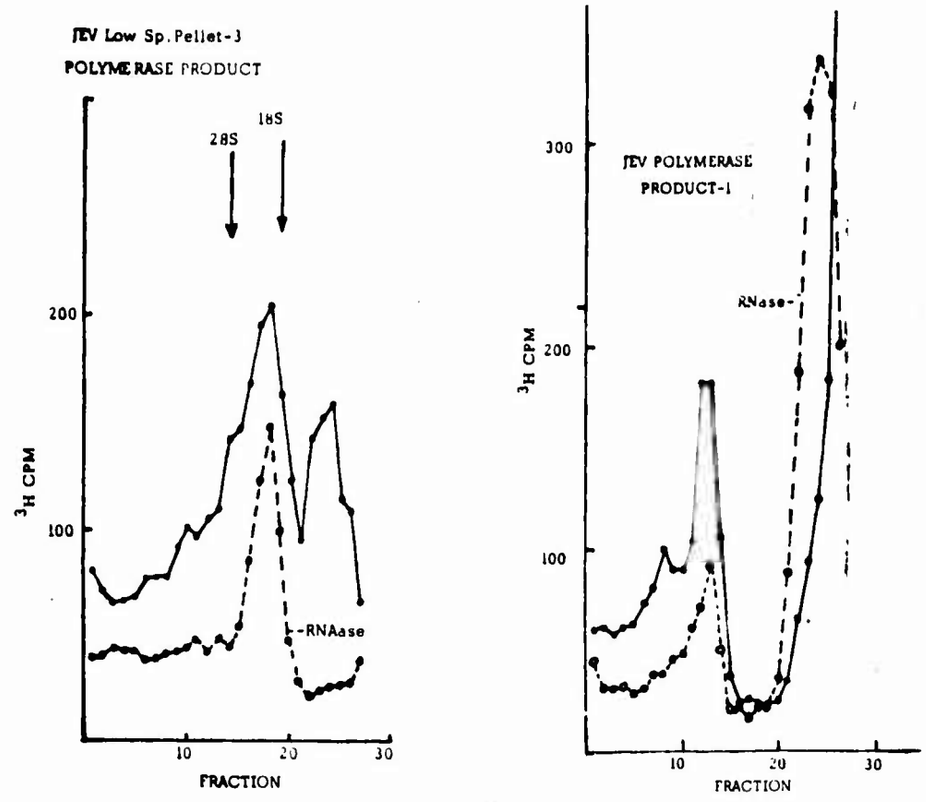


Figure 40. A. Sucrose gradient distribution of acid precipitable and RNase resistant JEV polymerase product from a 2000 x g enzyme preparation. 100 lambda of enzyme was reacted with complete media, SLS-phenol extracted and ethanol precipitated. The redissolved RNA was layered on a 5.1 ml 20-5% (w/v) sucrose gradient and centrifuged at 65,000 rpm for 90 minutes in a Beckman SW-65 rotor. Fractions were split into two 100 lambda aliquots which were either precipitated with 5% TCA or treated with RNase (200 gamma/ml) for 60 minutes at 37C before TCA precipitation. Arrows indicate relative position of marker chick cell RNA.

B. Sedimentation of JEV polymerase product in 5 ml 20-5% sucrose gradient. The sample was prepared as in "A" and layered on the gradient; centrifugation was at 65,000 rpm in the SW-65 rotor for 120 minutes. Individual fractions were split and TCA precipitated with or without RNase treatment.

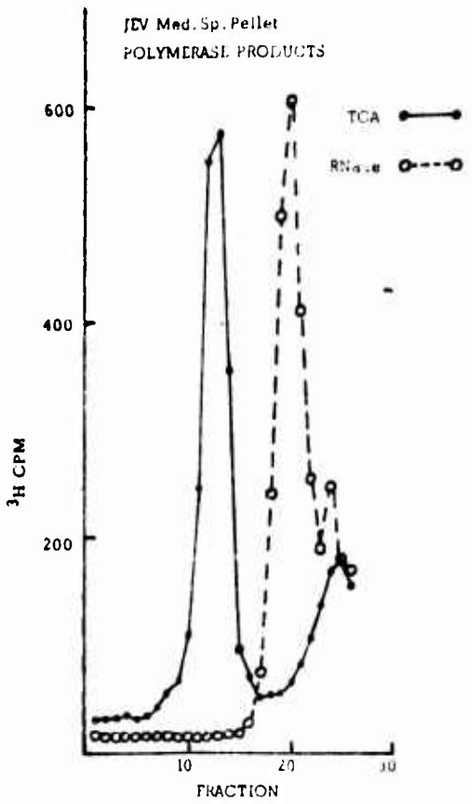


Figure 41. Distribution of JEV polymerase product when treated with RNase prior to centrifugation. The 22s peak from a sucrose gradient was collected, reprecipitated in ethanol, redissolved and split into two fractions. One fraction was diluted with distilled water and the second was diluted with an equal volume of RNase (200 gamma/ml) and incubated at 37C for 60 minutes. Both samples were placed in 5 ml 20-5% sucrose gradients and centrifuged in an SW-65 rotor at 65,000 rpm for 120 minutes, collected, and counted as acid precipitable counts.

Further confirmation of this suggestion was provided by gel electrophoresis. The agarose-acrylamide gels separate molecules on the basis of size rather than the complex flotation separation afforded by rate zonal centrifugation. The multistranded RI would be too large to enter the gel and the double stranded RF would migrate more slowly than the viral RNA. Figure 42 shows one such an electropherogram of JEV polymerase product showing the predicted distribution. Electrophoresis on more porous gels resulted in less apparent RF and (frequently) a trimodel distribution in the RF region (Figure 43A). When the same material was treated with RNase before electrophoresis, three rapidly migrating peaks were observed (Figure 43B). This was considered consistent with the results with rate zonal runs.

Dengue Products.

The dengue polymerase products have not been as extensively characterized as the JEV products. The general trend has indicated similar types of molecules exist in dengue polymerase product. The pattern in rate zonal centrifugation is biphasic with 22 and 26s peaks (Figure 44). Gel electrophoresis results in a biphasic distribution similar to JEV (Figure 45). However, we have been unable to find significant quantities of RNase resistant RNA in the dengue product (Figure 46).

Discussion

Our analysis of 2 group B polymerase enzymes indicates the ability to generate molecules consistent with a multistranded replicative intermediate and a double stranded replicative form. The replicative form is about a 22s molecule which is converted to a 15s molecule upon RNase digestion. The 22s molecule has also been described in dengue infected cells by Stollar (1967). A similar effect of RNase has been documented in Sindbis (Segal, et al, 1971), poliovirus (Baltimore, 1966), and several phage (Erickson, et al, 1964).

It is of considerable interest that upon denaturation with DMSO or heat the 15s molecule of Sindbis migrates as a single stranded 26s molecule. A heterodispersed replicative intermediate is also found in the group B polymerase products. These molecules tend to peak about 26s although this is variable. The presence of a 26s RI which should have a double stranded RNase resistant core may explain the observations of Trent, et al (1969) in St. Louis Virus, who found that the U. S. form was partially RNase resistant. Further the two molecules which can be in the 26s region of a gradient (i.e., RI and interadjacent RNA) of arbovirus-infected cells may explain the confusion in the literature on this subject. The results obtained by each investigator depends upon the relative amount of RI or single stranded 26 RNA present under the conditions used by each investigator.

At the present time we have little or no evidence of 42s RNA (Form incorporated into the virion) being produced in our reaction

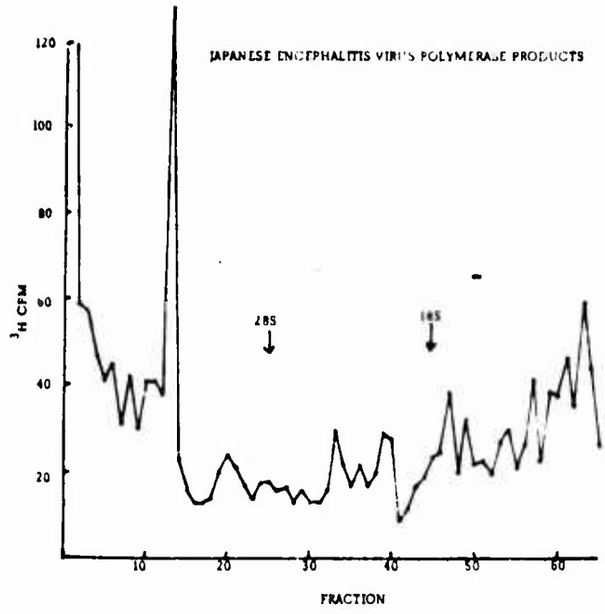


Figure 42. Electropherogram of JEV polymerase product from a 2000 x g pellet in a 2% polyacrylamide gel electrophoresed at 5 ma for 60 min. The gel was fractionated by lateral slicing and counted in NCS-liquifluor. Arrows indicate position of marker RNA in a parallel gel.

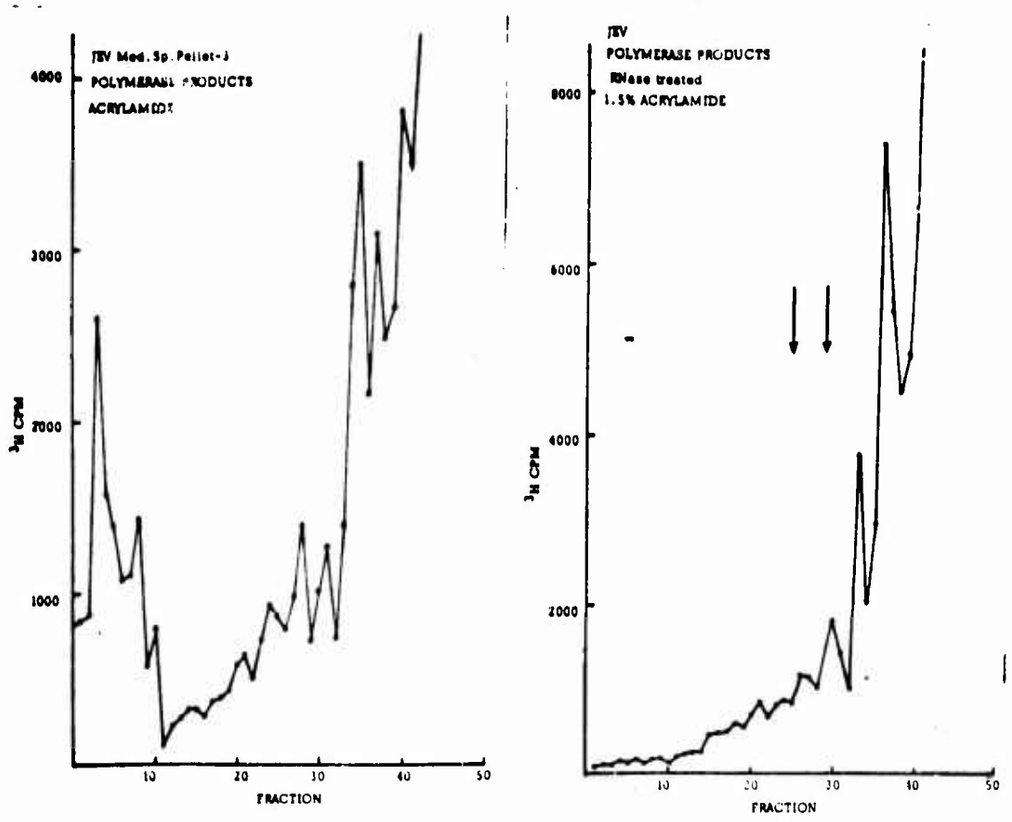


Figure 43. Electropherogram of JEV polymerase product in 1.5% polyacrylamide-agarose.

Electropherogram of JEV polymerase product in 1.5% polyacrylamide which was pretreated with RNase (200 gamma/ml). Arrows indicate relative position of marker R_{RNA}.

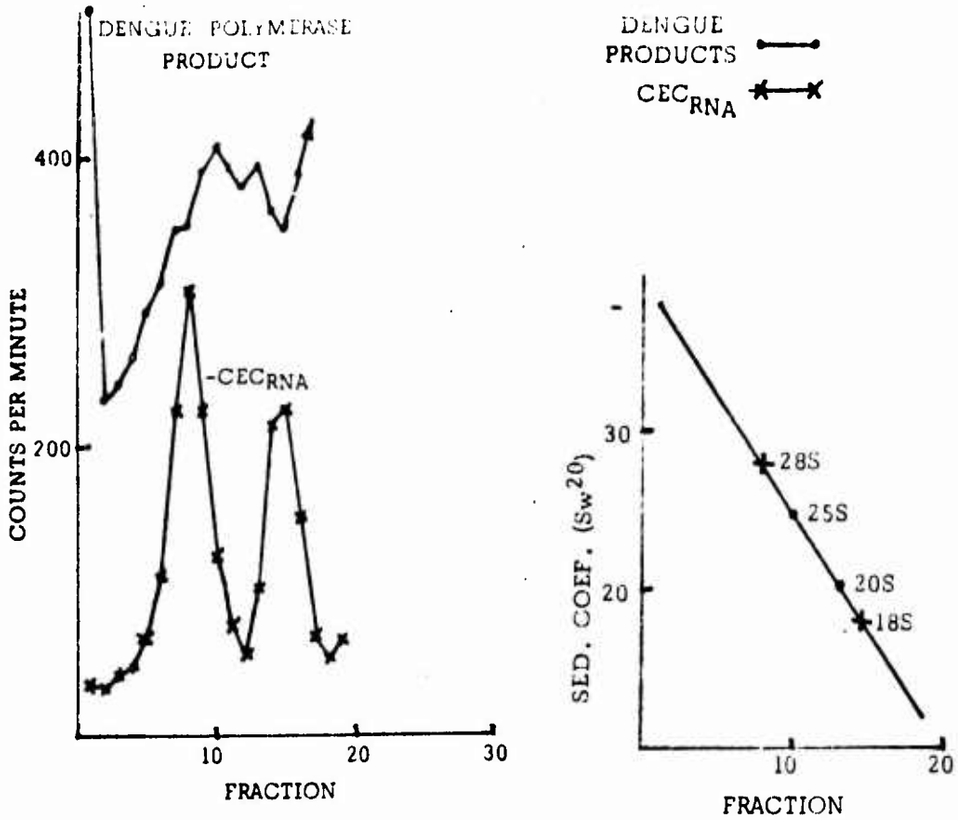


Figure 44. Sedimentation characteristics of dengue-2 polymerase product.

a. ^{3}H-GTP labeled dengue product RNA and ^{14}C-Chick cell RNA were co-run in a 5 ml 20-5% sucrose gradient in an SW-50.1 rotor at 50,000 rpm for 150 minutes.

b. Peak fractions of the polymerase product were plotted on a straight line graph established by the standard chick embryo cell RNA (CEC$_{RNA}$) sedimentation coefficients.

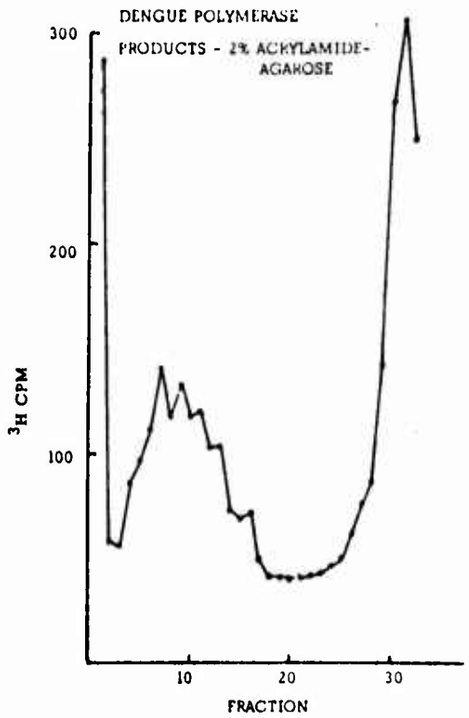


Figure 45. Electropherogram of dengue-2 polymerase products following electrophoresis for 60 minutes in 2% acrylamide.

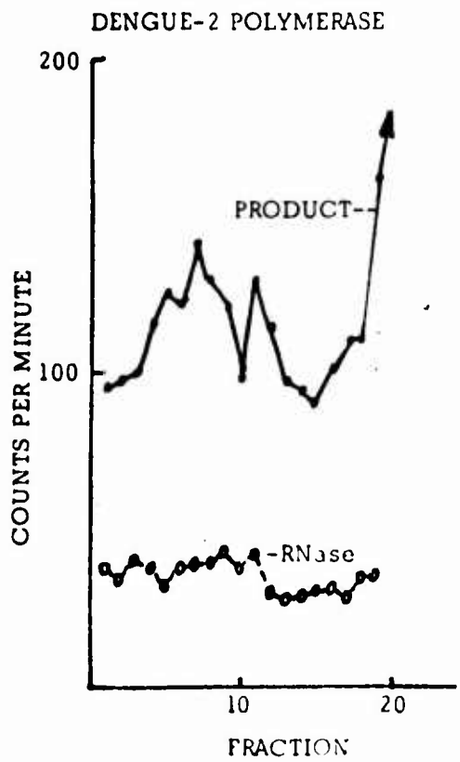


Figure 46. Rate zonal distribution of dengue polymerase product RNA. Polymerase product was prepared in complete media, extracted in SLS-phenol, precipitated in ethanol and redissolved in TNE buffer. The sample was layered on a 5 ml 20-5% sucrose gradient and centrifuged in a SW50.1 rotor at 50,000 rpm for 150 minutes. The 200 lambda fractions were divided into RNase treated and untreated samples. RNase was 20 gamma/ml and both aliquots were incubated at 37C for 60 minutes.

product. This could be due to nucleases in the enzyme preparation or uneven initiation and termination of chains such as in bacteriophage (Richardson, et al, 1970; Roberts, et al, 1969).

The information on RNA products generated by the polymerase system will be integrated into studies of sites of antigen and protein synthesis in infected cells. Species of RNA associated with the several types of membranes of infected cells described in a separate section above will be characterized by the methods described in this section. Finally, as an attempt to determine the function of nonstructural antigens which react in serological tests, antiserum against these antigens can now be tested for the capacity to inhibit the formation of polymerase products.

E. Comparative Structure of Arbovirus by Size Analysis of Virion Polypeptides and Nucleic Acids.

Group A and group B arboviruses have several morphological, physical-chemical, and biological similarities. However, the estimated sizes of their ribonucleic acid (RNA) and their structural polypeptides show marked differences. Further there is a lack of agreement among investigators regarding the number of structural polypeptides in the group B arboviruses. These conflicting data may be the result of either a true structural heterogeneity among arboviruses or may be due to variations in the techniques used in each laboratory. Thus, we have compared the RNA and structural polypeptides of several group A and group B arboviruses under standard experimental conditions in order to resolve the differences and to gain some insight into the structural basis for the serological groups. A third group of arboviruses, the Bunyamwera group, which contains California encephalitis virus, was examined under the same experimental conditions since little is known at the molecular level about this important group of viruses.

Methods.

Three of the group A viruses that were examined, Sindbis, eastern and western equine encephalitis, were propagated in primary chick embryo fibroblast cell culture while the fourth, chikungunya was propagated in an established line of baby hamster kidney cells. (Several of these agents were propagated in both cell types and no differences in their polypeptide composition was detected.) Cell cultures in 32 oz prescription bottles were infected at a multiplicity of infection (MOI) of approximately 10 during an adsorption period of one hour at 36°C. The inoculum was washed off the cell monolayers by rinsing them five times with Hanks' balanced salt solution and replaced with medium consisting of two per cent dialyzed fetal bovine serum in medium 199 with 1/20 the normal level of amino acids. After further

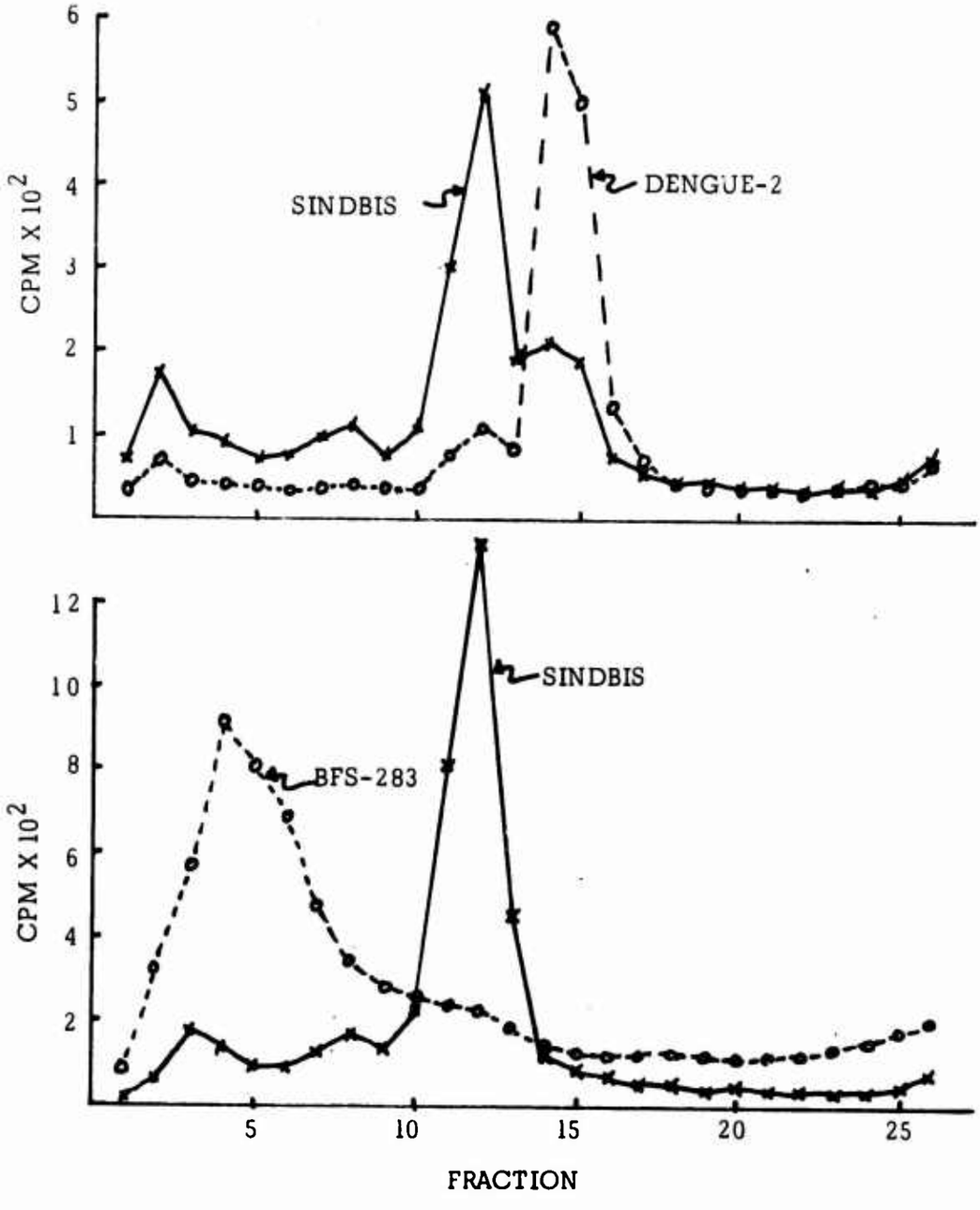


Figure 47. Rate-zonal centrifugation in 15-30 percent sucrose gradients of representatives of Group A (Sindbis), Group B (dengue-2) and California Group (BFS-283) arthropod-borne viruses.

incubation for two hours in order to allow for cessation of host protein synthesis, radioactive amino acids and/or uridine was added (3 microcuries/ml ^{14}C or 30 microcuries/ml ^{3}H). Maximum production of virus by the infected cells occurred at 12-15 hours. The radioactive, infected medium was harvested at 16 hours and mixed with an equal volume of saturated ammonium sulfate to precipitate the virus; 30-90 fold concentration was effected by resuspension of the precipitate in an appropriate volume of 0.01 M Tris 0.15 M NaCl-0.001 M EDTA buffer, pH 7.6. This material was layered onto a 15-30% sucrose gradient and centrifuged at 25,000 rpm for three hours. The radioactive peak just below the center of the gradient (Sindbis is used as an example in Figure 47A, top panel) was previously shown to contain the majority of infectious particles. Aliquots of this rate-zonal purified virus was degraded into its constituent polypeptides with 1% sodium lauryl sulfate (SLS) and 1% 2-mercaptoethanol (2-ME) at 100°C for 10 minutes and then analyzed by polyacrylamide gel electrophoresis as described above.

Radioactive group B viruses (Japanese encephalitis, dengue, the Asibi and French neurotropic strains of yellow fever, langat and St. Louis encephalitis) were propagated in a continuous line of monkey kidney cells (LLC-MK-2). Maximum production of group B viruses occurred by 30 hours after infection, taking about two to three times as long to replicate as those in group A. Further, sufficient quantities of radioactive virus could only be obtained by allowing replication to proceed for at least five days for some agents (dengue, yellow fever and Langat). Infected cells were maintained in 0.2% FBS in medium 199 with 1/10 the normal level of amino acids plus 30 uc/ml ^{3}H amino acids or 3.3 uc/ml ^{14}C amino acids. The group B viruses were pelleted from the culture medium (78,000 x g for three hours or 192,000 x g for 75 minutes) and concentrated 50 to 100 fold by resuspension in Tris-saline-EDTA buffer, pH 8.2. Resuspension was facilitated by sonication in a sealed tube at full power (10KC) in a Raytheon apparatus. Virions of each group B virus were isolated in 5-25% or 5-35% sucrose gradients centrifuged for 3.0 to 3.5 hours at 63,000 x g, similar to rate-zonal centrifugation shown in the JEV-protein section above. However, for purposes of comparing group A and group B sedimentation characteristics, dengue-2 (group B) was co-sedimented with Sindbis (group A) on a 15-30% sucrose gradient (Figure 47, top panel). Group A virions reproducibly sediment faster than those in group B. Degradation of the sucrose purified virions was carried out with SLS and 2-ME and analyzed on polyacrylamide gels as described above.

California encephalitis virus and the related agents, Tahyna and Bunyamwera, were propagated in chick embryo cell culture as were the group A agents except at an MOI of 0.1 to 1. Adsorption time was 1.5 hour after which the inoculum was washed out and replaced with amino acid deficient medium as described for the group A viruses. Radioactive amino acids were added four hours later rather than

immediately after the end of the absorption period because of the lower MOI. The radioactive, infected cell culture medium was harvested at the first sign of CPE, usually two to three days later, and viral products were concentrated and purified in 15-30% sucrose gradients in the same manner as the group A agents. Under these conditions (63,000 x g for three hours), the Bunyamwera group agents (Bunyamwera, California and Tahyna virions) sedimented near the bottom of the gradient while the group A agents sedimented much slower. An example is given in Figure 47, bottom panel) where California (BFS-283) virions were sedimented under the same conditions as Sindbis virions. Clearly, sedimentation characteristics alone compartmentalize at least some of the members of the major groups of arthropod-borne viruses.

Results.

Polyacrylamide gel electrophoresis of SLS-degraded, sucrose purified virions reveal similarities of each of the members of the major serogroups at the molecular level. The group A virions of WEE, EEE, Sindbis and Chikungunya preparations contain two polypeptides when analyzed under these conditions (Figure 48). Strauss and Burge (1968) determined that the two polypeptides of Sindbis virus had molecular weights of 50,000 and 30,000. Since these values are generally accepted as correct, the present data suggest that the four group A viruses examined here each contain two polypeptides with molecular weights of 50,000 and 30,000 daltons.

When sucrose-purified virions were treated with a nonionic detergent (NP-40) as described in the JEV section above, and resedimented through another sucrose gradient shown in the following section (VII A), a dense RNA-rich particle was obtained. Degradation of this particle with SLS and 2-ME followed by analysis on polyacrylamide gels resulted in only one peak of radioactivity; this peak was shown to co-electrophorese with the 30,000 MWT protein. Thus, the smaller of the two proteins is termed the "core" protein and the larger, 50,000 MWT protein, having been removed by the detergent, is called the "coat" protein.

Polyacrylamide gel electrophoresis of SLS degraded group B virions generally reveals three polypeptides (Figures 49 and 50). The small radioactive peaks to the left of the largest peak (and largest protein) of St. Louis encephalitis and yellow fever viruses probably represent aggregates that "hang up" near the top of the gel where the sample is applied. The largest and smallest of the three proteins of these group B virions are considered "coat" proteins since nonionic detergents remove them from the virion surface. Following the detergent treatment, a dense RNA-rich "core" isolated as described and shown in the JE protein section above, contains only the middle of the three peaks shown in Figures 49 and 50. This core protein has now been

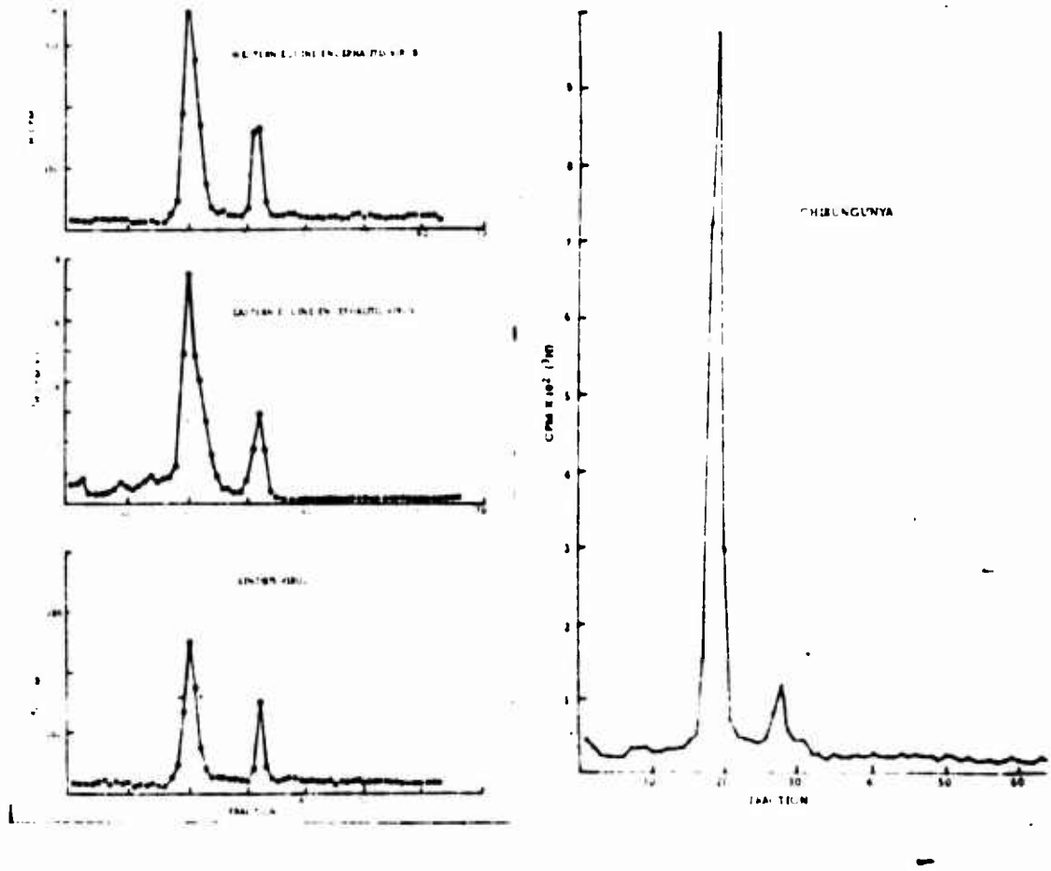


Figure 48. Polyacrylamide gel electrophoresis of sodium lauryl sulfate degraded, radioactive group A virions; migration is from left to right. The larger peak is the surface or "coat" protein (50,000 daltons) and the smaller peak is the internal or "core" protein (30,000 daltons).

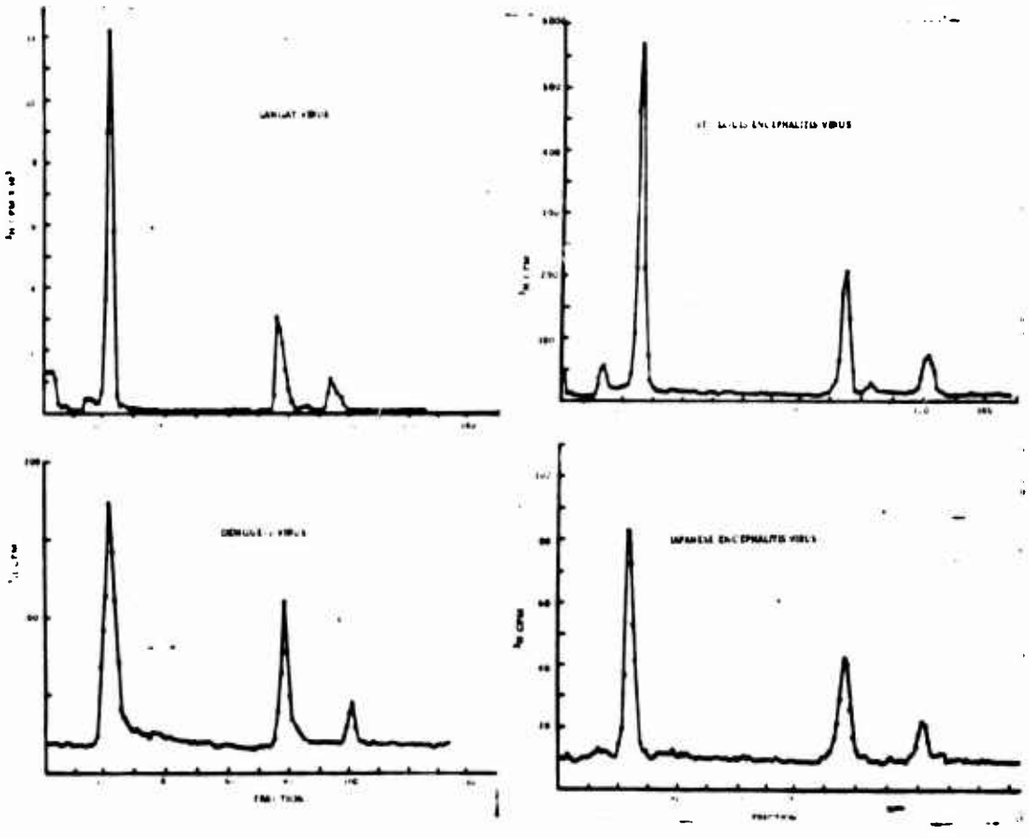


Figure 49. Polyacrylamide gel electrophoresis of SLS degraded, radio-active group B virions; migration is from left to right. The largest and smallest peaks are surface (coat) proteins and the center peak is the internal (core) protein.

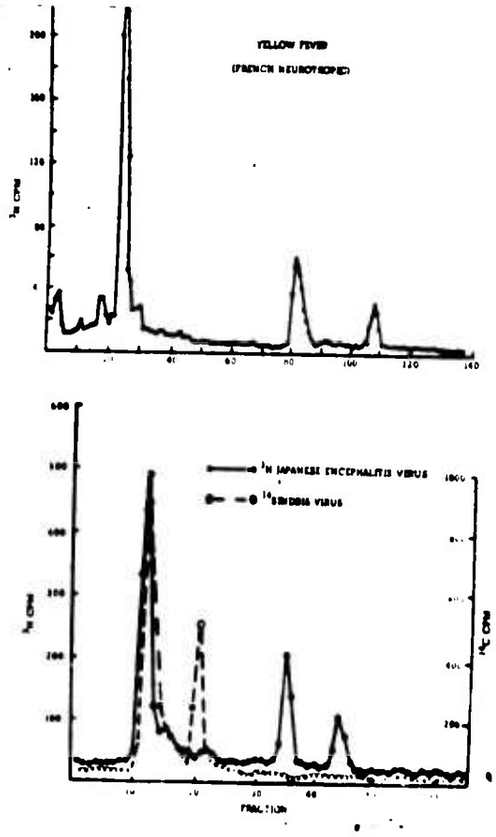


Figure 50. Polyacrylamide gel electrophoresis of SLS-degraded, group B virions. Japanese encephalitis virion proteins were coelectrophoresed with Sindbis virion proteins as standard markers (lower panel).

documented for dengue, JEV, SLE viruses; it is assumed that the other group B agents are the same.

Approximate molecular weights were assigned to the three structural polypeptides of JE by coelectrophoresis with the two structural polypeptides of Sindbis virus now accepted as standard molecular weight markers (Figure 50, lower panel). The size of the major JE coat protein is very close to the Sindbis coat protein, being slightly larger (53,000 daltons compared to the 50,000 Sindbis marker). However, the core protein of JE is very much smaller than the group A core protein (13,500 daltons compared to the 30,000 Sindbis marker). By extrapolation the minor JE coat protein is approximately 8,700 daltons.

The virion polypeptides of several group B arboviruses have been described by other investigators: Those of dengue-2 virus by Stollar (1969); Kunjin virus by Westaway (1969); and St. Louis encephalitis virus by Trent (1971). There are reproducibly three size classes of polypeptides, but the estimates of molecular weights for each size class vary considerably among the different laboratories: 7,500 to 13,000 daltons for the small surface protein, 13,000 to 18,000 daltons for the internal or "core" protein, and 53,000 to 65,000 daltons for the large surface protein. Because analogous findings for different strains of poliovirions have been taken as evidence for variable post-translational cleavage, it was of importance from both mechanistic and taxonomic views to determine the actual variation in group B virion polypeptides. The virions were dissociated with SLS and 2-ME, mixed two at a time and analyzed by coelectrophoresis on polyacrylamide gels. Differences in mobility were assumed to be related to changes in molecular weight, although recent studies have indicated that both charge and lipid binding can slightly influence mobility even in SLS-containing gels. Furthermore, since the large surface protein in a glycoprotein, the exact significance of mobility changes is unclear, but growth of all viruses in the same host cell would minimize effects due to carbohydrate variation.

Figure 51 represents coelectrophoresis of JEV with dengue-2 and SLE virion proteins. All three viruses have very similar size polypeptides with a variation in molecular weight of less than 10 per cent (Table 36); the polypeptides starting with the smallest are designated V (virion)-1, V-2 and V-3. By contrast, coelectrophoresis of a tick-borne group B arbovirus, Langat (TP-21), with JEV reproducibly reveals that the Langat small surface protein (V-1) migrates more slowly, indicating a 12 per cent increase in molecular weight (Figure 52, upper panel, and Table 36). Russian Spring Summer Encephalitis (RSSE) virus, another tick-borne agent, possessed a V-1 of essentially identical mobility to Langat (Figure 52, lower panel).

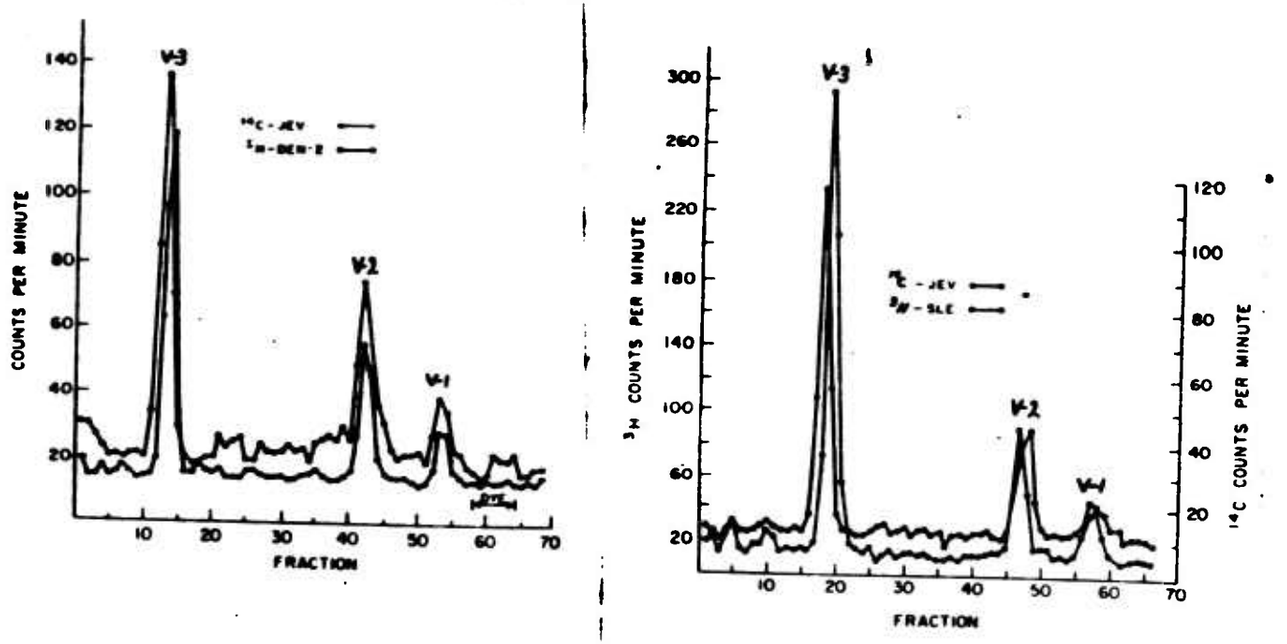


Figure 51. Coelectrophoresis of Japanese encephalitis virion (JEV) proteins with those of dengue-2 (DEN-2) and Saint Louis encephalitis (SLE) virions on polyacrylamide gels.

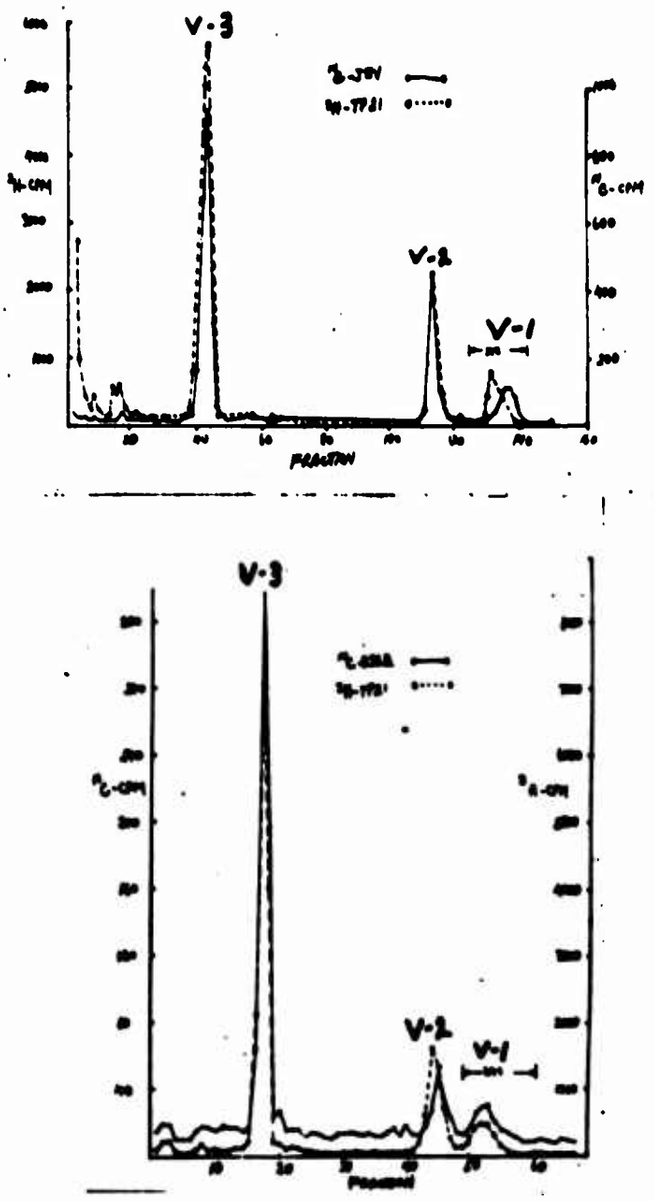


Figure 52. Coelectrophoresis of Japanese encephalitis virion (JEV) proteins with those of Langat (TP-21) (upper panel) and coelectrophoresis of Russian spring-summer encephalitis (RSSE) virion proteins with those of Langat (TP-21) (lower panel).

Table 36

Molecular Weight Estimates of
Group B Virion Polypeptide*

	V-1	V-2	V-3
JE	8.7	13.5	53
SLE	8.7	14.5	50
DEN-2	8.7	13.5	54
TP-21	9.7	13.5	54
RSSE	9.7	13.0	54
YF (FNT)	9.0	16.0	51
YF (Asib)	9.0	16.0	51

* Relative to JE polypeptides.

When a member of a different group B subgroup, yellow fever, was examined, a major difference was found in the size of V-2, the core polypeptide: coelectrophoresis of French neurotropic yellow fever, a nonvirulent strain, with JEV (Figure 53, upper panel) indicated that the former possessed a V-2 with significantly slower mobility, suggesting a molecular weight increase of 19 per cent (Table 36). Similarly, a virulent strain of yellow fever, Asibi, had a V-2 very similar to that of the French neurotropic strain (Figure 53, lower panel, and Table 36).

All group B arboviruses so far studied have three analogous virion polypeptides, indicating a fundamentally similar structure for all of them. Despite this, two major shifts in molecular weights have been found: (1) V-1, the small coat protein, is about 12% larger in tick-borne viruses than in nontick borne viruses; and (2) the core protein is about 20% larger in yellow fever viruses than in other group B agents. Thus, even subgroups within the group B complex may have a unique size class of polypeptide as reflected either in the core protein or one of the surface proteins. The virion polypeptides comprise about 75,000 daltons, corresponding to only about 25% of the coding capacity of the viral genome. Since there is little variation in the size of the major coat protein, the substantial variation in molecular weight of the smaller analogous polypeptides does not imply any changes in the size of the genome and is consistent with ambiguous post-translational cleavage.

Viral RNA.

In order to compare the relative size of group A and group B genomes, radiolabeled viral RNA was extracted from sucrose gradient

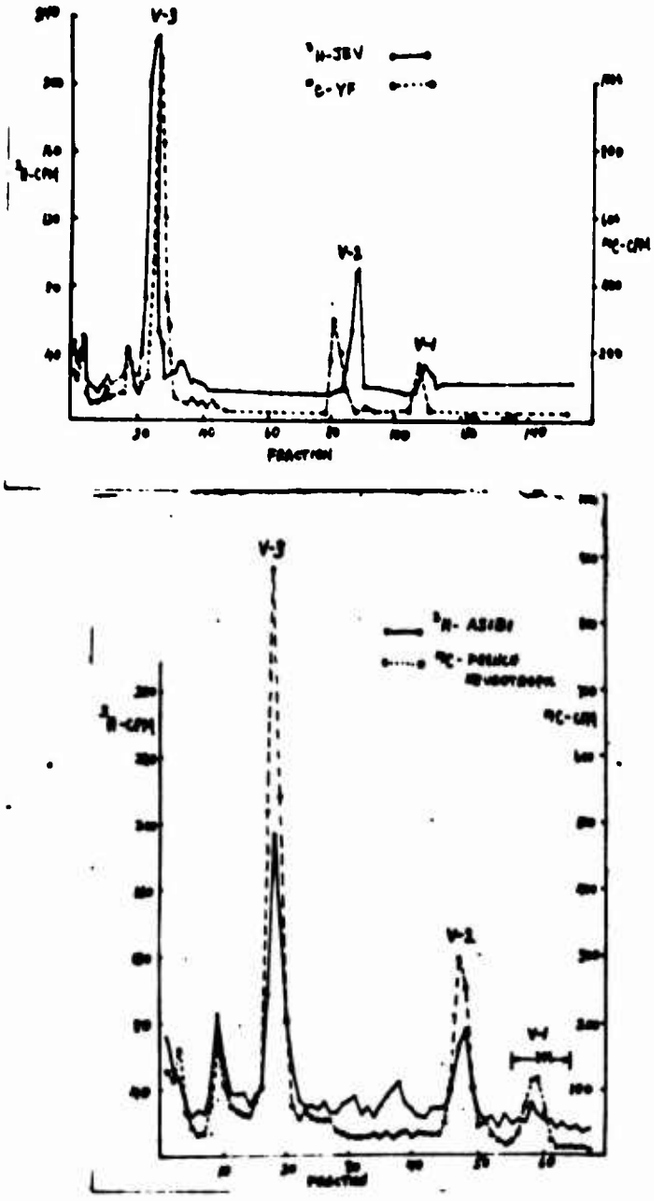


Figure 53. Coelectrophoresis of Japanese encephalitis virion (JEV) proteins with those of French neurotropic yellow fever (YF, upper panel) and coelectrophoresis of Asibi yellow fever virion proteins with those of French neurotropic strain (lower panel).

purified virions by the SLS-phenol method described in the polymerase section above. Extracted RNA's were mixed with similarly purified Sindbis RNA and electrophoresed in 2.0% polyacrylamide-agarose gels. The RNA's of all of the examples of group A and group B viruses migrated with the Sindbis RNA (Figure 54). This established that the genome of all of the arboviruses studied have genomes of similar size. Occasionally, some displacement of the peak fractions in relation to Sindbis occurred. This displacement, however, was not consistent and prolonged electrophoresis did not lead to further separation. While these experiments establish that the arboviruses have genomes of the same magnitude, they do not preclude the existence of minor differences which might be resolved with more sensitive techniques.

The molecular weight of Sindbis RNA, used in these studies as the reference arbovirus RNA, was determined by using chick cell ribosomal RNA as a marker (Peacock and Dingman, 1968). Our determinations ranged from 3.7×10^6 to 4.2×10^6 daltons with a mean of 3.9×10^6 daltons for the molecular weight of Sindbis RNA (Figure 55).

Finally, polyacrylamide gel electrophoresis of the Bunyamwera group virions is depicted in Figure 56). These viruses also have three structural polypeptides as do the group b viruses, but they are of a completely different size class. Sindbis marker proteins (group A) are shown in the upper panel and may be compared with the Sindbis-JEV(Group B) co-run in a previous figure. Which of these proteins is associated with the virion "coat" or nucleic acid "core" has yet to be determined.

These investigations have clearly shown that the virions of the major serogroups of arthropod-borne viruses have unique polypeptide compositions which can serve as a beginning for explaining at the molecular level the structural basis for the interrelationships among the members of these groups. These data confirm and extend the previous impressions of structural homogeniety within serologic groups gained from physical, chemical and serological techniques.

VII. Antigenic Analysis of Arboviruses

A. Radio-immune Precipitation of group A Arbovirus Antigens.

Arbovirus serology has been cumbersome in most laboratories; serological interpretation is often complicated by intra-group reactivity, closely related (antigenic) virus complexes within groups and a low degree of individual virus type specificity. Investigation of purified type specific and group reactive antigens of arboviruses was initiated to understand their immunological reactivity.

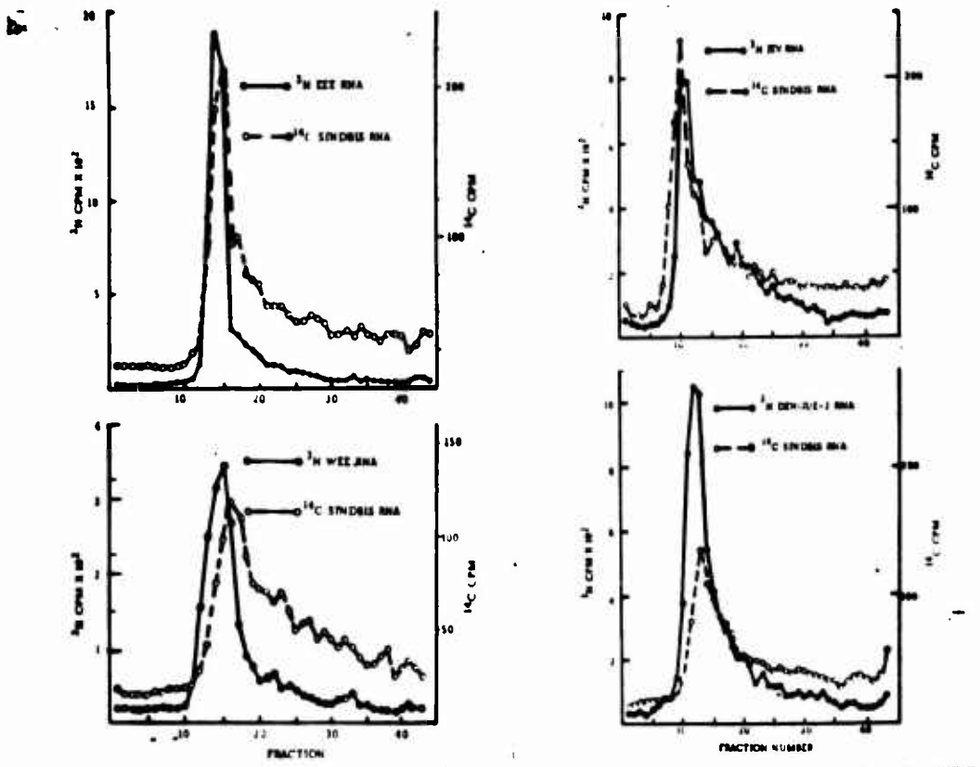


Figure 54. Comparison of the ribonucleic acid (RNA) extracted from purified Sindbis virions with:

a. Eastern equine encephalitis (EEE) RNA;

b. Japanese encephalitis virus (JEV) RNA;

c. Western equine encephalitis (WEE) RNA; and

d. Dengue-2 RNA.

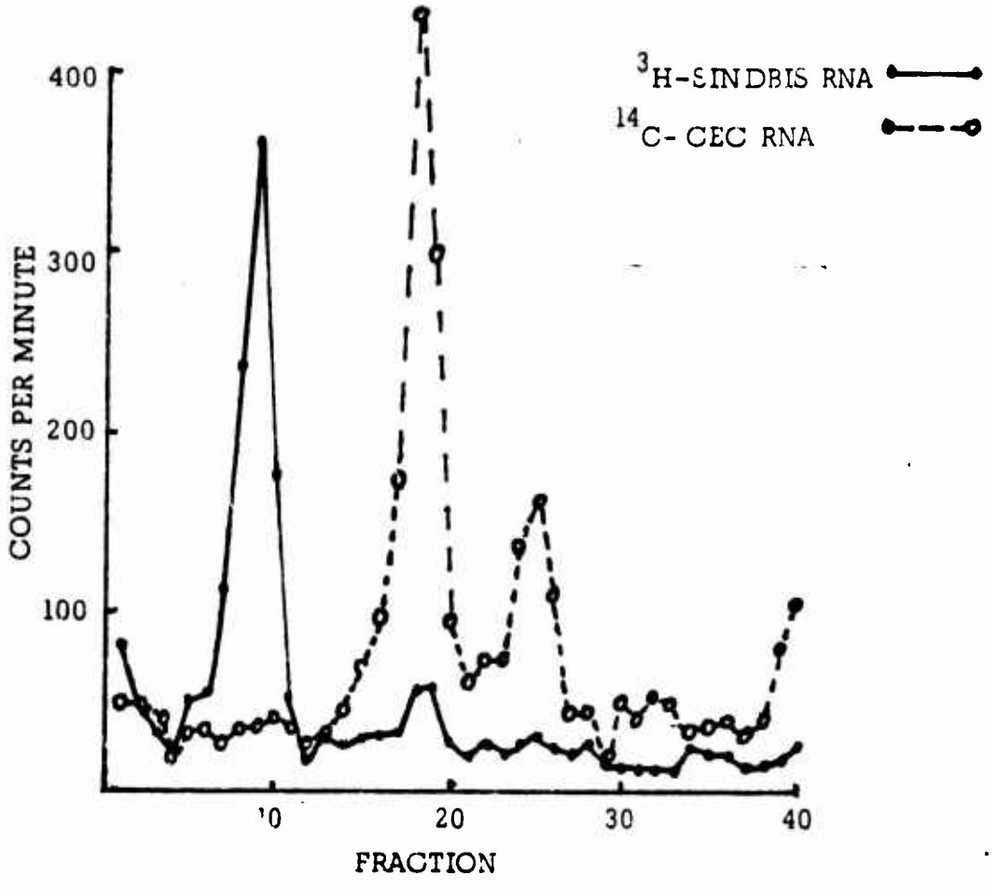


Figure 55. Coelectrophoresis on a polyacrylamide-agarose gel of Sindbis virus ribonucleic acid (RNA) and chick embryo cell (CEC) ribosomal RNA.

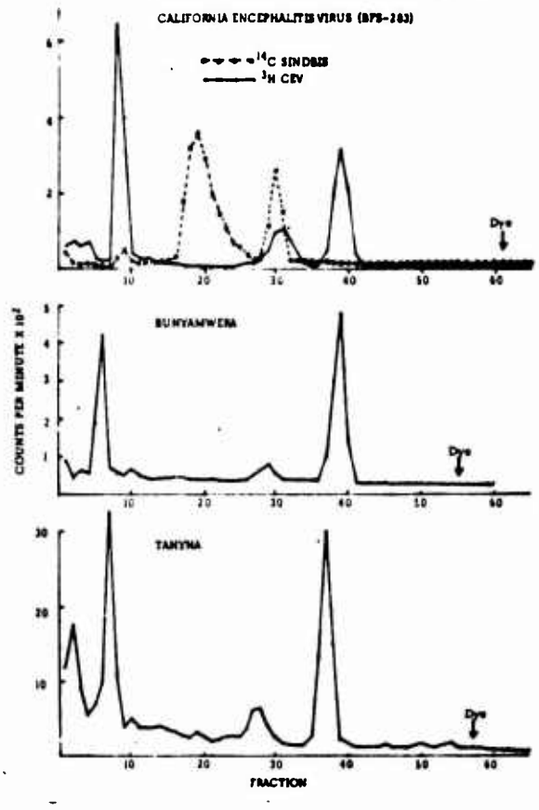


Figure 56. Polyacrylamide gel electrophoresis of SLS-degraded Bunyamwera group virions; migration is from left to right. Sindbis virion proteins (group A) were used as markers in a co-run with California encephalitis virion (CEV) proteins in the top panel.

Studies reported herein include an examination of the antigenic interrelationships of Sindbis (SIN), Western and Eastern equine encephalitis (WEE and EEE) viruses using radio-immune precipitation (RIP) techniques. These three representatives of the group A arboviruses have been previously demonstrated to contain only two structural proteins of similar molecular weights (Annual Report 1970) and share other common characteristics.

Materials and Methods

Virus Strains. All stock virus suspensions used as infective inoculum consisted of 20% infected suckling mouse brain suspensions. The Cambridge strain of EEE, MacMillan strain of WEE and Sindbis strain AR339 were used exclusively.

Cell Culture and Virus Propagation. Primary chick embryo cell fibroblast cultures were prepared from 10-day embryonated hen's eggs and grown in 32 oz prescription bottles using lactalbumin hydrodlydate medium with Earles' balanced salts, supplemented with 5% fetal bovine serum and antibiotics. Confluent monolayers were infected with a multiplicity of infection of 10, the infective inoculum removed after one hour adsorption and virus containing supernatant fluids harvested 14-16 hours post infection.

Radioisotopic Labeling. In preparing radioactive virus suspensions either carbon^{14} or tritium labeled L-amino acid mixtures (New England Nuclear, Boston, Mass.) were added to infected cultures containing Medium 199 without added amino acids and 2% dialyzed fetal bovine serum. Final concentrations of radioactivity added were 10 microcuries per ml for ^{3}H labeled preparations and 1 microcurie per ml for ^{14}C.

Virus Concentration and Purification. Infected cell culture harvests were clarified by centrifugation at 4000 x g for 20 minutes prior to concentration. Clarified virus suspensions were precipitated by the dropwise addition of sufficient cold saturated ammonium sulfate solution to yield a final concentration of 60%. Saturated ammonium sulfate solutions were adjusted to pH 7.4 with 1 M Tris prior to use. Precipitated virus suspensions were mixed for 30 minutes at 4°C and centrifuged at 16,000 x g for 30 minutes. The resultant precipitate was resuspended in TS. buffer (0.01M Tris-HCl pH 7.4, 0.15M Na Cl, 0.001M EDTA) to yield a final concentration of 30-50 times original.

Purified virions were obtained by rate zonal centrifugation of the concentrated virus in 30 ml 15-30% sucrose gradients on a 70% sucrose cushion; 1.2-2.0 ml samples were layered on top and centrifuged at 65,000 x g for three hours in the Beckman 25.1 rotor.

One ml fractions were collected through a puncture in the bottom of the tube and assayed for radioactivity and infectivity; peak infective virus was usually found in fractions 10 to 13.

Assay Methods. Infectivity was determined by plaque assay on primary chick embryo cell monolayers. Plaque reduction neutralization tests consisted of incubating approximately 100 plaque forming units with dilutions of antisera at 37°C for 30 minutes, and following plaque assay, calculating on profit paper the dilution of antisera that neutralized 50% of the plaque dose. Hemagglutination tests were performed by a microtiter modification of the method of Clark and Casals, using borate saline pH 9.0 and male goose erythrocytes. Sindbis virus hemagglutinin was assayed at pH 5.8 while WEE and EEE hemagglutinins were assayed at pH 6.0 and 6.2, respectively. Hemagglutination inhibition procedures utlized acetone extracted antisera. Complement-fixation tests were performed according to the method of the Department of Serology, WRAIR.

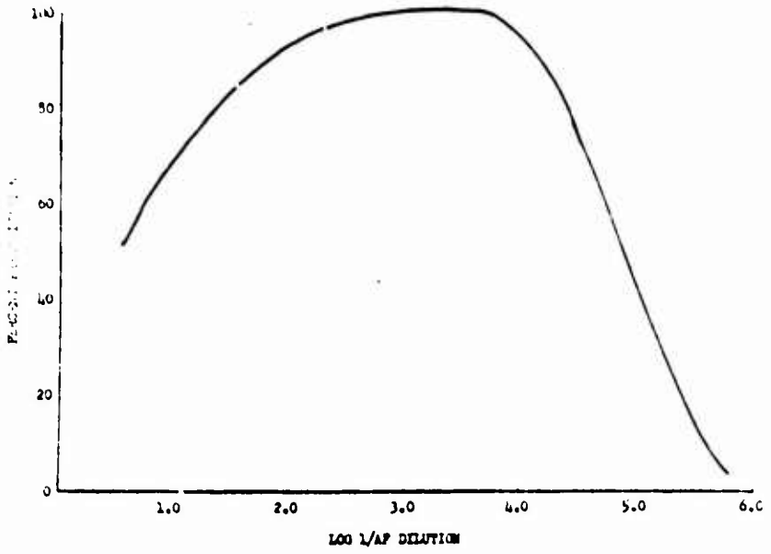
Preparation of Antisera. Mouse hyperimmune ascitic fluids (MHAF) were prepared as described in previous annual reports using Sarcoma 180 cells for the induction of ascites. Antiserum to mouse serum was prepared in rabbits by two intradermal 1 ml injections of mouse serum mixed in equal proportions with Freund's complete adjuvant. Injections were given one month apart and animals bled by cardiac puncture 10 days following the last injection.

Radio-immune Precipitation. Radio-immune precipitation procedures were essentially the same as those described in the previous annual report, employing 0.05 ml test antigen, 0.05 ml diluent or inhibitor, 0.05 ml anti-virus antibody and 0.05 ml anti-mouse serum. RIP inhibition tests utilized a two-hour preincubation of inhibitor and anti-virus antibody prior to the addition of the remaining reagents.

Results.

Homologous RIP Reactions. The precipitation of a constant amount of radioactive virion over a broad series of antiserum (hyperimmune mouse ascitic fluid) dilutions is shown in Figure 57A. Reduced precipitation observed at low ascitic fluid dilutions apparently resulted from ascitic fluid excess since precipitation at these dilutions could be increased upon the addition of higher concentrations of anti-mouse serum. A plateau of 100% precipitation indicated that all radioactivity was antigen associated. The linear decrease in per cent precipitation observed at higher dilutions of ascitic fluid reflected the antibody titer of the ascitic fluid and not anti-mouse serum excess since the addition of normal ascitic fluid did not increase precipitation.

A.



B.

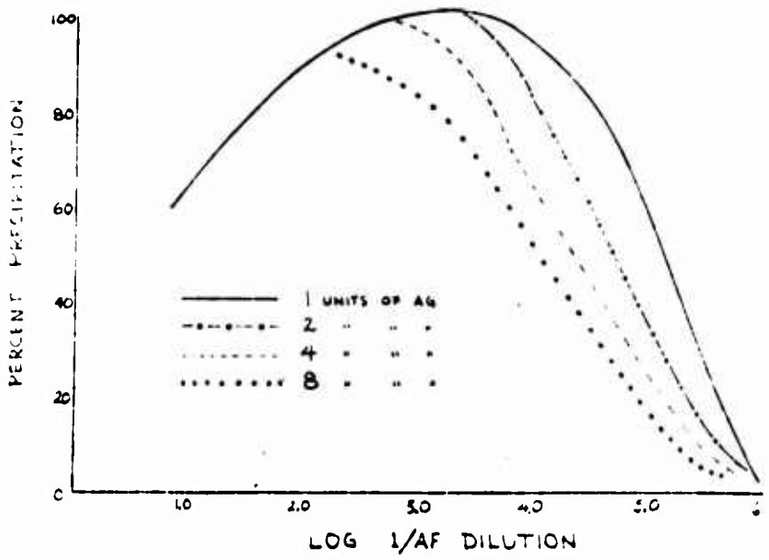


Figure 57. A. Radioimmune precipitation (RIP) of ^{3}H-WEE virus with WEE hyperimmune mouse ascitic fluid (HMAF) dilutions using 1:20 anti-mouse serum.

B. Effect of varied WEE virus concentration on RIP with WEE HMAF. Antigen concentrations shown consisted of undiluted, 1:2, 1:4 and 1:8 dilutions of a single WEE virion preparation.

The comparative sensitivity of the RIP test is shown in Table 37. The antibody titers of the three immune ascitic fluids were approximately equal by both CF and HAI. Both EEE and WEE virus immune ascitic fluids were tenfold higher titered than Sindbis immune ascitic fluids in both neutralization and RIP. The RIP test appeared the most sensitive and both neutralization and RIP resulted in hundred-fold increases in antibody titer over CF and HAI.

Table 37.

COMPARATIVE HOMOLOGOUS ANTIBODY TITERS OF MOUSE HYPERIMMUNE ASCITIC FLUIDS

	SEROLOGICAL TEST			
VIRUS	CF[1]	HAI[2]	NEUT[3]	RIP[4]
Sindbis	12.8	6.4	100	200
WEE	12.8	12.8	1000	2000
EEE	12.8	12.8	1000	2000

(All titers = 1/AF Dilution x 10^2)

1 Complement fixation.
2 Hemagglutination inhibition.
3 Plaque reduction neutralization (50%)
4 Radio-immune precipitation.

Increased sensitivity demonstrated by RIP was found to be dependent on the antigen concentration employed. The effect of varying antigen concentration on the per cent precipitation is shown in Figure 57B. Increasing antigen concentration served to effectively reduce both the plateau and the 50% RIP antibody endpoint of the immune ascitic fluid.

The relationship of antigen concentration and per cent precipitation at a constant antiserum dilution is shown in Figure 58A. Per cent precipitation decreased linearly with increasing virus concentration over a 100-fold range. The uniform slope of the resultant line indicated a direct relationship between the per cent RIP at a constant antiserum dilution and the antigenic mass of the virus preparation.

A homologous RIP inhibition using varying dilutions of unlabeled Sindbis virus as inhibitor is shown in Figure 58B. The 100% inhibition observed at low dilutions of inhibitor demonstrates that RIP reactions can be completely inhibited whereas the linear portion of the curve illustrates the relationship between per cent inhibition and inhibitor concentration.

Heterologous RIP Reactions. Antigenic relationships of the three viruses and their respective antisera were examined using RIP procedures (Figure 59). Homologous RIP titers were always evident far beyond the dilution range exhibiting cross-reactions. Cross-reactions were demonstrated between WEE antiserum and Sindbis virus and with Sindbis antiserum and WEE virus. However, neither of these antisera reacted with EEE virus preparations. Eastern immune ascitic fluid appeared specific inasmuch as no reaction with either Sindbis or WEE virus was detected. These data illustrate the closer antigenic relationship of WEE and Sindbis compared to EEE virus.

The effect of antigen concentration on RIP cross-reactions was investigated using Western immune ascitic fluid and the cross-reacting Sindbis virus (Figure 60). At low antigen concentrations (1 unit) precipitation approached 100% but decreased as antigen concentration was increased. At high concentrations of antigen (32 units) less than 20% precipitation was achieved over the entire dilution range. Of particular interest was the observation that at intermediate Sindbis virus concentrations, the precipitation curve appeared biphasic with maxima at ascitic fluid dilutions of 1:100 and 1:1000.

A detailed examination of the homologous WEE reaction and the heterologous reaction with Sindbis virus was conducted using multiple small increment dilutions of WEE immune ascitic fluid (Figure 61A). Three separable regions of antibody activity were demonstrated. The homologous reaction of Western immune ascitic fluid with WEE virus was detectable at a dilution range greater than that cross reacting with Sindbis virus. Precipitation in this region (Region III) appeared specific. The RIP reaction with the cross-reacting Sindbis virus yielded a region of reaction at low dilutions of the antiserum (Region I) which resembled the cross-reactions observed using equivalent antigen concentrations (Figure 59). The second peak of the biphasic cross-reaction curve (Region II) could only be demonstrated using selected concentrations of Sindbis virus.

The specificity of the antibody described in each of the three regions was examined by inhibition of the RIP reaction using unlabeled virion preparations (Figure 61B). WEE virus completely inhibited the homologous WEE RIP in Region III while Sindbis and EEE viruses inhibited only slightly. All three viruses inhibited greater than 50% of the cross-reaction in Region II yet only Sindbis and WEE viruses

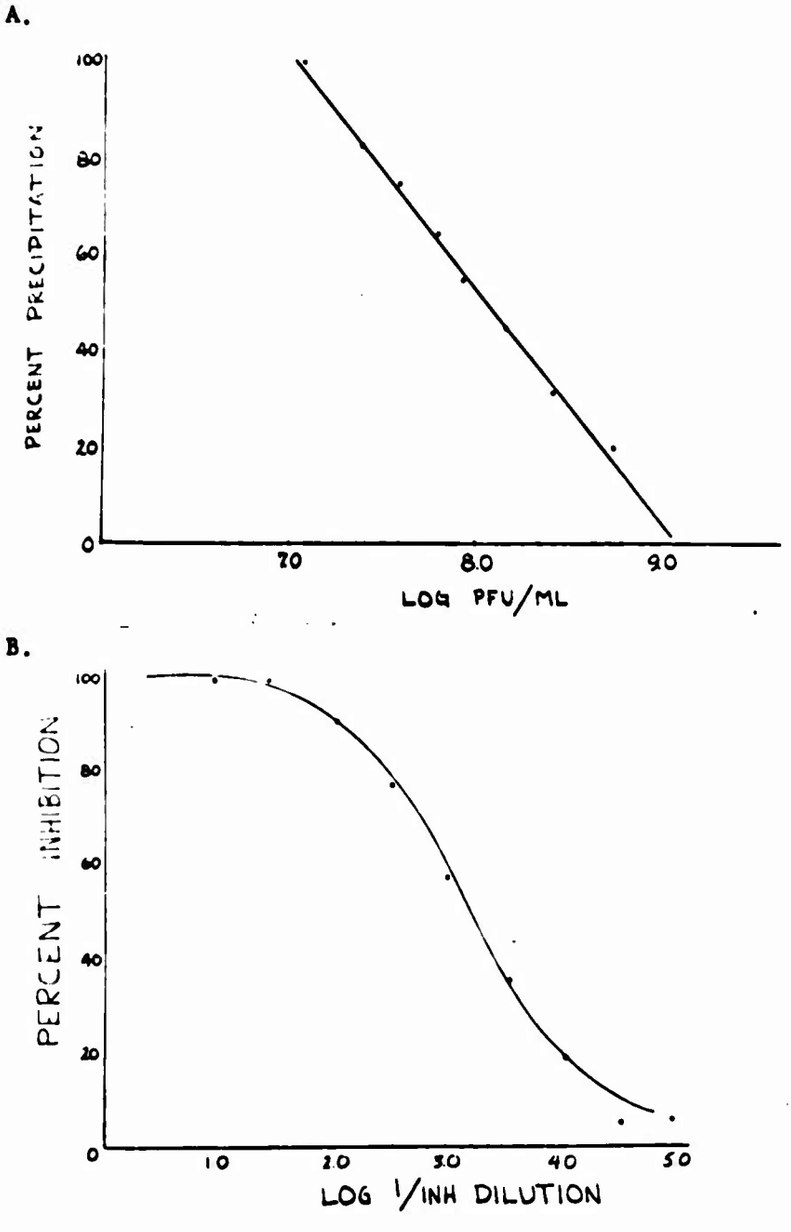


Figure 58. A. Relationship of virus infectivity (antigen concentration) and percent radioimmune precipitation. Sindbis hyperimmune mouse ascitic fluid (HMAF) at 1:10,000 dilution was constant in all RIP reactions.

B. Inhibition of homologous RIP. Inhibition of 50% precipitation of ^{3}H-Sindbis virus by dilutions of unlabelled Sindbis virus. Sindbis HMAF was used at a 1:10,000 dilution.

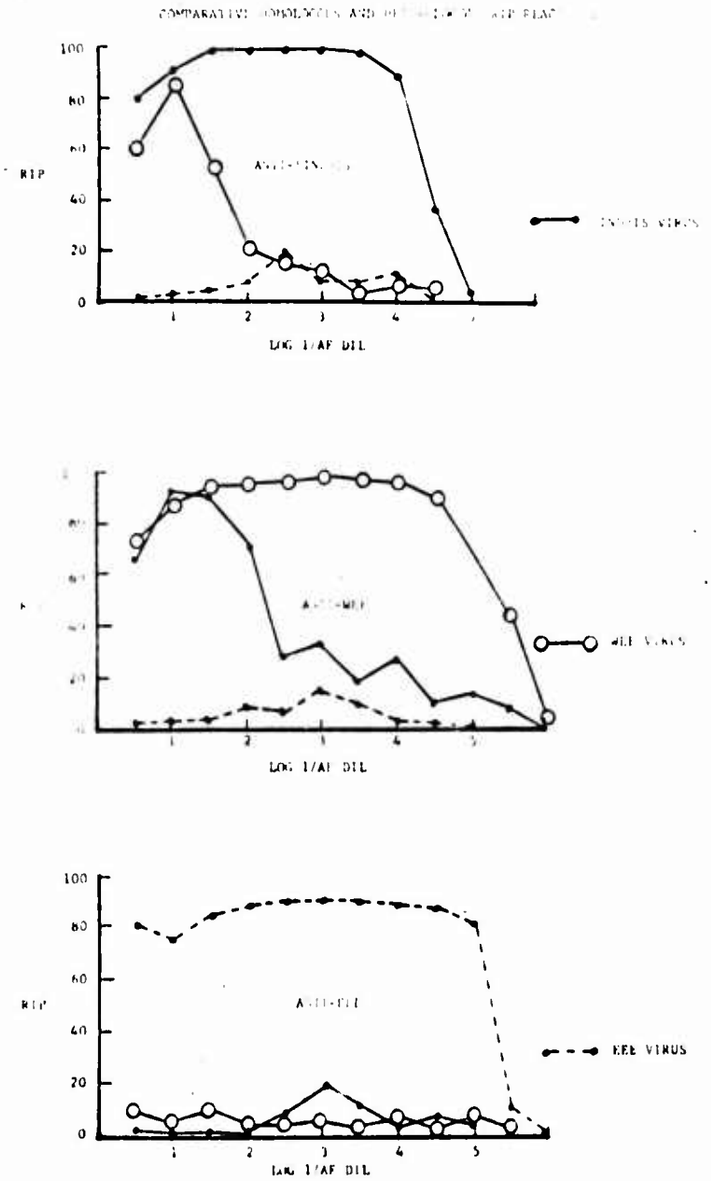


Figure 59. Comparative homologous and heterologous RIP reactions were performed using equivalent antigen concentrations of each of the three viruses indicated in each panel (4 X 10^6 PFU per test).

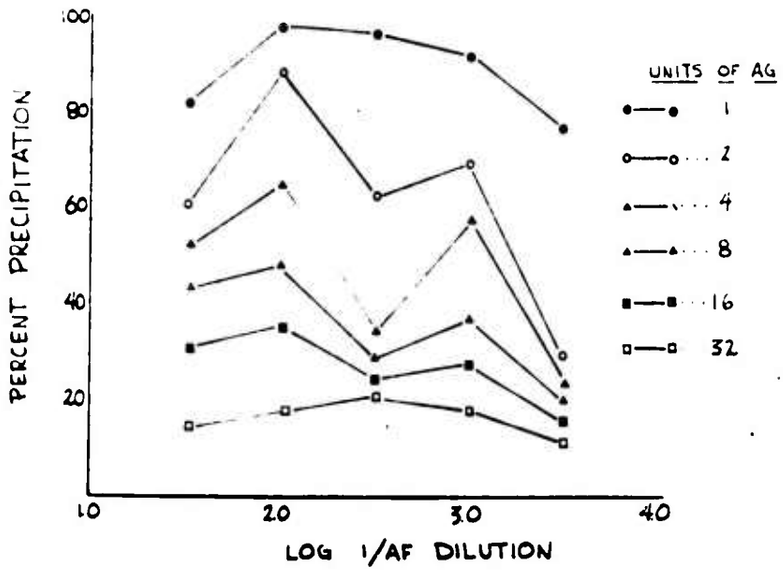


Figure 60. Effect of varying antigen concentrations on radioimmune precipitation cross reactions. WEE hyperimmune mouse ascitic fluid dilutions were reacted with two-fold dilutions of a Sindbis virion preparation.

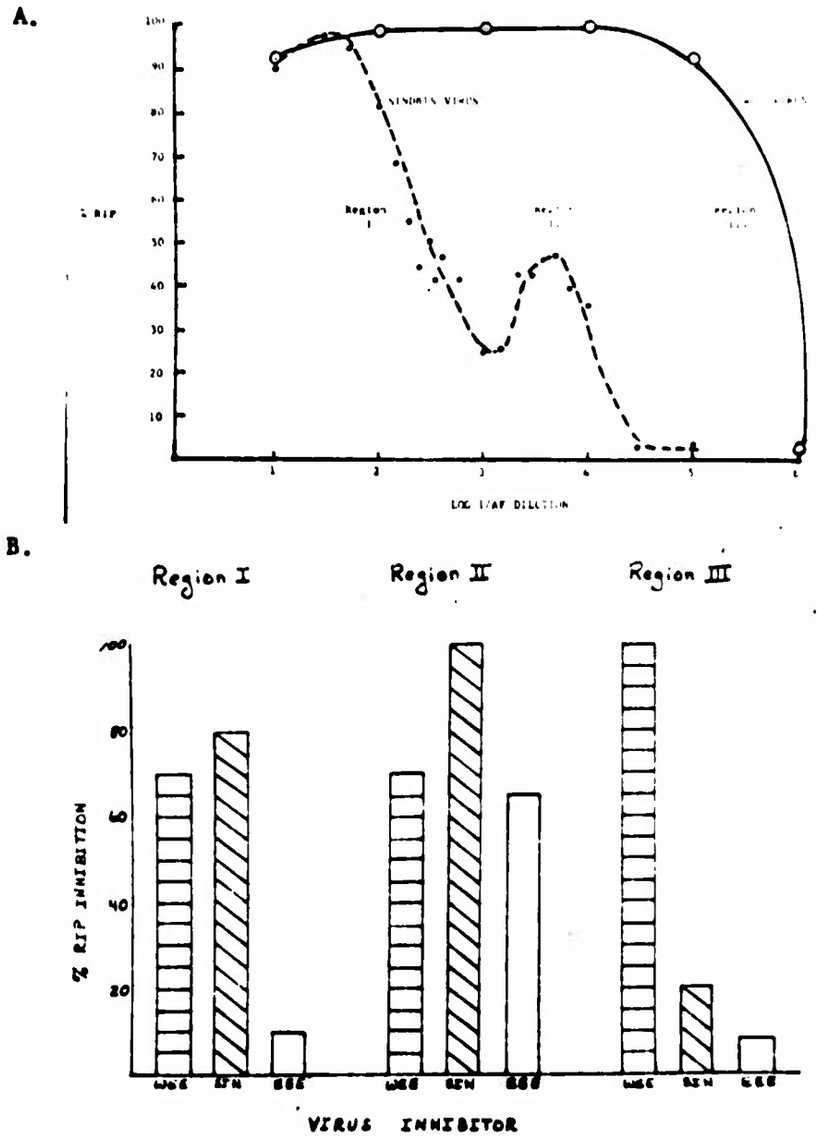


Figure 61. A. Three regions of antibody reactivity in WEE hyperimmune mouse ascitic fluid.

B. Radioimmune precipitation (RIP) inhibition of the three regions of antibody reactivity. Inhibition reactions employed the addition of unlabelled virion suspensions to the appropriate dilutions yielding 50% RIP in each of the three regions. Maximum inhibition resulting from the addition of from 10^7 to 10^{10} PFU of the respective cold (unlabelled) viruses is listed as a percentage.

exhibited significant inhibition in Region I. Inhibition of the Sindbis cross reaction in both regions I and II was unexpected since these dilutions of Western immune ascitic fluid contained considerable specific WEE virus antibody excess.

Differential RIP of Virus "Core" Particles and "Coat" Protein. Virus "cores" and "coat" (surface) protein were produced by treatment of purified virion suspensions with the nonionic detergent NP-40 and each component isolated in relatively pure form by rate zonal centrifugation in sucrose gradients (Figure 62A). The peak labeled "A" near the bottom of the gradient is probably residual undegraded virion since it contained most of the infectivity; further, polyacrylamide gel electrophoresis of this fraction revealed two polypeptides (Figure 62B) typical of the intact Sindbis virion composition described by Strauss, et al (1968). The sucrose gradient peak labeled "B" in the center of the gradient is probably the "core" since it is a sedimentable structure containing nucleic acid as measured by incorporation of radioactive uridine (not shown); acrylamide gel analysis of this peak revealed only a single polypeptide (Figure 62C) which migrated the same distance as the faster and smaller of the two virion proteins shown in Figure 62B. Thus, the smaller protein is considered to be the "core" protein. Sucrose peak "B" containing this protein was used directly for RIP tests. Radioactivity at the top of the sucrose gradient labeled "C" in Figure62A consisted of only one polypeptide by acrylamide analysis (Figure 62 D); it migrated the same distance as the slower and larger of the two virion proteins shown in Figure 62B. Thus, the larger protein is the single polypeptide constituting the "coat" or surface protein which was isolated at the top of sucrose gradients for RIP tests. All three viruses under study yielded essentially the same pattern of detergent degradation as described here for Sindbis virus.

Radioimmune precipitation of sucrose purified "cores" was attempted by using the same procedures as described for virion RIP. Virus cores appeared broadly cross reactive (Figure 63). Some specificity in core precipitation was observed with both Sindbis and Western immune ascitic fluids in that a higher percentage of the homologous cores precipitated at a slightly higher ascitic fluid dilution; however, heterologous cores were broadly cross reactive. Eastern immune ascitic fluid which previously appeared specific for EEE virion equally precipitated the cores of all three viruses.

The soluble coat protein which failed to enter the sucrose gradient following NP-40 treatment could also be precipitated with homologous antiserum (Figure 64). A maximum of 80% precipitation was achieved which did not change markedly upon dilution of the antigen. Endpoint titers of the ascitic fluid or 50% RIP titers were greatly reduced when compared to either core or virion RIP with these same antisera. Differences in RIP titer were evident when different antigen concentrations were compared, analogous to the antigen concentration effect described with virions.

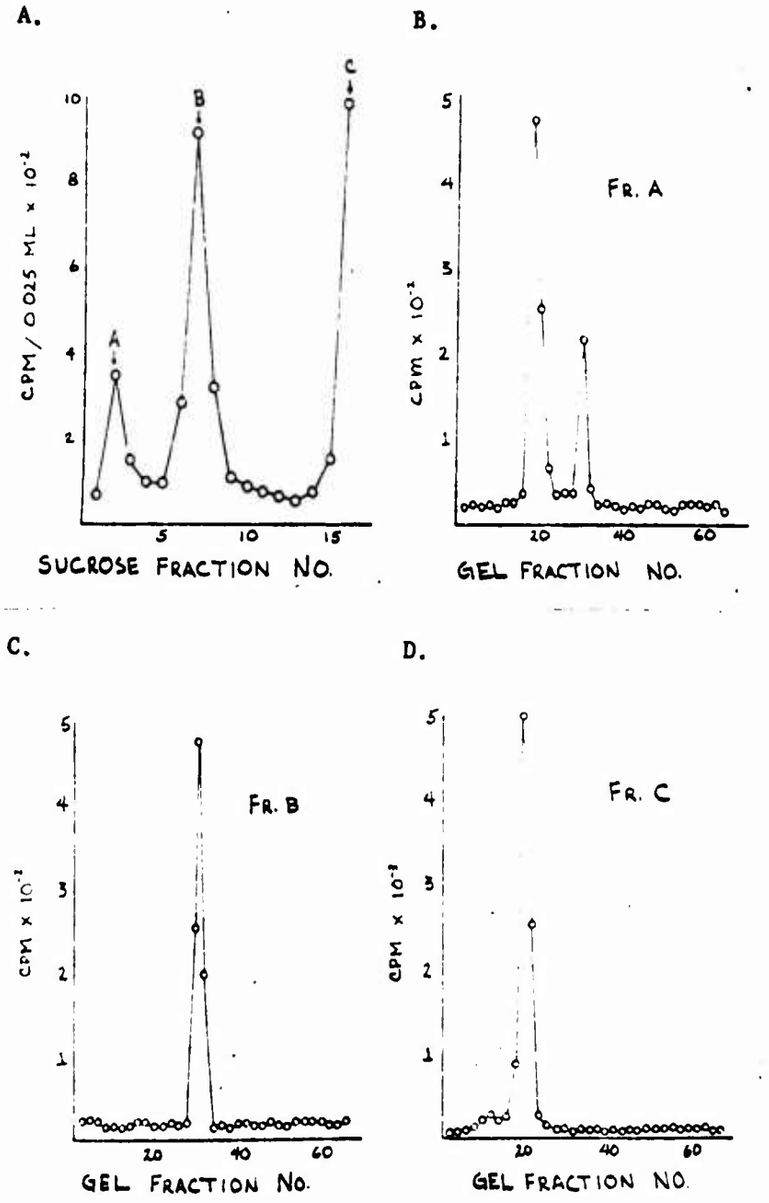


Figure 62. A. Rate zonal centrifugation of 1% NP-40 treated Sindbis virions through a 15-30% (w/v) sucrose gradient.

B. Polyacrylamide gel electrophoresis of Sindbis virion (fraction A in panel A).

C. Polyacrylamide gel electrophoresis of Sindbis core (fraction B in panel A).

D. Polyacrylamide gel electrophoresis of Sindbis soluble top component (fraction C in panel A).

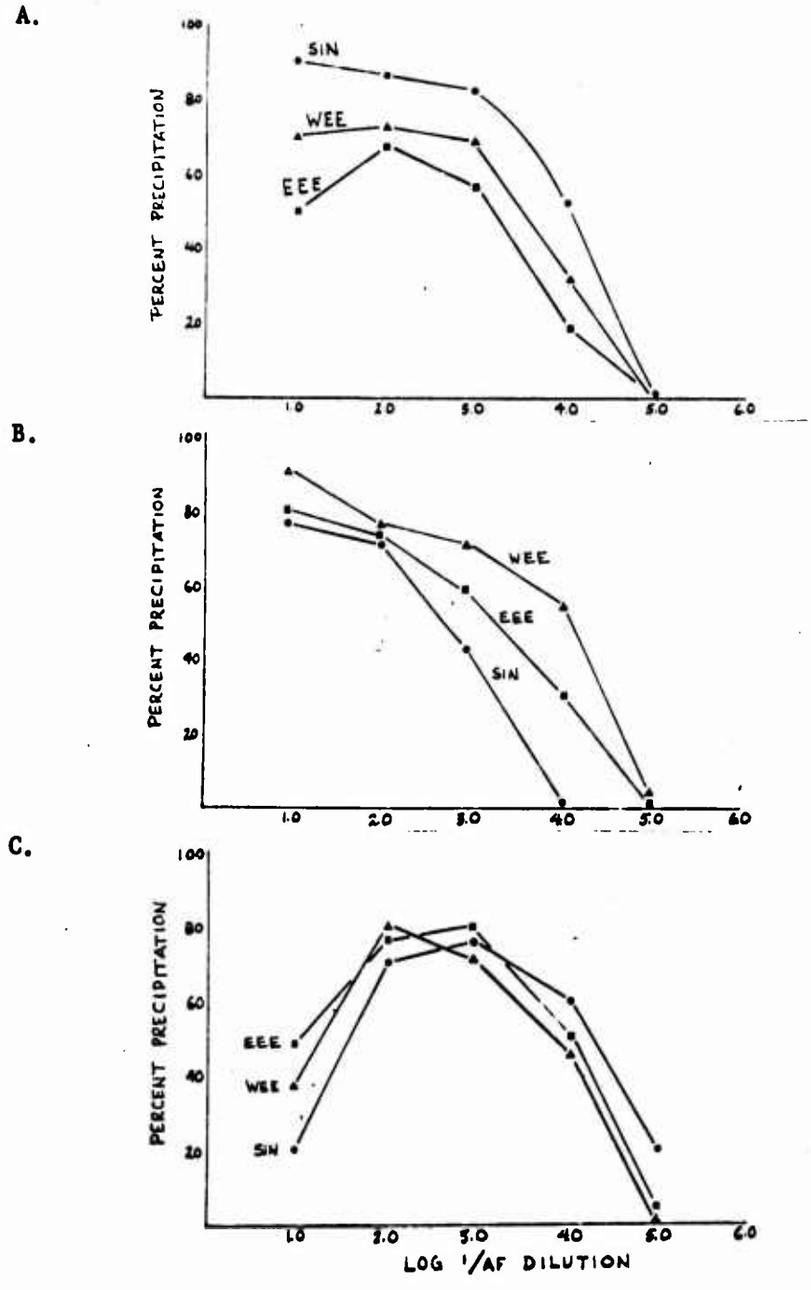


Figure 63. Radioimmune precipitation of virus "cores" with A. Sindbis, B. Western equine encephalitis (WEE), and C. Eastern equine encephalitis (EEE) hyperimmune mouse ascitic fluids.

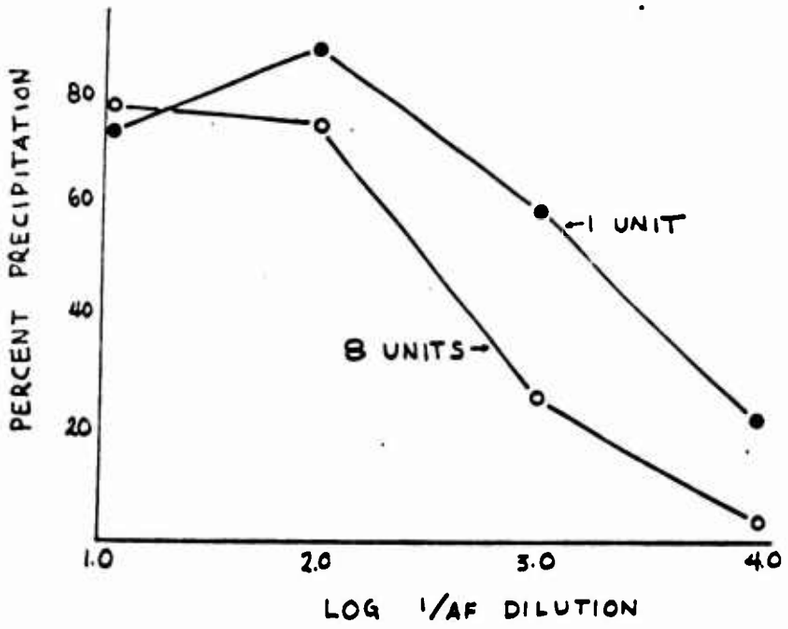


Figure 64. Effect of antigen concentration on radioimmune precipitation of Sindbis soluble coat protein.

The specificity of the coat protein was investigated by comparing homologous and cross-reactions using RIP of preparations from all three viruses (Figure 65). Although lower titers were observed, the cross-reactions resembled those described by [illegible] reactions using the complete virion. Antisera to Sindbis and WEE viruses recognized some common antigenic property of the coat protein of these viruses while EEE virus antiserum appeared specific.

Discussion.

The application of radio-immune precipitation (RIP) techniques to the study of certain group A arboviruses has revealed the role of certain structural antigens in cross-reactions exhibited by members of this group. Further, utilization of intrinsically labeled, purified virion suspensions enabled the determination of antibody titers with greater sensitivity than classical serological procedures.

Heterologous RIP reactions using virion antigens at equivalent concentrations based on infectivity demonstrated an antigenic relationship of Sindbis and WEE viruses while EEE virus remained antigenically distinct and antiserum to EEE virus appeared specific. The nature of the two-way cross reaction exhibited by Sindbis and WEE viruses depended to a great extent on the antigen concentrations employed in the RIP reaction. Concentrations could be selected which resulted in a biphasic cross-reaction curve.

RIP reactions of a single WEE virus antiserum allowed the resolution of three distinct antibody populations; Regions I and II (low dilutions of antiserum) representing the biphasic cross-reaction of this antiserum with Sindbis virion and Region III (high antiserum dilution) describing the homologous WEE virus reaction. Inhibition of the RIP reaction in each of these regions demonstrated a region of WEE-Sindbis antigenic similarity in Region I, a broadly cross-reactive antibody inhibited by all three viruses in Region II and a virus type specificity association with the homologous RIP reaction in Region III. That these three antibody populations were found in this single antiserum at different titers allowing their resolution was indeed fortunate; however, the three reactivities described (I) closely related virus complex, II group, and III virus type would be expected to exist in antisera prepared against these agents.

Disruption of virions with the nonionic detergent NP-40 followed by rate zonal sucrose gradient centrifugation revealed a core particle containing only the core protein and a soluble fraction containing only coat protein. RIP of the core particles differed markedly from that of complete virion in that broad cross reactions between all three viruses were observed. Little or no specificity was found associated with virus core particles presenting an analogy to the soluble group reactive antigen described for influenza viruses.

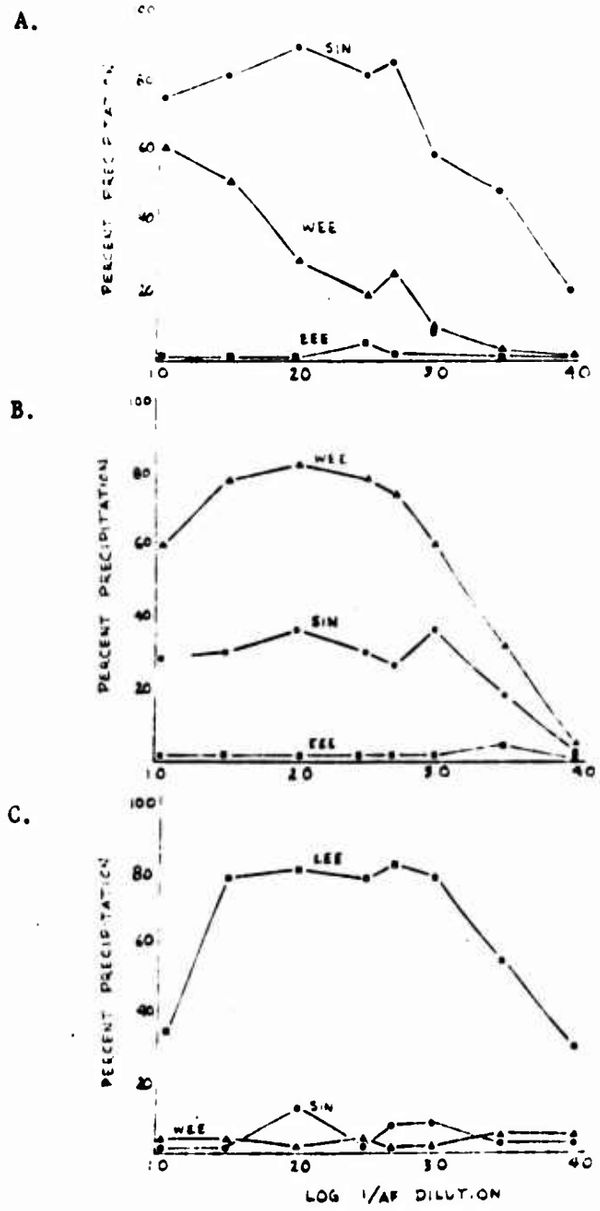


Figure 65. Radioimmune precipitation of soluble coat protein with A. Sindbis, B. Western equine encephalitis (WEE), and C. Eastern equine encephalitis (EEE) hyperimmune mouse ascitic fluids.

The broadly cross reactive nature of core particles and the RIP titers obtained suggested that these antigens contributed to the group reactive antibody population described in Region II. Our inability to successfully inhibit the precipitation of core using preparations of intact virions in either homologous or heterologous RIP reactions would indicate that this was not the case. The interpretation of these data implies that core antigens are not exposed in virion structure and do not participate in RIP reactions using intact virion. Conversely, antibodies resulting from free core in the mouse brain immunizing antigens must represent a separate antibody population lacking virus type specificity. The participation of this antibody population and virus core antigens in classical serological tests such as complement fixation could possibly help to explain the wide serological overlap demonstrable by these methods.

The observation that detergent solubilized coat protein could be precipitated by RIP procedures indicated that structured coat protein such as found on virion structure was not essential for antigenicity. Some structure, however, must be essential since virion degraded with sodium lauryl sulfate and B-2-mercaptoethanol preparatory to acrylamide analysis failed to precipitate. The specificity of the coat protein resembled that of virion with the exception of the relatively low dilutions of antisera required to effect precipitation. This observation could be interpreted as the effect of increased antigenic mass on the RIP reaction resulting from the degradation of virion structure since decreasing antigen concentration did result in higher RIP titers. If all of the antigenic properties described for virions can be attributed to specific antigens contained in the coat protein, further examination of the sequential degradation productions of this protein should allow the detection and separation of these specific antigens.

B. Immunogenicity of a Group A Arbovirus Vaccine in Man.

This report is concerned with the development, production and evaluation of either live-attenuated or formalin-inactivated vaccines against exotic viral agents, suitable for use in man. Evaluation studies on the formalin-killed, freeze-dried Chikungunya (CHIK) vaccine described in Annual Report 1970 are continuing. In order to determine the persistence of neutralizing antibody, the original group of vaccinees who received two 0.5 ml doses of CHIK vaccine, Lot E-20, 28 days apart, were bled at invervals of 56, 90, 180, 270, and 360 days after vaccination. Neutralization indices for these subjects are shown in Table 38.

Table 38.

Serum Neutralization Indices Observed in Chikungunya Vaccinees over a One-year Period

Subject	Log Neutralization Index on Day Post-Vaccination 56	90	180	270	360
BLW	2.0	2.0	1.7	1.7	1.7
CJM	2.7	2.7	2.4	2.4	2.0
CJW	3.0	2.7	2.0	2.0	2.4
CDD	2.7	2.4	2.7	2.7	2.4
DJ	1.4	1.7	N.D.	1.7	0.7
DRR	3.4	3.4	3.0	2.7	2.4
GRC	2.4	2.0	2.4	2.4	2.4
HSL	2.4	2.4	2.4	2.0	2.7
KCL	2.4	2.4	1.7	1.7	N.D.
LJF	2.4	2.4	2.0	2.4	2.4
LDR	2.0	2.4	1.4	1.4	1.4
MJR	3.0	4.0	3.6	3.3	3.0
MDM	3.6	3.3	3.3	3.6	3.3
MJL	3.3	3.6	3.0	3.0	3.0
RRB	3.0	3.0	2.6	2.6	2.3
SWF	3.3	3.6	N.D.	N.D.	N.D.

N.D. - Not done.

Five individuals from this group were given a 0.5 ml booster dose of CHIK vaccine after the 360 day bleeding. Neutralization indices were obtained for these subjects 14 days after receiving the booster dose. The results are shown in Table

Table 39.

Serologic Response of Chikungunya Vaccinees Receiving A Booster Dose One Year After The Primary Series

Subject	Pre-booster CF	HI	LNI	14 Days Post-booster CF	HI	LNI
CJM	<4	<10	2.0	4	80	>3.5
GRC	<4	<10	2.4	<4	40	3.7
MJR	<4	20	3.0	<4	40	>3.7
MJL	<4	<10	3.0	8	160	>3.5
MDM	<4	<10	3.3	4	80	>3.5

In parallel with the human studies the long-term efficacy of this vaccine is being evaluated by a live challenge assay in rhesus monkeys over a 24 month period. As reported in Annual Report 1970, vaccinated monkeys were solidly protected against viremia when challenged six months after vaccination. Results of a five challenge with

the CHIK virus on a second group 12 months after vaccination are shown in Table 40.

Table 40.

Long-term Protective Efficacy of CHIK Vaccine, Lot E-20 Observed in Rhesus Monkeys Challenged* 12 Months After Vaccination

MK #	Vaccine Status	1	2	3	4	5	6	7
105	yes	0	0	0	0	0	0	0
107	yes	0	0	0	0	0	0	0
120	yes	0	0	0	0	0	0	0
110	no	0.8**	1.5	4.6	3.4	0	0	0
112	no	0	3.5	4.1	2.3	0	0	0
123	no	0.8	2.3	3.8	3.5	0.5	0	0

* Challenge dose - 60,000 suckling mouse $ICLD_{50}$s.
** Log_{10} titer of circulating virus.

Results of the foregoing evaluation tests in man and lower primates clearly indicate that a formalin-killed, freeze-dried CHIK vaccine prepared in green monkey kidney tissue culture, confers protection for at least a year after a primary series. Results of the 18 and 24 month post-vaccination challenge of rhesus monkeys will be reported later.

Stability Studies on Tween-ether Extracted (TE) and Formalin-killed (HCHO) Chikungunya Vaccines:

Potency assays were performed using young adult mice on both, fluid and freeze-dried vaccines of the TE and HCHO types, at 3 and 12 month intervals. The freeze-dried vaccines were held at -20°C and the fluid vaccines at 4°C during the storage period.

It was of interest to note that the hemagglutinin content of the TE vaccines (both fluid and dried) remained remarkably stable over the observation period. Stability data for these vaccines are shown in Table 41.

Table 41.

Potency* of TE and HCHO CHIK Vaccines in the Fluid and Freeze-dried State over a 12-month Observation Period

Vaccine Type	Storage Temp °C.	ED_{50}/0.5ml Vaccine 1 mo.	3 mo.	12 mo.
HCHO-Fluid	4	0.03	0.04	0.03
HCHO-Dry	-20	0.05	0.10	0.02
TE - Fluid	4	0.17	0.04	0.17
TE - Dry	-20	0.13	0.05	0.09

* Young adult mice received 0.25 ml vaccine on day 0 and 7. Challenged on day 14 with 100 - 500 adult mouse $ICLD_{50}$/0.03, CHIK 168 virus.

The storage stability of these vaccines will again be monitored at 18 and 24 month intervals.

C. Production and Characterization of Arbovirus Reagents

1. Preparation of CHIK and Western Equine Encephalomyelitis (WEE) HA and CF Antigens by TE Extraction.

CHIK and WEE viruses were propagated in roller bottles containing baby hamster kidney, cell line 21 (BHK-21) cells until cytopathic effects were 90 - 100% complete. The supernatant fluids were harvested, centrifuged in the cold and concentrated 20X in an Amicon cell. The resultant concentrate was TE extracted, dispensed in screw cap vials and stored at 4°C. This material constituted the HA and CF antigens. Even though HA and CF titers of the concentrated fluids were high, TE extraction boosted titers 4 - 8X and also inactivated live virus. Titers for the HA and CF antigens for CHIK and WEE are shown in Table 42.

Table 42.

Titers of CHIK and WEE Antigens Prepared in BHK-21 Roller Flasks and TE Extracted

Antigen	1/HA	1/CF
CHIK, 15561	16,000	64
WEE, B-11	65,000	128

It was observed that TE antigens reacted more specifically with homologous antisera than did the unextracted antigens. Also, the same sera which reacted with normal (uninfected) mouse brain antigen in the CF tests, did not give these reactions when TE-extracted antigens were used. The TE-extracted antigens are stable at 4°C and have shown no loss in HA or CF activity over a one year period.

2. Preparation of Dengue-2 (D-2) and Japanese encephalitis (JE) Virus Soluble Complement Fixing (SCF) Antigens.

Soluble CF antigens have been routinely prepared for D-2 and members of the JE virus complex: Murray Valley encephalitis (MVE), West Nile encephalitis (WNE), St. Louis encephalitis (SLE), and Ilheus viruses. Infected suckling mouse brain homogenates are clarified with protamine sulfate, precipitated with ammonium sulfate and finally purified on Sephadex G-100 columns by gel filtration as described in previous annual reports. The SCF peak fractions are pooled and concentrated and used for immunodiffusion, CF box titrations, SLS/2-ME stability tests and protein analysis on polyacrylamide gel electrophoresis.

The molecular weights of the JE subgroup fall in the range of 45-55 X 10^3 daltons. The Dengue SCF antigen has a lower molecular weight in the range of 37-42 X 10^3. The Sephadex columns are calibrated using five marker proteins and reproducible calculations for mw are obtained using a graphic technique. Thus, there are reproducible differences in the physical properties of complement fixing antigens from these two serologically related group B arboviruses.

Soluble antigens have also been prepared in BHK-21 roller cultures using D-2 and JE viruses. The supernatant culture fluids are harvested after complete CPE. The fluids are then concentrated 100X by Amicon filtration and chromatographed on Sephadex G-100. The D-2 BHK-derived SCF has a mw of approximately 42 X 10^3 and the JE SCF prepared in this manner has a mw of approximately 60 x 10^3. The JE-BHK derived antigen has been shown to have at least a partial identity with JE suckling mouse brain SCF by immuno-diffusion in agarose.

To label the SCF antigen of JE, a mixture of tritiated amino acids was added to the medium of several JE virus infected BHK-21 roller cultures. The same procedure was followed as for the isolation of nonlabeled SCF antigen. A small radioactive peak corresponding to SCF activity was pooled and rechromatographed through Sephadex G-100 to further purify. Finally, a radio-immune precipitation (RIP) technique was used to precipitate labeled SCF antigen. After several attempts, a 2-4% precipitation was obtained. If a higher percentage of RIP is obtained, a co-run of the SCF will be done on polyacrylamide gel electrophoresis and comparison of the SCF protein to known JE virus and virus-infected cellular proteins will be made.

Attempts have been made to release soluble antigens from cell membranes using detergent treatment and digestion of smooth and rough membranes as well as nuclear membrane. Separation of nuclei and nuclear membranes from other cytoplasmic membranes can be done by Dounce homogenization in a sucrose-reticulocyte standard buffer. The CF activity of the nuclei and membrane tags are high and can be increased by TE treatment. After this digestion, precipitin lines are formed against homologous serum in immunodiffusion tests, and two peaks of CF activity are found after gel filtration of the digest. One peak has a mw of 70×10^3 and the other has a mw of 43×10^3. The soluble antigens formed this way are very unstable. Other digestion treatments are being tested.

3. Preparation of Hyperimmune Mouse Ascitic Fluids (HMAF) Against D-2 and JE virus SCF Antigens.

Purified SCF antigens for D-2, D-4, and JE viruses have been used to prepare HMAF. The antigens are first inactivated by either UV light or Betaprone treatment and given to mice in a four dose regimen; sarcoma cells are given with the fourth dose and ascitic fluids harvested approximately two weeks later as described in previous annual reports. Antisera made this way for D-2 and JE-SCF has resulted in high CF reactivity and non-neutralizing activity. Since the SCF antigen is thought to be a nonvirion antigen, it should not have neutralizing activity. Dengue-4 failed to produce CF titers in mice when the SCF antigen was administered.

Japanese encephalitis virus after Sephadex chromatography of infected mouse brain preparations, usually showed two peaks of CF activity after the void CF. These peaks, having mw's of approximately 70×10^3 and 50×10^3, were pooled separately and given to mice for production of HMAF. Dengue-4 also produced considerable tailing of CF activity before the major SCF peak when chromatographed. The high mw antigens were labeled "early coat," the "late coat" antigens eluted right before the SCF, and the SCF antigen was the last to elute. These were all given to mice for production of HMAF. Also, (WINE) virus has demonstrated two SCF peaks although HMAF has not yet been made for these antigens.

Indirect fluorescent antibody techniques using D-2 anti-SCF HMAF have demonstrated an early production of this antigen in an infected cell around the nuclear membrane (perinuclear fluorescense).

Post-fixation of JE virus infected cover slips with acetone or methanol has shown the JE SCF antigen to be acetone stable but methanol labile. This was also true for the JE coat antigen (higher mw). The D-2 SCF antigen is stable to both acetone and methanol.

Crude JE HMAF when reacted in this system with JE virus infected cells shows that the antigen it reacts with is methancl and acetone stable (see FA studies in Section VI C, above).

Other SCF antigen specific HMAF is being prepared to identify antigen synthesizing sites in the cell.

4. Specificity of SCF Antigens.

Illheus, JE, MVE, WNE, and SLE viruses were used to prepare mouse brain-SCF antigens. The antigens were used in cross CF box titrations and also in cross immunodiffusion tests to study antigenic specificity. The SCF antigens were compared to crude protamine sulfate clarified viral antigens in both systems.

The CF reactivity of the SCF antigens was more specific than the crude antigen reactions when these antigens were tested against homologous and heterologous crude HMAF. Post-zones were nonexistent in the SCF-HMAF boxes but usually were present when crude antigens were used.

Crude antigens when reacted against homologous and heterologous HMAF developed no precipitin lines in double diffusion in agarose. Under the same conditions, SCF antigens produced distinct single or double precipitin lines. Type specific antigens were formed for JE, MVE, and WNE viruses. On the same plate containing these antigens a second precipitin line formed that appeared to be group specific. For SLE and Ilheus viruses, a group specific line was the only one formed. The type and group precipitin lines were very close together but did not seem to spur. This suggests that they are separate and distinct antigenic determinants rather than "linked" as shown with the dengue subgroup.

A D-2 SCF preparation, showing similar double precipitin lines and known to have a high HA tier, was treated with sodium lauryl sulfate and 2-mercaptoethanol which should destroy the HA antigen. This did not remove either precipitin line, neither did heating at 60C for 30 minutes or adsorption with goose erythocytes. Whether this represents aggregates or separate detergent resistant antigens has yet to be determined.

Ouchterlony double diffusion plates are stained with Adler's stain and the resolution and intensity of the precipitin lines are enhanced. The dried agarose plate may then be stored for later reference.

5. Preparation of JE Virus HA Antigen. Virus is inoculated and adsorbed in BHK-21 roller cultures then thoroughly washed to remove any traces of residual seed virus. A 50 ml quantity of maintenance medium containing 0.25% human serum albumin and buffered to pH 8.0 is added to each flask. The infected roller cultures are harvested before any evidence of CPE at approximately 24-30 hours post infection. The supernatant culture fluids are passed through a 0.45μ Nalgene filter unit to clarify and precipitated in the cold with ammonium sulfate (60%). After centrifugation, the precipitate is resuspended to 1/100 of its original volume in TRIS buffer and purified by sucrose density gradient centrifugation.

A high degree of HA recovery approaching 100% has resulted when the supernatant culture fluids are ammonium sulfate precipitated. Sucrose gradient centrifugation revealed an RHA and SHA peak. Although these peaks are low in titer, the method could be utilized to make more virus with better yields. Since the virus is harvested before cell damage occurs, the resultant product is free from gross contamination.

VIII. Ecological Studies of Group A Arboviruses.

A. Persistence of serum Neutralizing Antibody to Wee Virus in Naturally Infected Quail.

Sentinel quail have been used since 1968 to monitor the seasonal transmission of EEE and WEE viruses in a swamp habitat on the eastern shore of Maryland. Each year since 1968 both viruses have been recovered from mosquitoes and birds. However, mosquito infection rates, antibody prevalence in wild birds and sero-conversion of sentinel quail show that the virus (EEE or WEE) predominating one year would be supplanted the following year by the other virus. Birds returning to the swamp each year have shown higher antibody prevalence rates to the virus which predominated the previous summer. Presumably, infections acquired during the previous summer resulted in a level of herd immunity to the homologous virus sufficient to retard transmission the next year. An important factor in this situation is the persistence of neutralizing antibody in birds following natural infection. To examine this aspect a number of uninfected, juvenile quail were exposed from 1 to 14 July 1969 in the swamp, removed to a mosquito-proof environment, and bled at selected intervals over a period of one year and the serum tested for N antibody.

Serum neutralizing antibody was assayed by a plaque reduction test in primary chick embryo cells. Plaque dose was approximately 100 pfu of the 46th mouse brain passage of MacMillan strain of WEE virus. Sera were heated at 56°C/30' and assayed at 1:20, 1:40, 1:160, 1:640, and 1:1280 dilutions. Fifty per cent endpoints were estimated by plotting per cent reductions on probit paper.

Antibody was not detected in pre-exposure sera. From 2 to 53 weeks post-exposure, all sera had neutralizing antibody. Highest titers were observed in 9 of 11 quail at 2 to 3 weeks after exposure. At one year after exposure titers varied from 1:20 to 1:200 (Table 43).

These data confirm the persistence of appreciable levels of WEE virus neutralizing antibody throughout a one-year period. Presumably these quail would remain refractory to mosquito challenge with WEE virus at one year or more post-infection. They remain at risk to EEE infection as the following observations indicate. During 1969, one sentinel quail was naturally infected in the swamp with EEE virus early in the summer, as shown by serological conversion. This quail was re-exposed as a sentinel and was infected during August by EEE virus, demonstrated by virus isolation and serological conversion.

Arbovirus Ecology - Pocomoke Cypress Swamp

During 1970, a program of arbovirus surveillance was conducted using the sentinel quail monitoring methods established in 1969. Of particular interest was the question concerning the alternating annual dominance of WEE and EEE viruses in the swamp habitat. Based on the high levels of WEE virus transmission observed in 1969, the 1970 study was predicted to demonstrate elevated EEE virus levels.

Figure 66 shows the percentage of the total quail exposed (70) that were infected with EEE virus during 1970. Although tests for WEE virus antibody in these specimens has not yet been completed, the high percentages of EEE virus activity observed suggests that EEE was hyperenzootic in 1970. Sentinel conversion rates for antibody to EEE virus were much greater during 1970 than had been observed during 1969. Most of the virus activity occurred during late September (80% positive specimens) which is the same time period as the low level virus transmission observed during 1969; however, this activity is in marked contrast to the WEE virus activity peak observed in early summer during 1969.

A comparison of the data obtained from quail exposed in or near the swamp (40) with those exposed at 1-3 mile distances from the nearest swamp habitat is shown in Figure 67. EEE virus transmission was detected at peripheral study areas. A small peak of activity was detected during late July; however, the major virus activity was observed during late September. Entomological examination of collections made in thes peripheral areas is in progress.

Table 43. PERSISTENCE OF SERUM NEUTRALIZING ANTIBODY TO WEE VIRUS IN NATURALLY INFECTED QUAIL

Weeks Post-Exposure		Reciprocal of Serum Antibody[1]						
		0[2]	2	3	12	27	39	53
Quail Number	1	< 20	20[3]	190	300	400	135	200
	2	< 20	520	N T	170	88	45	80
	3	< 20	76	20	20	20	20	20
	4	< 20	560	120	180	98	54	140
	5	< 20	500	280	160	200	78	110
	6	< 20	500	N T	430	340	135	220
	7	< 20	720	640	220	200	150	215
	8	N T	68	120	420	N T	90	88
	9	< 20	380	120	75	70	70	N T
	10	< 20	200	120	100	50	30	78
	11	< 20	80	50	95	85	50	66

1 Measured in plaque reduction neutralization test using approximately 100 plaque forming units 0.2 ml of MacMillan strain of Wee virus.

2 Serum obtained before exposure in nature to arboviruses.

3 Titer estimated by probit analysis plotting per cent reduction or plaque numbers at serum dilutions 1:40, 1:160, and 1:640.

4 N T - Not tested.

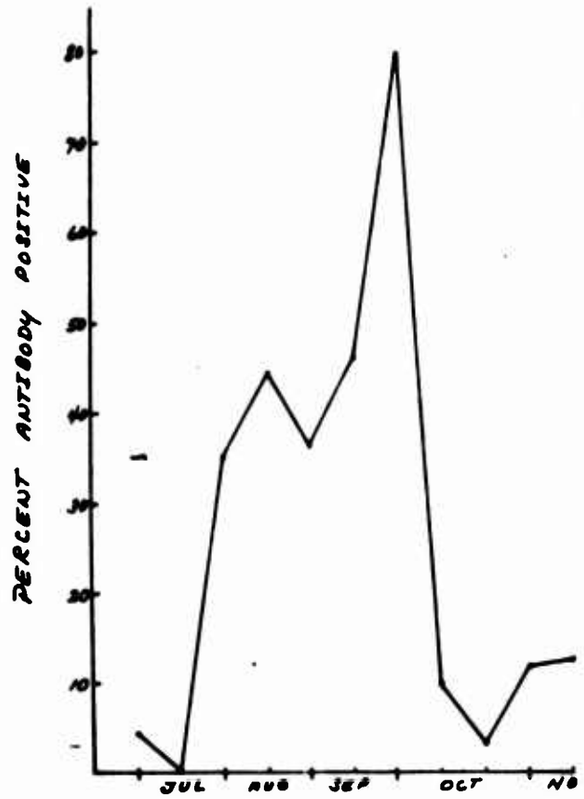


Figure 66. Percentage of exposed sentinal quail converting to eastern equine encephalitis antibody positive status during 1970. Ten quail in each of seven sites were exposed for two weeks and maintained in a mosquito proof environment for two subsequent weeks prior to testing.

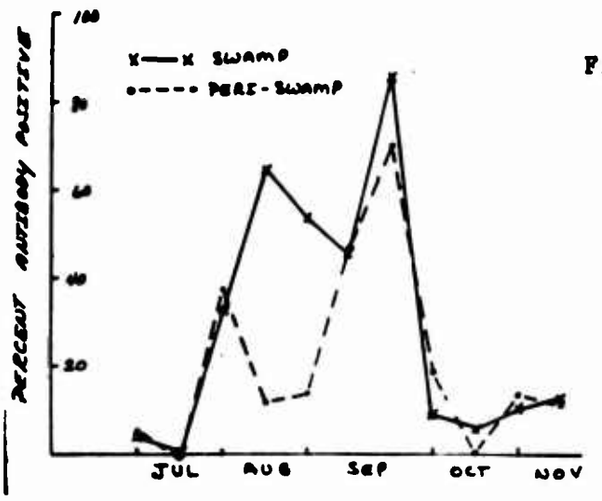


Figure 67. Eastern equine encephalitis virus activity as measured by antibody conversion in the swamp habitat (40 quail) compared to that in peripheral areas (30 quail) separated from swamp habitat by 1 to 3 miles.

Summary.

I. Simultaneous immunization of recruits in a basic combat training brigade with live adenovirus type 7 and type 4 (ADV-7, ADV-4) vaccines during an outbreak of acute respiratory disease (ARD) caused by both adenovirus types led to 95% suppression of ADV-7 associated ARD and 50% reduction in total ARD hospitalizations as compared with a brigade immunized with ADV-4 vaccine alone. A small excess of ADV-4 associated ARD hospitalizations which occurred in the study brigade may have been due in part to the relatively low potency of the ADV-4 vaccine used. The combined use of ADV-4 and ADV-7 vaccines proved to be a safe and effective means of controlling ARD in a military trainee population.

Detailed virologic, bacteriologic and serologic studies were undertaken among all ARD admissions from the study companies in the brigade immunized with both ADV-4 and 7 vaccines. Of 149 admissions, 131 were judged to be bona fide ARD. Three admissions occurred following shot reactions and 15 were the result of rubella virus infections. Of the 131 ARD admissions, 17 were complicated by pneumonia. Among the remaining 114 uncomplicated ARD admissions, 47 were associated with adenoviruses, 21 with nonadenovirus agents including Influenza A_2-7, rhinoviruses-10, group A streptococci-2 and Herpes virus hominis-2, and 11 admissions were associated with simultaneous evidence of infection by more than one agent. No etiologic agent was found for 35 admissions. Thus, in this group of immunized recruits, adenoviruses were still the most common cause of ARD hospitalizations, primarily ADV-4. Remaining ARD admissions were associated with a variety of other respiratory pathogens, multiple infections and disease of unknown cause.

II. ADV-4 and 7 vaccines were effective in interrupting epidemics of adenovirus associated ARD at Ft. Lewis and Ft. Leonard Wood in February, 1970. ARD rates at both posts decreased by 75% three to four weeks after immunization was begun. The impact of ADV-7 vaccine when given alone at Ft. Campbell in late February was less marked and fostered the emergence of ADV-4 associated ARD.

III. To determine whether living adenovirus Type 21 vaccine could be safely administered orally to susceptible young adults, an experiment was prepared in PROJECT WHITECOAT Volunteers at Ft. Detrick, Maryland, in September, 1970. Groups of susceptible volunteers were fed either $10^{6.4}$ tissue-culture infectious dose50 ($TCID_{50}$) of adenovirus Type 21 (ADV-21), vaccine virus or placebo tablets; all men were followed daily for 28 days after immunization for evidence of respiratory diseases, or other indisposition, and for evidence of infection by vaccine virus. Mild, afebrile respiratory and/or gastrointestinal illnesses were observed in three immunized and two control volunteers. In each instance, symptoms could not be related to either pharyngeal or stool excretion of ADV-21. Nine of 10 infected volunteers shed ADV-21 in

stools (mean duration 10.1 days; range 4-17 days). No pharyngeal excretion of ADV-21 was observed in any of these volunteers. Each of the nine developed specific N antibody to ADV-21. No evidence for person to person transmission of vaccine was obtained. The experiment establishes that ADV-21 vaccine strain, can be safely administered orally to susceptible volunteers, and that in doses of approximately $10^{6.0}$ $TCID_{50}$/man, approximately 90% of susceptibles can be infected.

Antibody activity of serum and secretory immunoglobulins was assayed using a C^{14} labeled ADV-21 antigen in a radio-immunodiffusion system. Increases in IgM, IgA and IgG activity were demonstrated at 28 days in sera from vaccinees but not from controls. Copro IgA antibody activity was also shown in vaccinees but not in controls. Nasal secretions showed no detectable IgA antibody responses by this method. These studies show marked differences in serum and local IgA antibody activity in induced enteric adenovirus infection compared to previously reported responses following natural infection. The protective role of secretory IgA in adenovirus infections is obscure. However, absence of nasal IgA responses may indicate that protection against disease with enteric ADV vaccines depends primarily upon humoral antibody.

IV. Preliminary experiments with column chromatographic procedures used in purification of Australian Antigen (HAA) indicate the presence of soluble antigen-antibody complexes in HAA positive sera. The hypothesis that HAA in serum as a firmly bound complex and that its antigenic makeup depends in part on bound antibody is being investigated.

The immunoelectro osmophoresis precipitation test is at present the only technically and logistically feasible procedure available for large scale screening for HAA. Comparative testing of commercially available test kits was carried out to aid in selection of equipment for use in military blood donor centers. It is apparent that the IEOP method has limited sensitivity due to the minimum antigen concentrations which will produce visible precipitin reactions.

Complement-fixation and agar gel diffusion tests were used to estimate the HAA carrier rate in military blood donors. A rate of 2.6/1000 was estimated from 1,112 samples tested with this detection method. Routine monthly testing of patients and employees in hemodialysis unit revealed that two patients (3.8%) and two employees (8.7%) had HAA.

Clinical and laboratory studies provided additional evidence that hepatitis may cause different pathologic manifestations in different people depending upon age, length of exposure and their immune response.

V. Strains of dengue-2 and dengue-3 viruses of diverse geographic origins including southeast Asia, the Caribbean region, Africa and

Tahiti were compared by plaque reduction neutralization tests using hyperimmune mouse ascitic fluids and human convalescent sera. The dengue-2 strains all appeared similar. The dengue-3 strains from the Caribbean and from Tahiti were similar to each other and differed significantly from the southeast Asian strains. A subtype of dengue-3 virus is defined.

Intratypic variation of the nonstructural soluble complement-fixing (SCF) antigens of dengue-1 strains (Hawaii and TH-Sman) and dengue-2 strains (new Guinea C and TH-36) were examined by immunological and biophysical techniques. The SCF antigen of Hawaii could be separated from that of TH-Sman by subtle differences in CF cross-reactions, but by distinct differences in their relative mobilities when tested by disc gel electrophoresis. SCF antigens of dengue-2 strains could not be distinguished by any of the methods used. It is suggested that the SCF antigen is responsible for the strain variation observed in the two dengue-1 strains.

VI. Polyacrylamide gel electrophoresis of Japanese encephalitis virus (JEV) grown in both LLC-MK_2 and chick embryo cell culture revealed three principal polypeptides with molecular weights of 8,700, 13,500, and 53,000 (V-1, V-2, and V-3, respectively). Infected chick cells that were treated with actinomycin D and cycloheximide contained seven polypeptides not present in uninfected cells. In addition to V-2 and V-3, polypeptides with molecular weights of 10,500, 19,000, 45,000, 71,000, and 93,000 (NV-1 through NV-5) were found; V-1 was not regularly detected. A similar pattern of polypeptides was obtained by radioimmune precipitation of soluble antigens from cytoplasmic extracts of infected, actinomycin-D treated, chick cells. When virions were treated with NP-40, a dense, RNA-rich structure was detected which contained V-2. An extracellular, slowly sedimenting, RNA-poor, hemagglutinating particle with a density comparable to the virion was present in virus preparations from cell culture and contained V-1, V-3, and NV-2.

Membranes from radioactive Japanese encephalitis virus infected chick cells were separated into light and dense fractions on sucrose step gradients, corresponding to predominantly smooth and rough membranes. Approximately 95% of radioactivity was associated with these membranes, and all of the JEV specified radioactive proteins were found attached to them. Approximately 5% of the radioactivity was soluble (not attached to membranes) and did not contain all of the specified proteins as tested by coelectrophoresis on polyacrylamide gels. The major protein associated with light membranes was the largest of the viral specified proteins which is not a glycoprotein. The data suggest that morphogenesis of JEV occurs exclusively on membranes.

Fluorescent antibody results with a dengue-2 antigen-antibody system were found to vary with the fixative employed. Hemagglutinating antigens (HA) were destroyed by methanol whereas the soluble complement

fixing antigen (SCF) was unaffected. In general, aldehyde fixation led to diminished fluorescence but the effect could be overcome by using cold lipid solvents. SCF was found to be strictly limited to the perinuclear region of the cell by using anti-SCF; HA was found to be distributed throughout the cytoplasm by using anti-virion hyperimmune mouse ascitic fluid. Perinuclear fluorescence produced by anti-SCF is the first to appear (10-12 hours post infection) in infected cells. The perinuclear region of dengue-2 or Japanese encephalitis virus infected cells was examined in the electron microscope. Large accumulations of virions were seen in the cisternae of smooth and rough endoplasmic reticulum and many cells contained widely dialated vesicles which contained electron dense particles resembling virus particles with aberrent shapes.

The RNA polymerases of JEV and dengue-2 were extracted and characterized. Both were found to be magnesium dependent and concentrated in the "mitochondrial fraction." Both polymerase reactions progressed with linear kenetics over short periods of time. The products generated were heterodispersed on sucrose gradients with major 26s and 22s peaks and some 4s material. The JEV 22s peak was partially double stranded. The 26s peak of JEV and all of the dengue product was single stranded.

The basic polypeptide composition of the virion was determined for the following arboviruses: Sindbis (SIN), eastern equine encephalitis (EEE), western equine encephalitis (WEE), chikungunya (CHIK), Japanese encephalitis (JE), St. Louis encephalitis (SLE), Dengue-2 (DEN-2), Yellow Fever (YF), Langat (LAN), Russian spring-summer encephalitis (RSSE), Bunyamwera (BUN), California encephalitis (CE), and Tahyna (TAH). Radio-labeled (^{14}C or ^{3}H) virions were degraded using SLS and 2 ME. Polyacrylamide gel electrophoresis and simultaneous co-runs with a single standard (SIN) allowed molecular weight comparisons. The results indicate that members of any major serogroup may be identified by the number and size of the polypeptides comprising the virion. The four group A viruses each have two polypeptides and little variation in the molecular weights of polypeptides was seen between viruses. BUN, CE, and TAH have three structural polypeptides; again, no significant differences were found between these three agents by these methods. Within the group, however, three subgroups were identified on the basis of some size differences of one of the polypeptides. The polypeptides of JE, SLE, and DEN-2 were similar in size but two strains of Yellow Fever virus (Asibi and French Neurotropic) clearly differed from the JE-SLE-DEN group by a significant (19%) difference in the size of the V-2 ("core") polypeptide. LAN and RSSE viruses had a distinctively larger (12%) V-1 polypeptide compared to the mosquito-borne group B viruses. The mechanism or biological expression of these structural differences is as yet undetermined, but the correlation of structure with biologic differences appears to be of fundamental importance.

VII. The application of radio-immune precipitation (RIP) techniques to the study of certain group A arboviruses has revealed the role of certain structural antigens in cross-reactions exhibited by group members. RIP reactions with Sindbis (SIN) and western (WEE) and eastern equine encephalitis (EEE) viruses suggested three separate antibody populations in antiserum prepared against WEE virus. Antibody populations defined by cross reactions and RIP inhibition consisted of a WEE specific, a WEE-SIN complex reacting and broadly cross reactive population-inhibited by all three viruses. Virion degradation products which were produced by detergent treatment remained antigenic and could be monitored by RIP. Envelope protein containing components reacted with essentially the same specificity as virion while virus cores appeared broadly cross reactive and lacked any virus type specificity.

Evaluation of a formalin-killed, freeze-dried chikungunya (CHIK) vaccine carried out in man and lower primates indicate that this vaccine, prepared in green monkey kidney cell culture, confers protection for at least a year after a primary immunization series. Stability studies on Tween-ether extracted and formalin-killed CHIK vaccines show that both are stable for at least one year and will be tested at 12 and 18 month intervals. Hemagglutinin titers of Tween-ether vaccines (both fluid and dried) remained remarkably stable over the observation period.

VIII. Laboratory studies of antibody longevity in naturally infected sentinel quail showed that maximum neutralizing antibody titers were observed two to three weeks post infection but antibody could be detected for greater than a year.

Arbovirus ecology studies in the Pocomoke Cypress Swamp during 1970 indicated considerable EEE virus transmission to sentinel quail during the late summer. Virus transmission did not appear to be restricted to the swamp habitat proper but was detected in sentinels placed one to three miles from the study area.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00 Communicable Diseases and Immunology

Work Unit 166, Viral Infections of Man

Literature Cited.

References:

1. Acheson and Tamm, Virology 41:321.
2. Aterman. Amer. J. Dis. Child. 105:395, 1963.
3. Baltimore. J. Mol. Biol. 18:421, 1966.
4. Bhamarpravati, et al. Arch. Path. 77:538, 1964.
5. Buescher, E. L. Med. Clin. N. Amer. 51 769, 1967.
6. Burrell, et al. J. Gen. Virol. 6 319, 1970.
7. Carey, et al. Lancet 1:105, 1971.
8. Cooper, et al. Virology 41:408, 1970.
9. Edmundston, et al. J.A.M.A. 195:453, 1966.
10. Erickson, et al. J. Mol. Biol. 10:519, 1964.
11. Grimley, et al. J. Virol. 2:1326, 1968.
12. Hammon, et al. Science, 131:1102, 1960.
13. Hammon and Sather. Am. J. Trop. Med. Hyg. 13:629, 1964.
14. Ibrahim, et al. Proc. Soc. Exper. Biol. Med. 128:80, 1968.
15. Ibrahim and Hammon. J. Immunol. 100:86, 1968.
16. Jacobson and Baltimore. J. Mol. Biol. 33:369, 1968.
17. Katz and Moss. Proc. Nat. Acad. Sci. 66:677, 1970.
18. Levin and Friedman. J. Virol. 7:504, 1971.
19. Lowry, et al. J. Biol. Chem. 193:265, 1951.
20. Martin and Sonnabend. J. Virol. 1:97, 1967.

21. Murphy, et al. Lab. Invest. 19:372, 1968.

22. Ota. Virology 25:372, 1967.

23. Peacock and Dingman. Biochemistry 7:668, 1968.

24. Peters and Ashcovai. Gastroenterology 58:284, 1970.

25. Prince and Burke. Science 169:593, 1970.

26. Richardson, et al. Nature 225:1109, 1970.

27. Robb and Martin. Virology 41:751, 1970.

28. Roberts, et al. Nature 224:1168, 1969.

29. Rosen. Jap. J. Med. Sci. Biol. 20:67, 1967.

30. Russell and Nisalak. J. Immunol. 99:291, 1967.

31. Sabin and Schlesinger. Science 101.640, 1945.

32. Scheele and Pfefferkorn. J. Virol. 3:369, 1969.

33. Segal, et al. Bact. Proc., p. 205, 1971.

34. Spiegelman, et al. J. Cell Physiology 70:35, 1967.

35. Sreevalsan and Yin. J. Virol. 3:599, 1969.

36. Stollar, et al. Virology 30:303, 1966.

37. Stollar, et al. Virology 33:650, 1967.

38. Stollar. Virology 39:426, 1969.

39. Strauss, et al. Proc. Nat. Acad. Sci. 59:533-537, 1968.

40. Strauss, et al. Virology 37:367, 1969.

41. Strauss and Kaesberg. Virology 42:437, 1970.

42. Trent, et al. J. Virol. 3:385, 1969.

43. Trent and Qureshi. J. Virol. 7:379, 1971.

44. Vander Veen, et al. J. Hyg. Camb. 66:499, 1968.

45. Westaway and Reedman. J. Virology 4:688, 1969.

Publications:

1. Bancroft, W., Warkel, R., Talbert, A., and Russell, P. K. A spectrum of liver pathology in family members with hepatitis associated antigen (HAA) (Abstract). Clinical Research 19:454, 1971.

2. Brandt, W. E., Cardiff, R. D., and Russell, P. K. Dengue virions and antigens in brain and serum of infected mice. J. Virol. 6:500-506, 1970.

3. Brandt, W. E., Chiew ilp, D., Harris, D. L., and Russell, P. K. Partial purification and characterization of a dengue virus soluble complement-fixing antigen. J. Immunol. 105:1565-1568, 1970.

4. Cardiff, R. D., Brandt, W. E., McCloud, T. G., Shapiro, D., and Russell, P. K. Immunological and biophysical separation of dengue-2 antigens. J. Virol. 7:15-23, 1971.

5. Cardiff, R. D., McCloud, T. G., Brandt, W. E., and Russell, P. K. Molecular size and charge relationships of the soluble complement-fixing antigens of dengue viruses. Virology 41:569-572, 1970.

6. Conrad, M., Young, A., Conrad, P., Park, R., Bancroft, W., and Bernier, G. Prophylactic use of gamma globulin to prevent endemic viral hepatitis (Abstract). Proc. Am. Soc. Clin. Invest. p.21a, 1971.

7. Dalrymple, J. M., Teramoto, A. Y., Cardiff, R. D., and Russell, P. K. Radioimmune precipitation of group A arboviruses (Abstract). Bacteriol. Proc., p.168, 1971.

8. Rose, H. M., Lamson, T. H., and Buescher, E. L. Adenoviral infection in military recruits: Emergence of type 7 and type 21 infections in recruits immunized with type 4 oral vaccine. Arch Environ. Health 21:356-361, 1970.

9. Russell, P. K., Chiewsilp, D., and Brandt, W. E. Immunoprecipitation analysis of soluble complement-fixing antigens of dengue viruses. J. Immunol. 105:838-845, 1970.

10. Scott, R. M., Dudding, B. A., Romano, S. V., and Russell, P. K. Immunological responses following live enteric adenovirus type 21 vaccine (Abstract). Bacteriol. Proc. p.194, 1971.

11. Shapiro, D., Brandt, W. E., Cardiff, R. D., and Russell, P. K. The proteins of Japanese encephalitis virus. Virology 44: 108-124, 1971.

12. Shapiro, D., Cardiff, R. D., Brandt, W. E., and Russell, P. K. Properties of dengue-2 and Japanese encephalitis virus slow sedimenting hemagglutinins (Abstract). Bacteriol. Proc., p.182, 1971.

13. Smith, T. J., Buescher, E. L., Top, F. H., Jr., Altemeier, W. A., and McCown, J. M. Experimental respiratory infection with type 4 adenovirus vaccine in volunteers: Clinical and immunological responses. J. Inf. Dis. 122:239-248, 1970.

14. Yuill, T. M., Brandt, W. E., and Buescher, E. L. Non-specific arbovirus hemagglutinin inhibitors in turtle sera. J. Immunol. 106:1413-1415, 1971.

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. ORG'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	3A061102B71Q	00	167
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11. TITLE (Procede with Security Classification Code)

(U) Rickettsial Diseases of Military Personnel (09)

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13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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FISCAL YEAR PRECEDING: 71	3	150
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19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence Not Considered

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(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. Prevention of scrub typhus in troops in Southeast Asia. 2. Evaluation of military importance of R. canada, a new member of the Typhus Group of Rickettsia.

24. (U) 1. (a) Formulation of a polyvalent scrub typhus vaccine to immunize against antigenically heterogeneous strains in nature. 1. (b) Development of a tissue culture neutralization test to assay immunity in experimental animals and man. 1. (c) Study of genetic stability of scrub typhus during transovarial transmission in a naturally infected colony of L. (L.) akamushi. 2. (a) Identification of etiologic agents causing Typhus and Spotted Fever Group infections in troops in South Vietnam. 2. (b) Investigation of enzootic rickettsial disease in the Fort Bragg area.

25. (U) 70 07-71 06. 1. (a) Major and minor antigen components of prototype strains are being identified by indirect and direct immunofluorescence and with complement fixation with yolk sac and tissue culture derived antigens. A method for plaque cloning of candidate vaccine strains to insure purity is being developed. The relative importance of humoral and cellular factors responsible for induction of scrub typhus immunity in mice is being characterized. 1. (b) A method for quantitative enumeration of scrub typhus rickettsiae in tissue culture has been developed. 1. (c) Changes in the antigenic composition of the mite colony strain was found when isolations from siblings of different generations were compared. 2. (a) Serologic cross-reactivity between Typhus and Spotted Fever Group antibodies have been characterized by indirect immunofluorescene. 2. (b) Serologic evidence of enzootic R. rickettsi but not R. canada has been found in wild animals at Fort Bragg. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

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Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 167, Rickettsial diseases of military personnel

Investigators.

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Description.

During the current reporting period, research activities have been concerned with: (1) the scrub typhus vaccine development program including: (a) antigenic analysis of the prototype Karp, Gilliam and Kato strains of Rickettsia tsutsugamushi and 6 other distinctive strains recovered in Thailand using complement fixation tests with antigens prepared from infected yolk sacs of embryonated hens' eggs and from infected tissue culture cells, as well as by indirect immunofluorescence with rabbit immune sera in order to characterize the antigens common to the different strains; (b) development of a plaque assay system for R. tsutsugamushi which could be used for cloning of candidate vaccine strains to insure purity; (c) characterization of the immune response after primary infection and after subsequent challenge with R. tsutsugamushi; (d) development of a tissue culture neutralization test for use in evaluation of the immunity resulting from infection and immunization with experimental vaccines; and (e) study of the genetic stability of R. tsutsugamushi in vector mites; (2) evaluation of the existing and potential military importance of R. canada by: (a) attempts to isolate and identify the agents causing rickettsial disease among military personnel and their dependents at Fort Bragg, North Carolina; and (b) investigations of the etiology of Typhus and Spotted Fever Group infections occurring in military personnel in South Vietnam; and (3) evaluation of the status of Q fever infection in dogs in Bangkok, Thailand.

Progress.

1. Scrub Typhus Vaccine Development

a. Antigenic Analysis of Prototype Strains

(1) Scrub Typhus Complement-Fixing Antigens from Infected Yolk Sacs. The production of strain-specific complement-fixing

antigens is a necessary prerequisite for determining the extent of antigenic diversity that exists among strains of Rickettsia tsutsugamushi in nature. Attempts to demonstrate the antigenic relationship of 78 strains of scrub typhus recovered in Thailand to the prototype Karp, Gilliam and Kato strains resulted in the recognition of 5 other distinctive strains, TA678, TA686, TA716, TA763 and TH1817. However, it still has not been possible to define the distribution of these candidate prototype strains among the Thai agents recovered from man, wild mammals and Leptotrombidium chiggers. Until this is accomplished, other antigenic types of R. tsutsugamushi that are suspected of being present in the collection cannot be identified.

A polyvalent killed vaccine that can be expected to provide a reasonable degree of protection against disease must contain antigens representative of all those present in the different antigenic types. Complement fixation tests with strain-specific antigens and serum produced by the intracerebral inoculation of guinea pigs identified the major antigenic component of the candidate vaccine strain. The success of the indirect immunofluorescent test for the diagnosis of human disease using a mixture of the Karp, Gilliam and Kato strains as antigens indicated that there was considerable duplicity in the antigenic composition of wild strains. Therefore, before a polyvalent vaccine can be formulated, the minor antigenic components of candidate vaccine strains must be identified so that the fewest number of strains can be used to provide the broadest degree of protection.

Karp, Gilliam and Kato strain-specific antigens have been prepared from yolk sacs of infected eggs with a reasonable amount of certainty by a method that employed adsorption with amberlite IRF-97, sedimentation through 20% sucrose, extraction with potassium acetate, and differential centrifugation. Continued passage of the 5 candidate prototype strains in embryonated eggs during the past year still has not produced yolk sacs that contain the numbers of rickettsiae required for antigen preparation. Repeated attempts to produce antigens with the most heavily infected yolk sacs that could be obtained were unsuccessful. In most instances, anti-complementary activity obscured specific reactivity of the preparations. At best, antigens that titered 1:10 with homologous serum were produced, but the volumes of these were inadequate to determine their reactivity with all of the sera from guinea pigs infected with different passage levels of the other prototype and candidate prototype strains. Serial passage of these strains in embryonated eggs will be continued with the hope that variants more suitable for cultivation in eggs will emerge. It is also planned to use newly developed technics of zonal centrifugation in an attempt to recover from lightly infected eggs the concentrations of rickettsiae required for antigen production.

In order to obtain information about the composition of the minor antigens in the 8 prototype and candidate prototype strains, attempts were made to produce broadly-reactive antigens. Review of previous reports of Japanese workers showed that their Karp, Gilliam and Kato complement-fixing antigens exhibited a considerable degree of cross-reactivity with heterologous immune sera. Several antigens processed by their method of preparation (1) failed to reproduce their results. In each instance, the antigens were anticomplementary and could not be used. Further efforts will not be made to produce broadly-reactive complement-fixing antigens from infected yolk sacs. The more promising results obtained with the production of antigens from infected cell cultures is described elsewhere in this report.

(2) Scrub Typhus Complement-Fixing Antigens from Cell Cultures. Recent improvements in techniques for the growth of cells on the relatively large surface areas of roller bottles led to attempts to prepare strain-specific, as well as broadly-reactive scrub typhus antigens, from heavily infected cells produced in vitro. Initially, monolayers of Vero cells, a continuous cell line derived from African Green monkey kidney tissue were used. These were grown at 37 C in roller bottles with 690 cm^2 of growth area, nurtured with 100 ml of medium consisting of Minimum Essential Medium (MEM) and 10% fetal bovine serum (FBS) and rotated at 0.5 rpm. The cells were infected by removing 75 ml of the growth medium and adding 0.5 - 1.0 ml of an infected yolk sac seed suspension containing 10^7 - 10^8 50% mouse infectious doses. After an absorption period of 1 to 2 hr on the roller apparatus, the inoculum was removed, and 100 ml of maintenance medium (MM) comprised of MEM and 2% FBS was added. The MM was changed every 3 to 4 days until a cytopathic effect involving 90 to 100% of the cells was present throughout the cell sheet. At that time, usually 18 to 21 days after infection, the MM containing detached cells and rickettsiae was poured off and saved. A 25 ml volume of 0.25% trypsin was added to each bottle and incubated for 30 min to remove the remaining attached cells. Both fluids were combined and merthiolate was added to a final concentration of 1:10,000. The suspension was stored at 4 C for 14 to 28 days prior to processing. Initially, the antigens were prepared by centrifugation of the suspension for 1 hr at 7,500 rpm at 4 C. The sediment was resuspended in 40 ml of veronal buffered saline (VBS). After another centrifugation, the sedimented rickettsiae and cellular debris were suspended in 10 ml of VBS containing 0.1% formaldehyde. Testing of these antigens revealed that most of them were markedly anticomplementary (AC). Rewashing of the organisms in a variety of diluents failed to reduce the AC reactivity without also reducing the antigen titer correspondingly. Some AC reactivity could be removed by layering 1 to 3 ml of antigen on 25 to 30 ml of 30% w/w sucrose solution and centrifuging the

mixture at 3,500 rpm for 30 min in a horizontal head at 4 C. The supernatant sucrose solution containing most of the rickettsiae was harvested from the sedimented cellular debris and centrifuged at 10,000 rpm for 20 min. After a wash in Shishido's sucrose PGy diluent (2) the rickettsiae were resuspended in VBS equivalent to one-tenth of the original volume of antigen. Although there was a considerable loss in volume of antigen, this procedure was necessary to remove the AC cellular debris.

Because most of the Vero-derived antigens were AC, two other cell lines were tried, viz, BS-C-1, another continuous line of African Green monkey kidney and 14pf, a fibroblast derived from areolar tissue of a normal rat. The procedure for preparing antigens from these cell lines was essentially the same as previously described.

Only small volumes of antigens have been prepared from these cell lines, but they have less AC activity than the Vero-derived antigens and with centrifugation through 20% w/w sucrose most of the AC activity could be removed.

Table 1 presents the complement-fixing titers of immune sera from guinea pigs infected with the 3 prototype Karp, Gilliam and Kato strains, and with the 5 candidate prototype strains, TA678, TA686, TA716, TA763 and TH1817 tested with 2 units of satisfactory tissue culture antigens that have been produced. Immune serum from a guinea pig infected with TC586 which was being evaluated as a substitute for the Gilliam strain in experimental vaccine was included also. Antigens produced in different cell lines by inoculation of the same seed suspension of TA686, TA763 and TH1817 exhibited different patterns of reactivity with heterologous immune sera. In contrast, Gilliam antigens fixed complement only in the presence of the homologous and TC586 immune sera, irrespective of the cell line used. Because of the limited experience with tissue culture antigens, the variation in reactivity cannot be explained definitively. These findings may be due to the duration of the cultivation period and method of processing the antigen and the effect these factors have upon the state of degeneration or breakdown of the major and minor antigenic components of the respective rickettsial strains. Use of the cell culture-derived antigens in studies employing immunofluorescence showed them to be unsatisfactory because of distorted morphology and the levels of reactivity were markedly lower than was found with antigen smears prepared from suspensions of infected yolk sacs. Until this question is resolved, it will not be possible to explain the differences between reactivity of antigens prepared with different passage levels of the same strain as was evident with the 17th and 39th yolk sac passage of TA686. Alternatively, the possibility that some of the candidate prototype strains are not pure but are comprised

TABLE 1

COMPLEMENT-FIXING ANTIBODY TITER OF SERA OF GUINEA PIGS INFECTED WITH STRAINS OF R. TSUTSUGAMUSHI WHEN TESTED WITH CERTAIN TISSUE CULTURE ANTIGENS

Tissue Culture Antigen (2 units)	Immune Guinea Pig Sera								
	TC586	TA678	TA686	TA716	TA763	TH1817	Karp	Gilliam	Kato
TA686-Vero #700902 A8:TC1:E17*	ND**	-+	160	-	40	80	-		-
TA686-14pf #710125 A8:TC1:E17	-	-	320	80	-	-	-	-	-
TA686-BS-C-1 #710312 A8:TC1:E39	-	20	160	160	320	160	160	320	1280
TA716-Vero #700911 A8:TC1:E35A	ND	-	-	2560	160	-	-	-	40
TA763-Vero #700904 A7:E54	ND	-	-	40	1280	80	-	-	160
TA763-14pf #710212 A7:E54	-	-	-	-	320	-	-	-	-
TH1817-Vero #700831 A4:E34C	ND	-	-	-	-	>2560	-	-	-
TH1817-14pf #710208 A4:E34C	-	40	-	80	160	320	-	80	40
TC586-14pf #710302 A6:E27B	640	-	-	-	-	-	-	>1280	-
Gilliam-BSC #710212 E136	>1280	-	-	-	-	-	-	>1280	-
Gilliam-14pf #710217 E138	>1280	-	-	-	-	-	-	>1280	-
Gilliam-Vero #701230 E138	>1280	-	-	-	-	-	-	>1280	-
Gilliam-14pf #710125 E138	320	-	-	-	-	-	-	2560	-
Karp-BSC-1 #701112 F50	-	20	40	40	320	80	160	80	80

* Cells infected with TA686 strain that had been passed 8 times in mice, once in tissue culture and 13 times in eggs

** Not done

† Complement was not fixed at an initial 1:10 dilution

of mixtures of two or more antigenic types cannot be excluded at this time. Immune sera from several animals infected with the same passage level as well as with material representative of earlier stages in the passage history of the candidate prototype strains have been produced to determine the effect of continued proprogation upon antigen composition. Efforts are continuing to produce larger volumes of cell culture antigens that are type-specific and others that are broadly reactive in order to complete the antigenic analysis of the strains of R. tsutsugamushi under study.

(3) Antigenic Interrelationship Among Prototype Scrub Typhus Strains. The requirement for defining the minor antigen components of candidate strains with respect to the production of a polyvalent scrub typhus vaccine has been discussed in previous sections of this report. Since attempts to produce broadly-reactive antigens from infected yolk sacs for the purpose of identifying antigens common to the different strains failed, the antigenic interrelationship among the strains was determined by indirect immunofluorescence.

Groups of 3 rabbits were infected with yolk sac suspensions of each of the 8 strains of R. tsutsugamushi. Two rabbits were infected with the Gilliam homotype TC586. Serum was collected from the rabbits 28 days after inoculation. Indirect fluorescent antibody tests were carried out with the serial 4-fold dilutions of the rabbit sera and antigens comprised of smears of suspensions of yolk sacs infected with the respective strains. A goat anti-rabbit fluorescein conjugate was used to detect the antirickettsial antibodies.

Table 2 summarizes the results obtained. Some variation in the response of rabbits receiving the same inoculum was apparent. Differences were found in the height of the antibodies reacting with the different antigens. Also, serum from one or two of the rabbits reacted with more heterologous antigens than the others. The values presented are the geometric means of the titers of each rabbit serum in the group, with the following exceptions. None of the rabbits infected with TA678 and TH1817 developed significant levels of homologous antibody. The results presented in Table 2 corresponding to these sera were obtained with specimens collected from rabbits 28 days after administering the last of a series of 3 inoculations, 2 to 4 weeks apart. The result for TA678 is the geometric mean of the titers of 2 rabbit sera and that for TH1817 was obtained in tests with only 1 serum.

Although the results clearly display the extent of the antigenic interrelationship among the prototype and candidate prototype strains, it is not possible to determine precisely which antigens are shared.

TABLE 2

ANTIGENIC ANALYSIS OF CANDIDATE PROTOTYPE SCRUB

TYPHUS STRAINS BY INDIRECT IMMUNOFLUORESCENCE

Immune Rabbit Sera	Geometric Mean Antibody Titers and Antigen: Karp	Gilliam	Kato	TA678	TA686	TA716	TA763	TH1817	TC586
Karp	2560	254	1015	16	403	403	16	96	25
Gilliam	640	10240	640	640	1613	2560	403	1015	10240
Kato	64	40	403	16	108	64	25	16	25
TA678	-+	160	-	320	-	-	-	-	-
TA686	108	40	40	16	403	160	108	40	40
TA716	1015	640	1015	640	4064	10240	2560	40	254
TA763	64	40	108	25	1613	1015	1015	46	40
TH1817	160	-	160	-	-	40	-	640	-
TC586	-	1280	20	-	-	-	-	-	1280

+ All sera from immune animal negative at initial 1:40 dilution

The occurrence of one way cross-reactivity is best explained by differences in the concentration of the antigens shared by the respective strains. For example, the antigen in TA716 which is shared with TA678 was high enough in concentration in that strain to elicit antibodies, but the same component in TA678 was not, although its presence was detected by immunofluorescent staining. The profile of the reactions obtained by immunofluorescence also serves to reveal differences between the strains. Although TA716 and TA763 share many antigens in common, there are significant differences in their respective antigenic composition. It was not previously known whether strains classified as homotypes of prototype strains on the basis of complement fixation tests had the same or different minor antigenic components. The results of the tests with TC586, which had been considered a Gilliam homotype, show it to be markedly different.

It is possible that TC586 may contain another distinctive antigen component in addition to the antigens shared with Gilliam. Studies are in progress to determine if homotypes of other prototype strains also have different antigenic components.

If it is assumed that the cross-reactivity detected by indirect immunofluorescence is indicative of the amount of heterologous antigen present in the strain, it is possible to use these data to select strains for a polyvalent vaccine. Table 3 lists the composition of the vaccine and presents the relative antibody titers that might be expected from the individual components and the composite. The theoretical antibody responses were calculated from the values in the previous table by adjusting them to correspond to a maximum homologous response of 1000. Combining 4 strains, TA763, TH1817, TC586 and TA678 would be expected to elicit antibody levels equal to or greater than homologous titers obtained with monovalent vaccines of 7 of the prototype strains. Although antibodies against Karp and Kato would be noticeably deficient, it is not known if this would appreciably affect the performance of the vaccine. The addition of the Karp strain to the polyvalent vaccine would be expected to afford appreciable protection against all 9 prototypes.

The antigenic interrelationship among these strains will be determined by indirect immunofluorescence with guinea pig immune sera. Since guinea pigs have been immunized with previous passage levels of the prototype strains, comparison of the results of tests should show whether or not any of the candidate strains have been mixtures of 2 or more antigenic types. If the pattern of heterologous reactivity of sera from guinea pigs infected with material from different passage levels is constant, then the strain can be considered to be antigenically stable and probably pure. When the studies with

TABLE 3

EXPECTED ANTIBODY RESPONSE TO COMPONENTS AND COMPOSITE

POLYVALENT SCRUB TYPHUS VACCINE

Candidate Vaccine Strain	Relative Antibody Titers to Prototype Strains								
	Karp	Gilliam	Kato	TA678	TA686	TA716	TA763	TH1817	TC586
TA763	63	39	106	25	1589	1000	1000	45	39
TH1817	250		250			63		1000	
TC586		1000	16						1000
TA678		500		1000					
Subtotal	313	1539	372	1025	1589	1063	1000	1045	1039
Karp	1000	99	396	6	157	157		38	10
TOTAL	1313	1638	768	1031	1746	1220	1006	1084	1049

the guinea pig sera are completed, polyvalent vaccines will be prepared and their efficacy tested.

b. Plaque Assay System for R. tsutsugamushi

Weinberg et al. (3) using Rickettsia rickettsi and primary chick embryo fibroblasts developed the first successful plaquing technic for assay of infectivity of rickettsial suspensions. Employing the same procedures, McDade et al. (4, 5, 6) reported the method to be applicable to the plaque assay of other members of the Spotted Fever Group, as well as for R. prowazeki, R. mooseri, Q fever and scrub typhus rickettsiae. The principal requirement of the Department of Rickettsial Diseases for a plaque assay procedure was (a) to clone candidate prototype strains of R. tsutsugamushi to be included in experimental vaccines in order to insure their purity; and (b) to confirm the existence of mixtures of different antigenic types in certain scrub typhus strains recovered from man, rodents and vector chiggers in Thailand.

Attempts were made to duplicate the results reported using the following basic procedure. Monolayers of primary chick embryo fibroblasts were produced from minced 10-day-old chick embryos treated with 0.25% Difco 1:250 trypsin for 30 min at room temperature (RT). After enzyme treatment, the cell suspensions were strained through cheese cloth, and centrifuged at 1,500 rpm for 10 min. The sediment was resuspended in a nutrient medium consisting of 95% M199 and 5% calf serum, 20 ml per embryo. Five ml portions of the cell suspensions were dispensed into Falcon plastic T30 flasks and incubated at 37 C. The following day the medium was poured off and 0.1 ml of serial 10-fold dilutions of the rickettsial seed suspension was pipetted onto the cells. After 15 min at RT, the infected monolayers of cells were covered with 5 ml of an overlay medium consisting of 5% calf serum and 0.5% agarose in M199. The flasks were then incubated at 32 C. The plaques were visualized after staining the monolayer of cells by adding a second overlay containing 0.01% neutral red in M199 and returning the flasks to the 32 C incubator. The duration of the incubation period and the time of the staining was dependent upon the species of the infecting organism. According to the reports, Spotted Fever Group rickettsiae usually produced plaques in 5 to 6 days, Typhus Group organisms took 10 to 11 days, and 16 to 18 days were required for strains of R. tsutsugamushi.

Many attempts to reproduce the results of the other workers have been only occasionally and unpredictably successful. In 14 of 25 experiments, plaques were obtained in one or more flasks infected with the Bitterroot strain of R. rickettsi, and in 2 of 15 trials, R. mooseri plaques were formed. Contrary to the impression given

in the published accounts, it was impossible to identify plaques prior to staining because of the lack of specific cytopathologic changes in infected cells. The only microscopic difference between cells in the plaques and the surrounding areas was seen after staining. Intracytoplasmic granules of neutral red were not found in the cells comprising the plaque. Table 1 shows the range of numbers of plaques obtained in different [illegible] and compares plaque titers with titers obtained by inoculation [illegible] the yolk sac of embryonated eggs. It should be pointed out that the table is a compilation of all experiments which were [illegible] partially successful. In certain titrations where replicate testing was done, plaques were found in only one of the flasks. Although the numbers of plaques resulting from the inoculation of the same dilution varied considerably, the Plaque Forming Units (PFU) calculated from the mean values corresponding to the 10^{-6} and 10^{-7} dilutions of the R. rickettsi seed are comparable. The plaque titers obtained with both R. rickettsi and R. mooseri were at least 10-fold greater than the yolk sac 50% lethal dose ($YSLD_{50}$), indicative of the greater sensitivity of the plaque assay method for determining infectivity.

Efforts to produce plaques with strains of R. tsutsugamushi were all essentially unsuccessful. In 2 of 16 experiments, innumerable tiny plaques were observed in monolayers infected with the Gilliam strain. In one instance, the inoculum was the 10^{-5} dilution, and in the other, the 10^{-6} dilution of Gilliam seed suspension which had a mouse 50% infectious dose titer of $10^{8.8}$. However, in both trials, plaques were not found in flasks inoculated with higher dilutions.

Initially, a concerted effort was made to duplicate exactly, step by step, the procedure used by McDade et al. which he kindly provided in detail. After many negative experiments, however, all conceivable factors that may have influenced plaque formation were evaluated. Cell culture media and agarose were purchased from the same source used by the authors, as well as from other manufacturers. Embryonated white and brown eggs from different breeding lines were obtained from several hatcheries. Various times and temperatures of incubation, with and without 5% CO_2 in the atmosphere, other types of nutrient animal sera, salt solutions and neutral red were investigated. Because it appeared that the cells did not survive long enough, particularly during the prolonged incubation period needed for plaquing of R. mooseri and R. tsutsugamushi, the concentration of serum in the first agarose overlay was increased to 20%. Glass 2 ounce prescription bottles and plastic [illegible] were substituted for the plastic petri dishes.

TABLE 4

COMPARISON OF PLAQUE TITERS AND EMBRYONATED EGG LD_{50} TITERS OF SUSPENSIONS OF R. RICKETTSI AND R. MOOSERI

Seed Suspension	$YSLD_{50}$* Log_{10}/ml	Inoculum Dilution Log_{10}	No. Expts	No. Plaques Range	No. Plaques Mean	PFU** Log_{10}/ml
R. rickettsi		-5	4	TNTC+	-	-
Bitterroot Strain	6.95	-6	10	13-80	44	8.64
E55 ys pool		-7	8	1-16	7	8.85
		-5	4	TNTC	-	
E55 ys pool	ND++	-6	4	41-80	66	8.82
		-7	4	4-21	8	8.90
R. mooseri		-6	2	TNTC		
Wilmington Strain	8.15	-7	2	TNTC		
		-8	2	18-26	22	10.34

* Yolk sac LD_{50}

** Plaque forming unit

\+ To numerous to count

++ Not done

On many occasions apparently healthy monolayers of cells became uniformly shiny red after applying the neutral red overlay instead of assuming the expected stippled pink appearance usually seen when normal cells become filled with granules of the dye. Also cells in large irregular areas of the flask detached spontaneously during the incubation period after adding the neutral red overlay. It was found that various lots of plastic flasks behaved differently. A crystal violet dye adherence test obtained from the manufacturer was used to determine the wetability of the growth surface of the plastic. If a dilute solution of the dye separated from the surface in less than 8 seconds, the hydrophilic quality of the surface was unsatisfactory. This may explain a portion of the failure experienced in producing plaques by rickettsiae, since testing showed that 3 of the lots used did not pass the dye test. However, only slightly better results were obtained when other flasks that were acceptable were used. Although plaques could regularly be produced in 5 to 6 days with Spotted Fever Group strains, the chick embryo fibroblasts still would not survive for the 14 to 18 days required for production of plaques with scrub typhus rickettsiae. Attempts are already in progress using nutrients containing newer salt solutions with better buffering capacity that have resolved similar problems encountered by others for plaque production with viruses. In addition, the suitability of certain established cell lines for plaque assay is under investigation.

c. Characterization of the Immune Response of Mice after Infection and Challenge with Scrub Typhus

Previous workers have shown that killed scrub typhus vaccines protect mice primarily against infection with the homologous strain. However, mice convalescent from infection with one scrub typhus strain resist lethal challenge with all other strains (7). In contrast, when man is infected with scrub typhus heterologous protection is incomplete and short-lived. After 2 to 3 months he is fully susceptible to infection with other antigenic types. Understanding the basis of the immunity provided the mouse by infection may lead to better means of protecting man against disease than might be achieved by a polyvalent killed vaccine. Previous attempts were made to characterize the antibody response in mice following initial infection with the prototype Karp, Gilliam and Kato strains, and after subsequent heterologous challenge. The effect of route of inoculation, size of infectious dose and antibiotic therapy upon the antibody response after primary infection, as well as the effect of the size of the challenge dose upon the antibody response after challenge were studied. The time period for treatment and the interval between infection and challenge were those routinely used in immunity-challenge experiments for the definitive identification of scrub typhus strains. It was postulated that after initial infection,

the animal developed homologous immunity evoked by the dominant antigenic component of the infecting strain. The common minor antigenic components primed the animal so that after exposure to the heterologous strain, a booster response occurred which prevented death, but not infection. However, review of the data showed that on the 36th day after primary infection when the animals were challenged, both homologous and heterologous antibodies were still rising. The rate of antibody production and the ultimate titers attained in animals after challenge were indistinguishable from the antibody response of unchallenged controls (8).

In the experiment to be reported here, the interval between primary infection and challenge was increased in order to detect a booster response if it occurred. In addition, an attempt was to be made to identify the immunoglobulin class of the homologous and heterologous antibody resulting from primary infection and challenge.

Thirty white mice weighing 10 to 12 gm were inoculated intraperitoneally with 0.2 ml of 10^2 LD_{50} of the Karp strain and challenged 49 days after the primary inoculation with $10^{5.5}$ LD_{50} of the Kato strain. Previous experiments had shown that both homologous and heterologous antibody titers would be either constant or starting to wane at that time. All animals were treated with chloramphenicol by administering the antibiotic in the drinking water (2.5 mg/ml) from the 3rd day through the 21st day after inoculation. Serum pools were made from blood collected from the retroorbital venous plexus the day after inoculation and at 2 to 4 day intervals thereafter, through the 78th day.

The antibody titers of the serum pools were determined by indirect immunofluorescence (IF) and complement fixation (CF). In the IF tests, 4-fold serial dilutions were tested with antigen smears comprised of yolk sac suspensions of the Karp, Kato and Gilliam strains. The fluorescein conjugate employed was a horse-derived anti-mouse whole serum. Prior IF tests, in which Karp, Kato and Gilliam mouse immune sera were tested with each of the corresponding antigens, displayed considerable heterologous activity. In general, only 4-fold differences were found between homologous and heterologous titers in reciprocal tests with Kato and Karp sera and antigens. The titer of both Karp and Kato immune sera with the Gilliam antigen was about 16-fold lower than the homologous titers.

The procedure for complement fixation used was the CF-52 test developed by the Department of Serology, WRAIR, adapted to the microtiter system. Two-fold serial dilutions of the mouse serum pools were tested with partially purified suspensions of the prototype Karp, Kato and Gilliam strains. These antigens were type-specific and did not fix complement with 32 to 128 units of heterologous antibody in immune sera collected 28 days after infection.

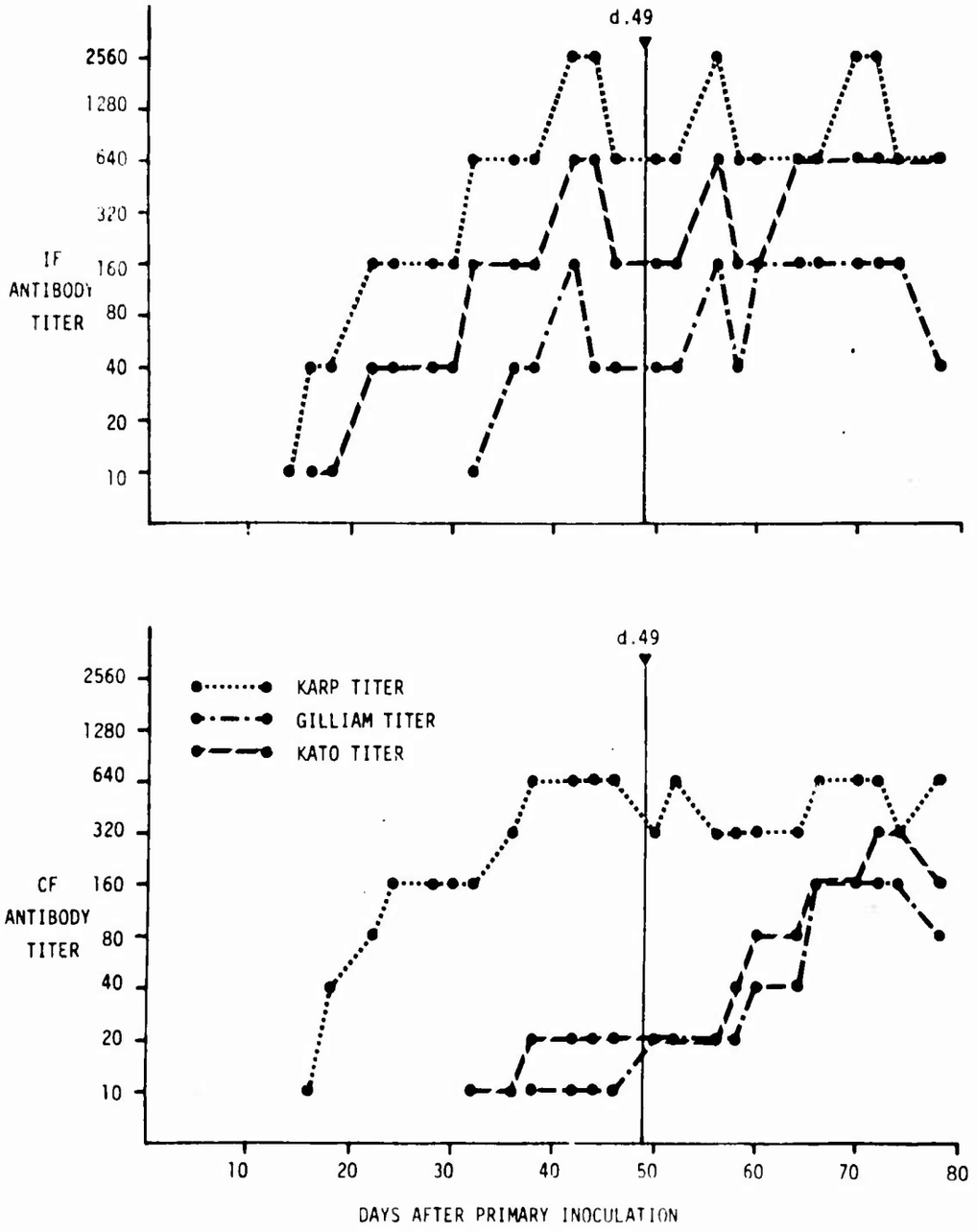
Separation of antibodies of the IgM and IgG class in each serum pool was done by density gradient centrifugation. Continuous linear gradients, from 10 to 40% were prepared at 4 C with sucrose (ribonuclease-free) in tris-buffered saline. A portion of the serum pool was diluted with an equal volume of 10% sucrose solution and a 0.2 ml sample was layered on top of the 4.4 ml gradient (9). Ultracentrifugation was carried out in the Model L Spinco at 4 C using an SW 39 rotor at 35,000 rpm (100,000 x g) for 18 to 22 hr. Thirteen serial fractions, each approximately 0.38 ml in volume, were collected from the bottom of the gradient under controlled pressure.

The effectiveness of the separation of antibodies was confirmed by determining the total protein and immunoglobulin content of each of the fractions. The Lowry method (10) was used to determine the concentration of proteins in the fractions. In all instances, the characteristic double-peaked curve was obtained when the results were plotted graphically. The first low peak corresponded to the fourth fraction collected and the second high peak to fraction 10. The immunoglobulin content of each fraction was identified with the Ouchterlony double diffusion technic (11) performed on alcohol-cleaned slides to which 4 ml of 2% purified agar had been placed. A sample of each fraction was tested against both goat anti-mouse IgM and IgG at room temperature in a humidified chamber for at least 18 hr. Precipitin lines formed between wells containing fractions 3 and 4 and the anti-mouse IgM, and between fractions 7 through 10 and the anti-mouse IgG. The anti-IgM - IgM precipitin lines were generally much fainter than the anti-IgG - IgG lines. These reactions were greatly enhanced by treatment with 0.125% cadmium cations after the lines had formed (12).

The results of the IF and CF tests with Karp, Gilliam and Kato antigens and the pools of mouse serum collected after primary infection with the Karp strain, and subsequent challenge with the Kato strain are illustrated in Figure 1. Homologous Karp IF antibody was first detected 14 days after inoculation and attained peak levels on the 42nd day. Thereafter, 4-fold fluctuations in titer occurred until the 78th day when the experiment was terminated. The heterologous Kato antibody appeared on the 16th day, and the Gilliam antibody on the 32nd day. The Kato and Gilliam antibody titers paralleled the Karp antibody titers at 4-fold and 16-fold lower levels respectively, until the 60th day of the experiment. On this day, which corresponded to the 11th day after challenge with the Kato strain, and afterwards, there was obvious dissociation of the patterns of the respective antibody curves. Kato antibody titers were equal to the Karp titers on day 60 and 64, and remained constant for the next 2 weeks.

FIGURE 1

ANTIBODY RESPONSE OF MICE FOLLOWING INTRAPERITONEAL INOCULATION OF $10^{2.0}$ LD_{50} KARP STRAIN DURING TREATMENT WITH CHLORAMPHENICOL AND AFTER SUBSEQUENT CHALLENGE WITH $10^{5.5}$ LD_{50} KATO STRAIN



Similarly, Gilliam antibody titers remained at least from the 60th to the 74th day at 4-fold lower levels than the Kato antibody. On the 78th day, a 4-fold drop in Gilliam titer was observed. These findings can be explained by attributing the heterologous Kato and Gilliam antibody activity before the 60th day to cross-reactivity of Karp antibody with the Kato and Gilliam antigens always displayed by the IF test. On the 60th day and thereafter, antibodies resulting from the challenge with the Kato strain exceeded the level of cross-reactivity of the Karp antibodies and were independently manifested.

It was necessary to qualitatively characterize the class of immunoglobulin responsible for the IF antibody activity. Monospecific anti-mouse IgM and IgG fluorescein conjugates were not available from commercial sources. Attempts were made to devise a 3-step indirect immunofluorescent procedure. The density gradient fractions were applied to the antigen smears and the following reagents were added in sequence after removal of the previous one: either rabbit anti-mouse IgM or rabbit anti-mouse IgG, and then goat anti-rabbit globulin fluorescein conjugate. The results indicated that the rabbit anti-mouse IgM and IgG sera were not as monospecific in the fluorescent antibody system as they were when used in Ouchterlony double diffusion and immunoelectrophoretic techniques.

Tests with the horse anti-mouse whole serum fluorescein conjugate and the density gradient fractions showed the reagent was reacting with both IgG and IgM antibodies. However, antibody could not be detected in any of the fractions unless the antibody titer of the whole serum was 1:160 or greater. The results of tests of the fractions from the 46th, 60th and 64th day specimens did provide some information about the nature of the immune response after infection and subsequent challenge. The serum pools collected on these days had Karp antibody titers of 1:640. Fractions 3 and 4 of the 46th day specimen in which presumably only IgM was present, titered 1:10 with the Karp antigen only. The range of antibody titers found in fractions 7, 8 and 9 of the same specimen in which presumably only IgG was present, were 1:40 to 1:160 with the Karp antigen, and 1:10 to 1:40, with the Kato antigen. Reactivity with the Gilliam antigen was not seen. In the 60th day specimen collected 11 days after the Kato challenge, fractions 3, 4 and 5 containing IgM titered 1:10 with the Karp antigen, and from 1:10 to 1:40 with the Kato antigen. The range of titers in fractions 7, 8 and 9 containing IgG with the Karp, Kato and Gilliam antigens were respectively, 1:40 to 1:160, 1:40, and 1:10 to 1:40. The same pattern of results was obtained with the fractions from the 64th day specimen. It was evident that Karp IgM antibody was decreasing while Kato IgM antibody was increasing. The Kato and Gilliam IgG antibody remained the same as was found four days earlier, but the amount of Kato IgG antibody had increased.

The more specific complement fixation test provided a clearer picture of the antibody response. Homologous Karp antibody was first detected on day 10, attained peak levels on the 36th day after infection, and remained essentially constant until the end of the experiment. Low levels of Kato and Gilliam antibody were found initially in the 32nd and 38th day specimens, respectively. Prior to challenge on the 49th day, the heterologous titers were 16-fold lower than the level of Karp antibody. The Kato antibody began to increase 9 days after challenge, followed 2 days later by the Gilliam antibody. The Kato antibody reached peak levels on the 72nd day of the experiment, 23 days after challenge.

When complement fixation tests were performed on the density gradient fractions of the mouse serum pools, it was found initially that fractions 1 through 5 were anticomplementary. The anticomplementary activity was removed by dialysis of the fractions against physiologic saline at 4 C for 24 hr. Complement-fixing antibodies were found only in fractions 7 through 9 which presumably contained only IgG immunoglobulins. The relative Karp, Kato and Gilliam antibody titers paralleled exactly the reactivity of the whole serum pool. Tests with fractions 3 through 5 of all of the serum pools were negative. Other workers have found that antigen-antibody reactions with mouse IgM immunoglobulins do not fix complement.

Although it is not possible to state unequivocally that the antibody response to the Kato challenge was of the primary type, there is little evidence to indicate that it was a secondary response. It had been reported previously that the time of appearance and the rapidity with which peak levels are attained are influenced by the dose of the inoculum and the administration of antibiotics. In an earlier experiment, after the intraperitoneal inoculation of 16 LD_{50} of the Karp strain, which does not kill all of the mice, CF antibodies were first detected 14 days after inoculation and peak titers were attained around the 17th day. The administration of antibiotics delayed the appearance of antibodies until the 21st day, and peak levels were not reached until day 35. Thus, the difference between the Karp antibody response after primary infection and the Kato antibody response after challenge could be attributed to the influence of the chloramphenicol treatment from day 3 through day 21. When $10^{5.6}$ LD_{50} of Kato rickettsiae were inoculated intraperitoneally, and the mice treated with antibiotic, CF antibodies appeared on the 7th day and were at maximal levels on day 35. When similar large doses of either Karp and Kato were used to infect mice and antibiotic was not administered, CF antibodies were found on day 7, and the homologous titers on day 10 were 1:80. Between the 10th and 14th day all of the mice died. Comparable levels of Kato antibody were found in the present experiment in the mice 10 days after challenge.

This evidence, when considered with the results of the IF tests which suggested that the mice developed Kato IgM antibodies after challenge, led to the conclusion that a secondary or booster antibody response was not the basis of the protection afforded the animals. When a method for quantitating the rickettsial neutralizing capacity of serum is available, it will be used to evaluate further the mechanism of immunity. Future plans include also an evaluation of the role of cellular immunity.

d. Development of a Tissue Culture Scrub Typhus Neutralization Test

Studies of the immunity produced by either infection with R. tsutsugamushi or the administration of experimental vaccines have been handicapped by the lack of satisfactory assay methods. The only serologic means currently available to quantitate scrub typhus antibodies are based upon indirect immunofluorescence (IF) and complement fixation (CF). The results of trials with killed rat lung-spleen vaccines already reported failed to show a relationship between the presence or absence, or the level of serum antibodies found in CF and IF tests, and the ability of the mouse to survive challenge with the homologous strain (13). At the present time, the only way to obtain valid information about the immune status of man or experimental animals is to determine the response of the host to infectious challenge. Previous technics demonstrating in vitro neutralization of scrub typhus rickettsiae using mice (14) and tissue culture (15) have been imperfect and required the use of hyperimmune sera. Although a plaque assay for rickettsiae has been reported (3, 4, 5, 6), its usefulness for demonstrating neutralization was not evaluated. Recently other investigators have reported a method for the quantitative assay of Chlamydia psittaci in cell culture and its application to neutralization tests (16, 17, 18). Incubation of the seed suspension with antibody in this procedure resulted in a significant reduction in the number of infected foci found in the cell monolayer. These authors found also that sensitivity and reproducibility were increased by the addition of antiglobulin prepared against the IgG immunoglobulins in the serum being tested to the immune complex. Infected foci in monolayers of cells were visualized microscopically after immunofluorescent staining.

In August 1970, efforts were initiated to develop a similar neutralization test for R. tsutsugamushi. This report is concerned with results of attempts to enumerate quantitatively scrub typhus rickettsiae in cell cultures. Difficulties were encountered initially in the selection of a cell line. Preliminary trials with primary chick embryo fibroblasts and Vero African Green monkey kidney cells were unsatisfactory. These cells tended to overgrow rapidly, making

it difficult to visualize intracellular scrub typhus rickettsiae in compacted and overlapping cytoplasm. It was not possible to control the rate of growth of the cells by varying the concentration of protein in the nutrient fluids. In addition, considerable nonspecific immunofluorescent staining of cellular granules complicated the counting of rickettsiae.

During the early trials, antibiotics were not included in the nutrient medium because its possible effect upon low concentrations of microorganisms was unknown. Prior experience in this laboratory had shown that 100 units of penicillin and 20 ugm of streptomycin per ml did not inhibit the growth of R. tsutsugamushi when the multiplicity of organisms to cells was relatively high; i. e., 1 to 5 rickettsiae per cell. Recently, the frequency of bacterial contamination has required the use of these antibiotics.

The results of experiments reported here were obtained with BS-C-1 cells, another continuous line of African Green monkey kidney cells. The primary purpose of these experiments was to define the sensitivity of the assay method; i. e., to determine the lowest concentration of rickettsiae that could be enumerated accurately.

The procedure was essentially the same in all the experiments. Monolayers of BS-C-1 cells were removed from stock bottles by treatment with 0.25% trypsin. The concentration of the cells in growth medium, consisting of 10% fetal bovine serum (FBS) and 2mM glutamine in M199, was adjusted to 0.5 to 1.0 x 10^5 cells per ml. The suspension was distributed in 1 ml amounts to flat-bottom cylindrical vials each containing a 12 mm circular coverslip. The tubes were incubated at 37 C until there was 80 to 100% confluence of cells in the monolayer, usually 1 to 3 days. The growth medium was removed and each of 2 vials were inoculated with 0.2 ml of varying concentrations of a yolk sac suspension of the Karp strain diluted in maintenance medium (2% FBS and 2mM glutamine in M199). The vials were centrifuged in a horizontal head at 500 x g at room temperature for 30 min to facilitate infection of the cells. The inoculum was removed, and the monolayer washed twice with maintenance medium. Finally, 1 ml of maintenance medium was added, and the vials incubated at 37 C for the duration of the experiment.

Rickettsiae in the cell monolayers were enumerated in the following manner. After removal of the maintenance medium, the monolayer of cells on the coverslip was washed twice with phosphate buffered saline (PBS) and air-dried. It was not possible to identify rickettsiae in cells with certainty when either the Giemsa stain or the May-Grunwald modification was used. In preparation for immunofluorescent staining, the monolayer of cells on the coverslips were fixed with

acetone for 10 min and then dried in the air. The coverslips were treated with 0.05 M HCl, washed, air-dried, and then stained for 30 min at 37 C with a mixture of a 1:20 dilution of the rabbit anti-Karp fluorescein conjugate and 5% rhodamine bovine albumin. The fluorescent dyes were removed, the coverslips washed twice with PBS and allowed to air dry. The coverslips were mounted in buffered glycerin, pH 7.2, on a slide and examined with the dark-field fluorescent microscope at 450x magnification. Uninoculated monolayers, included in each experiment as controls, were processed in the same manner.

Table 5 presents the results of a series of experiments in which attempts were made to enumerate R. tsutsugamushi in monolayers of BS-C-1 cells 21 hr after infection with decreasing concentrations of the Karp strain. The results are grouped according to the dilution of the seed suspension to facilitate comparison of reproducibility from experiment to experiment. The number of infected cells per coverslip was calculated from the average number of cells counted in the indicated number of high-powered fields (HPF), multiplied by the total number of HPF included on the surface area of the coverslip. The per cent of infected cells denotes the ratio of the average number of infected cells to the average number of total cells counted in the indicated HPF. In the course of carrying out these experiments, it was apparent that as the concentration of rickettsiae in the inoculum was reduced, more HPF had to be examined in order to obtain meaningful values. Thus, the 12 HPF counted in early experiments was increased to 50 HPF later. A certain amount of nonspecific staining of particles about the same size and shape as scrub typhus rickettsiae also was noted. The magnitude of this problem became evident when cultures were infected with the $10^{-5.7}$ and 10^{-6} dilutions of the Karp suspension. In certain instances in these trials, when the numbers of cells thought to contain rickettsiae in infected cultures were compared with the number of cells in uninoculated controls exhibiting similarly fluorescing particles, virtually no difference was found. Therefore, the results of most of the experiments with the highest dilutions of the Karp seed are probably invalid.

There was considerable variation in the number of infected cells counted on coverslips inoculated with the same dilution of seed. Differences were noted among the experiments, as well as within the same experiment (Table 5). The greatest discrepancies occurred in the earlier experiments, and more recent trials agreed more closely. Nevertheless, an evaluation of sensitivity of the assay system can be made. The titer of the Karp suspension expressed as tissue culture cell infectious units calculated from the mean values

TABLE 5

ENUMERATION OF R. TSUTSUGAMUSHI IN BS-C-1 CELLS

Seed Dilution	Expt	HPF Counted	Infected Cells Per Coverslip: Percent	Number	Mean (Range ± 1 SD)	Log_{10} Number Infected Cells (± 1 SD)
10^{-4}	10	12	3.5	2,584		
		12	2.7	2,128		
10^{-4}	11	12	24.6	10,336	7,296 (2,959-11,639)	3.86 (3.47-4.07)
		12	13.5	6,384		
10^{-4}	12	20	16.8	12,768		
		20	14.5	9,576		
$10^{-4.7}$	12	20	2.3	1,368		
		20	1.4	760		
$10^{-4.7}$	13	50	2.9	2,128	1,737 (764-2,710)	3.24 (2.88-3.43)
		50	0.9	517		
$10^{-4.7}$	19	50	4.8	1,388		
		50	5.6	1,611		
$10^{-4.7}$	20	50	5.6	2,888		
		50	5.6	3,283		
10^{-5}	12	20	0.5	304		
		20	1.1	684		
10^{-5}	13	50	0.7	426	1,100 (253-1,947)	3.04 (2.40-3.29)
		50	0.8	426		
10^{-5}	19	50	4.6	1,155		
		50	2.7	1,124		
10^{-5}	20	50	3.9	2,006		
		50	5.2	2,675		
$10^{-5.7}$	13	50	0.3	213		
		50	0.4	243		
$10^{-5.7}$	19	50	2.3	942	888 (235-1,541)	2.95 (2.37-3.19)
$10^{-5.7}$	20	50	2.6	1,642		
		50	3.3	1,398		
10^{-6}	20	50	1.9	1,064	897 (661-1,133)	2.95 (2.82-3.05)
		50	1.5	730		

obtained with the 10^{-4}, 10^{-5} [illegible] were calculated to be $10^{7.86}$, $10^{7.94}$ and $10^{8.04}$, respectively. The 50% tissue infectious dose (ID_{50}) of this suspension was $10^{8.35}$.

The tissue culture assay system was [illegible] the rate of multiplication of R. tsutsugamushi in [illegible] Table 6 summarizes the results of several experiments in which [illegible] the Karp suspension varying from 1/10,000 to 1/[illegible] were titrated. After 21 hr incubation, the numbers of [illegible] per HPF, and rickettsiae in infected cells were [illegible]. The calculation of the multiplication rate was predicated on the assumption that the number of infected cells was an index of the number of rickettsiae inoculated into the culture the previous day. The total number of rickettsiae in the infected cells, divided by the number of infected cells, gave the multiplication factor. There appeared to be a tendency for the rate of multiplication to increase with the inoculation of greater numbers of rickettsiae. The validity of these observations must be confirmed because the differences may be due to sampling error resulting from counting [illegible] HPF. Previous investigations in this laboratory using HE fixed cells showed that R. tsutsugamushi increased 3-fold [illegible].

Table 7 summarizes results obtained [illegible] maintained for 1, 2 and 3 day periods. It was anticipated that increases in numbers of rickettsiae due to multiplication during the extended incubation period would facilitate enumeration of infected cells. The absence of values in the table denotes the occurrence of bacterial contamination. The results indicate that within the limits of sampling error, there was no change in the number of infected cells over the 3 day period. Although rickettsial counts were not made, there was no observable increase in the number of organisms in each cell. Indeed, there appeared to be a decrease in the number of infected cells during the period of incubation in the cells inoculated with the 10^{-6} dilution. Other experiments are in progress to evaluate the relationship between the dose of the inoculum and the ability of the organisms to multiply in tissue culture cells.

Since these studies were completed, work with the Ld-C-1 cell has been discontinued. Preliminary trials with 4p1, a continuous line of rat fibroblast, have shown this cell to contain fewer intracytoplasmic particles resembling rickettsiae. In addition, the acid treatment-rhodamine bovine albumin procedure was discontinued and 0.005% Evans' blue was adopted as a counterstain. These changes have almost completely eliminated the problems of nonspecific fluorescence of normal cellular [illegible] granules.

TABLE 6

GROWTH OF R. TSUTSUGAMUSHI IN BS-C-1 CELLS

Seed Dilution	HPF Counted	Multiplication Factor Observed	Mean	SD
1/10,000	12	3.4	4.43	1.33
	12	3.1		
	12	6.6		
	12	5.4		
	20	3.9		
	20	4.2		
1/20,000	12	5.0	4.75	0.35
	12	4.5		
1/30,000	12	3.4	3.45	0.07
	12	3.5		
1/40,000	12	3.3	3.55	0.35
	12	3.8		
1/50,000	20	3.5	2.65	1.20
	20	1.8		
1/100,000	20	2.8	2.60	0.28
	20	2.4		

TABLE 7

INFLUENCE OF DURATION OF INCUBATION PERIOD UPON

ENUMERATION OF R. TSUTSUGAMUSHI IN BS-C-1 CELLS

Seed Dilution	Expt. No.	HPF Counted	No. Infected Cells Per Coverslip Incubation Period Day 1	Day 2	Day 3
1/2,000	10	10	5,320*	10,032**	7,904
			5,016		11,696
1/10,000	10	12	2,584	5,624	6,840
			2,128	4,408	5,928
1/100,000	20	50	2,006	2,736	
			2,675	2,219	
1/500,000	19	50	942	912	
				699	
1/500,000	20	50	1,642	1,155	
			1,398	1,034	
1/1,000,000	20	50	1,064	364	395
			730	334	

* 15 HPF counted
** 20 HPF counted

Recent studies by other investigators of the interaction of R. prowazeki and human macrophages have shown that rickettsiae coated with antibody are destroyed within several days after phagocytosis. On the other hand, unsensitized rickettsiae quickly escape from the phagosome and multiply intracytoplasmically until the phagocyte was destroyed (19). Earlier this year, studies were initiated to determine if guinea pig peritoneal macrophages could be used for quantitative enumeration of scrub typhus rickettsiae and for the development of a neutralization test.

Young adult guinea pigs were inoculated intraperitoneally with 10 ml of trypticase soy broth. Three days later, after rapid euthenasia by the intracardiac injection of 20 ml of pentobarbital, 50 ml of M199 with 20% FBS, 10 units/ml heparin, 100 units/ml penicillin and 20 µgm/ml streptomycin were inoculated into the peritoneal cavity. After gentle kneading, the abdomen was opened, the fluid removed aseptically and kept at 4 C while the cell concentration was determined. The cells were sedimented at 200 x g for 10 min. The supernatant fluid was discarded and the cells resuspended to original volume in the same nutrient solution without the heparin. After centrifugation, the cells were resuspended in nutrient medium to a final concentration of 2 to 3 x 10^6 cells/ml. The cell suspension was distributed in 1.0 ml amounts to vials containing 12 mm diameter coverslips and incubated at 37 C. The following day the monolayer of macrophages was about 80% confluent. The procedures for infection with dilutions of the Karp strain, and the subsequent processing for immunofluorescent staining, was as previously described for experiments with BS-C-1 cells, except Evans' blue was used as the counterstain.

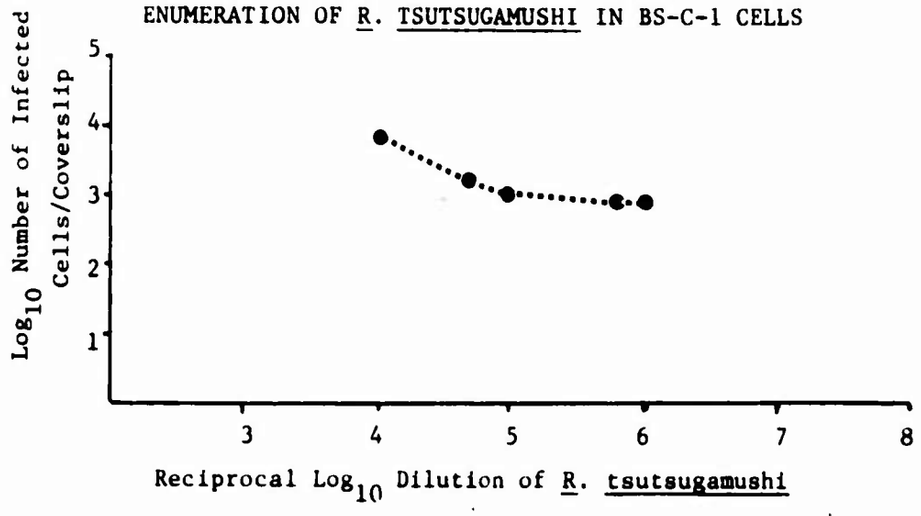
Table 8 summarizes the results of two experiments in which R. tsutsugamushi was enumerated in guinea pig peritoneal macrophages 21 hr after inoculation of a 10^{-3} and 10^{-4} dilution of the Karp seed suspension. The indicated values were calculated as previously described. With the single exception obvious in experiment 26 with the 10^{-3} dilution, the results of replicate tests were very similar. The titer of the Karp suspension calculated from the mean values, expressed as tissue culture cell infectious units would be $10^{7.5}$. The mouse LD_{50} titer of this seed suspension was $10^{8.35}$. The results of the experiments to quantitate R. tsutsugamushi in BS-C-1 and the guinea pig macrophages are compared graphically in Figure 2. The slopes of the two curves are comparable if the points corresponding to the $10^{-5.7}$ and 10^{-6} dilutions in BS-C-1 cells are disregarded. The reasons for considering these values invalid have been presented earlier.

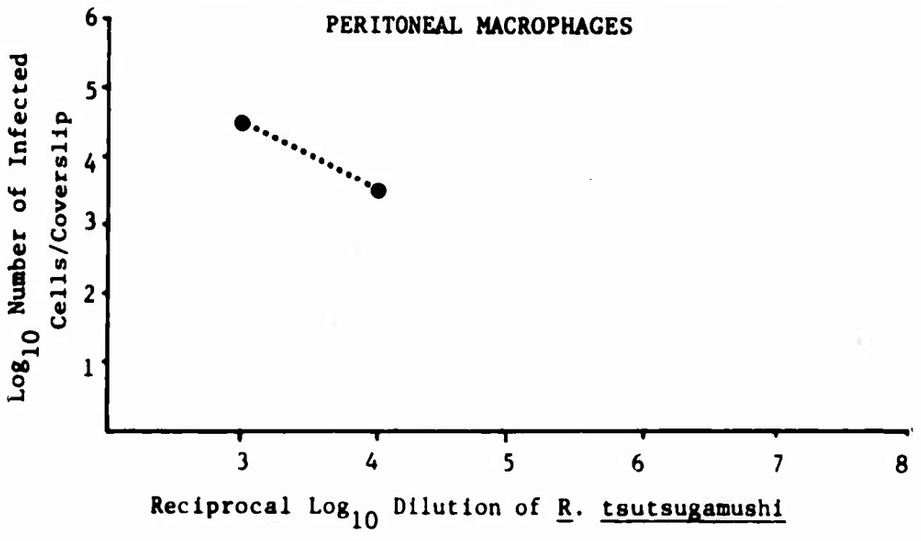
TABLE 8

ENUMERATION OF R. TSUTSUGAMUSHI IN GUINEA PIG PERITONEAL MACROPHAGES

Seed Dilution	Expt	PMF Counted	Infected Cells Per Coverslip: Percent	Number	Mean (Range ± 1 SD)	Log_{10} Number Infected Cells (± 1 SD)
10^{-3}	26	25	18	14,957		
		25	27	28,576		
10^{-3}	28	25	38	31,993	29,913 (21,803-38,023)	4.48 (4.34-4.58)
		25	37.9	38,663		
		25	33	30,699		
		25	35	34,590		
10^{-4}	26	25	3.4	2,918		
		25	4.6	3,526		
10^{-4}	28	25	2.7	2,614	3,060 (2,562-3,558)	3.49 (3.41-3.55)
		25	4.5	3,465		
		25	2.8	2,371		
		25	2.4	3,465		

FIGURE 2


ENUMERATION OF R. TSUTSUGAMUSHI IN BS-C-1 CELLS
Log10 Number of Infected Cells/Coverslip
5
4
3
2
1
3
4
5
6
7
8
Reciprocal Log10 Dilution of R. tsutsugamushi


ENUMERATION OF R. TSUTSUGAMUSHI IN GUINEA PIG PERITONEAL MACROPHAGES
Log10 Number of Infected Cells/Coverslip
6
5
4
3
2
1
3
4
5
6
7
8
Reciprocal Log10 Dilution of R. tsutsugamushi

When the sensitivity of the two cell systems are defined, and enumeration of rickettsiae can be accomplished with the required degree of reproducibility, attempts will be made to use the assay system for scrub typhus neutralization tests. In addition, the usefulness of the fluorometer as a means for rapid, accurate, objective measurement of the number of fluorescing rickettsiae in infected monolayers on coverslips will be evaluated.

e. Study of Genetic Stability of R. tsutsugamushi in Vector Mites

(1) Preparation and Performance of Type-Specific Anti-rickettsial Serum Fluorescein Conjugates. Current methods for the identification of an organism as a species of R. tsutsugamushi requires: (a) recovery of the agent in mice; (b) determination of characteristic microscopic morphology in Giemsa-stained smears of mouse peritoneum and spleen; and (c) establishing through immunity-challenge the ability of mice convalescent from infection with the isolate to survive lethal challenge with a recognized strain of R. tsutsugamushi. At best this procedure requires a minimum of 10 weeks. Characterization of the antigenic relationship of an unknown strain to subspecies of scrub typhus rickettsiae previously established as prototypes or candidate prototypes entails: (a) its adaption to cultivation in the yolk sac of embryonated hens' eggs; (b) preparation of strain-specific complement-fixing antigens; (c) production of immune sera by intracerebral inoculation of guinea pigs with suspensions of infected mouse spleen and (d) carrying out cross-complement fixation tests with an array of the prototype antigens and the corresponding immune guinea pig sera. The principal difficulty in this procedure is to obtain the heavy concentration of rickettsiae in infected yolk sacs that are required for the production of satisfactory strain-specific complement-fixing antigens. Even after many years of continuous cultivation of most of the candidate prototype strains under study by the Department of Rickettsial Diseases, it still has not been possible to complete their antigenic characterization. Similar difficult and length procedures are required for the specific identification of other species belonging to the Typhus Group and Spotted Fever Group of rickettsiae.

Proposed studies to determine if changes in antigenic composition of R. tsutsugamushi occurred during transstadial and transovarial transmission in vector mites required development of methods for rapid, specific identification of strains of scrub typhus. Consequently attempts were made to conjugate immune sera with fluorescing dyes that react specifically with the homologous organism but permit species identification under the fluorescent microscope. The principal effort has been devoted to the preparation of reagents for direct immunofluorescent staining of strains of R. tsutsugamushi, and to a lesser extent with species of rickettsiae belonging to the Spotted Fever and Typhus Groups.

In the Annual Report from 1969-1970, preliminary results were described indicating that direct-staining conjugates prepared from sera collected from rabbits 25 days after infection with either Karp, Gilliam or Kato and conjugated with fluorescein isothiocyanate were sufficiently specific to distinguish the respective strain from the other two prototypes. It is known that antibodies in sera collected from rabbits early after immunization, which are principally of the IgM class of immunoglobulins, exhibit more specific serologic reactivity than antibodies in sera collected later that are almost wholly IgG immunoglobulin. Thus, groups of 3 rabbits were infected intraperitoneally with about $10^{8.3}$ 50% mouse infectious doses of each of the 5 Thai candidate prototype strains, TA678, TA686, TA716, TA763 and TH1817. Serum was obtained from each animal 14 days and 28 days after inoculation. The antibody titer of each specimen was determined by indirect immunofluorescence with smears of yolk sac suspensions of the homologous organisms. In no instance did the 14-day serum contain sufficient levels of antibodies to warrant attempts at conjugation. Previous experience had shown that rabbit serum had to have an immunofluorescent titer of 1:2,560 or greater in order to prepare a satisfactory conjugate. Even after 28 days, none of the rabbits infected with TA678 or TH1817 had developed the required levels of antibodies. Work is in progress to produce the necessary antibody titers in other rabbits by administering repeated booster doses of these two strains.

The method used for conjugating fluorescein to antibody was as follows: To 10 ml of cold TA686, TA716 and TA763 rabbit antiserum, an equal volume of cold 3.2 M ammonium sulfate was added slowly. A precipitate formed when about half of the sulfate solution had been added. The suspension was mixed continuously for 4 hr at 4 C, after which the precipitated globulins were sedimented by centrifugation at 10,000 rpm for 10 min. The supernatant fluid was discarded and the globulins were dissolved in 10 ml of distilled water. The globulins were precipitated again with 10 ml of 3.2 M ammonium sulfate, sedimented by centrifugation, and then dissolved in 4 to 5 ml distilled water. Residual ammonium sulfate was removed by dialysis against saline overnight. The protein content of the globulin solution was determined by the Biuret reaction. A sodium bicarbonate-sodium carbonate buffer was added to form 10% of the final volume and the solution was adjusted to pH 10. To the cold alkaline globulin solution was added 1 mg fluorescein isothiocyanate (FITC) for each 20 mg of protein. The mixture was stirred overnight at 4 C. Unlabeled FITC was removed by passing the conjugate through a Sephadex G50 column.

Table 9 presents the results obtained when 2-fold serial dilutions of the conjugates for TA686, TA716 and TA763 as well as those previously

TABLE 9

DIRECT IMMUNOFLUORESCENT STAINING OF STRAINS OF R. TSUTSUGAMUSHI

	Highest Dilution of Conjugate Producing Minimal (1+) Fluorescence of Rickettsial Strain*								
	Karp	Gilliam	Kato	TC 86	TA678	TA686	TA716	TA763	TH1817
Karp #700515	512	-**	128	-	-	128	128	128	64
Gilliam #700515	-	>512	-	512	-	-	-	-	-
Kato #700803	64	-	512	-	-	128	256	256	-
TA686 #710127B	-	-	-	-	-	128	-	-	-
TA716 #701030	256	-	512	-	256	512	>512	64	-
TA763 #701030	-	-	128	-	-	>512	>512	>512	-

* Antigen comprised of smears of infected yolk sac suspensions

** No staining at a dilution of 1:64

made for Karp, Gilliam and Kato were applied to smears of yolk sac suspensions of the 3 prototype and 5 candidate prototype strains of R. tsutsugamushi. Smears of TC586 were included also because of its interest as a substitute for Gilliam in experimental vaccines. Minimal reaction (1⁻) denotes that definite rickettsial morphology was recognizable although the intensity of fluorescence was slight. All conjugates were diluted in a diluent prepared by ether extraction of a 10% normal yolk sac suspension. The addition of Evans' blue in final concentration of 0.005% to the diluent greatly decreased background staining and autofluorescence, thereby enhancing the fluorescence of the rickettsiae.

The TA686 conjugate exhibited reactivity only with the homologous antigen. The Gilliam conjugate stained its corresponding antigen and TC586 which is considered to be a homotype of the Gilliam strain on the basis of complement fixation tests reported previously. The heterologous reactivity evident with the Karp conjugate did not preclude its use for specific identification because at a dilution of 1:256, which produced 2+ homologous fluorescence, it did not stain the other strains. Although the Kato conjugate could be used at a 1:256 dilution to differentiate it from Karp, TA686 and 4 other strains, the reagent would not distinguish between Kato, TA716 and TA763. The TA716 and TA763 conjugates were much less specific in reactivity and would differentiate the homologous strain from 4 other, and 6 other strains, respectively. If all of the prototype and candidate prototype strains are comprised of single antigenic types, then the heterologous reactivity exhibited by the conjugates was due to the sharing of common antigens. The antigenic interrelationship among these strains of scrub typhus that was apparent when the unconjugated rabbit immune sera were tested with homologous and heterologous antigens by indirect immunofluorescence has been described previously in this report. In general, the process of conjugating the antibody with fluorescein reduced or eliminated some of the heterologous reactivity evident in indirect fluorescent antibody tests. Attempts are continuing to produce more specific reagents by selection of rabbit immune sera containing higher levels of homologous antibody than used previously for conjugation and by adsorption with heterologous strain antigens.

Rabbit immune sera have been collected in order to prepare direct conjugates labeled with tetramethylrhodamine isothiocyanate (TMRITC) for use with fluorescein conjugates to identify the components of mixtures of strains by coincident staining. Thus far, only a Gilliam TMRITC-labeled reagent has been prepared and tested against the array of 9 prototype and candidate prototype antigens. The homologous titer was 1:128. The only heterologous reactivity was with the Gilliam homotype TC586 and the titer was 2-fold lower.

Work is in progress also to prepare conjugates for members of the Spotted Fever and Typhus Groups. To date, FI C-conjugated antisera for 2 strains of R. rickettsi and one strain of R. mooseri have been made. These reagents are group-reactive and will not differentiate among the different species in the respective groups. As time permits, efforts will continue to prepare direct immunofluorescent staining reagents that can be used for species identification of groups of rickettsiae other than scrub typhus.

(2) Influence of Transovarial Transmission in Leptotrombidum (L.) akamushi upon the Antigenic Composition of Scrub Typhus Rickettsiae. Because little is known about the extent of antigenic diversity that exists among strains of scrub typhus, the feasibility of developing a polyvalent killed vaccine that would afford significant protection against the disease can be questioned. This phenomenon of antigenic heterogeneity is unique since it has not been observed with other species of rickettsiae causing human disease. In order to obtain some insight into the complexity and magnitude of the problem a collaborative project was initiated with the U. S. Army Medical Research Unit (USAMRU), Malaysia, to determine if the antigenic composition of strains of R. tsutsugamushi remains constant through developmental stages and transovarial transmission in vector mites, as well as during the period of chronic infection in mammalian hosts. This report is concerned with the initial observations made in Malaysia and at WRAIR of the antigenic composition of the scrub typhus rickettsiae in a naturally-infected colony of Leptotrombidium (L.) akamushi developed and maintained by USAMRU in Malaysia.

The colony was derived in the following manner. Initially, 100 unengorged L.(L.) akamushi larvae collected in nature were fed together on a single normal white laboratory mouse. One adult male and female mite developing from the larvae were selected and mated. During the feeding the mouse was naturally infected with R. tsutsugamushi and the strain recovered from it was designated the parent strain, MF-1854. The relationship of the parent strain to the rickettsial organisms subsequently recovered from successive generations is unknown. It is unlikely that the female mite used to initiate the colony developed from the only larva in the group that was infected. The possibility that two or more of the original larvae were infected and transmitted their respective strains to the mouse at the time of feeding cannot be excluded. Indeed, the parent strain MF-1854 may be a mixture of antigenic diverse strains of which only one is the agent carried in the colony. Alternatively, the infected larva from which the colony was derived may not have infected the mouse with its agent. In this case the parent strain would then be completely unrelated to the scrub typhus rickettsiae in the colony.

Larvae emerging from eggs produced as the result of the first mating were fed individually on separate white mice, collected and reared to the adult stage whenever possible. Strains of R. tsutsugamushi were recovered from each of the mice used to feed these first generation larvae (F-1). All of the larvae from the first and next several generations developed into female mites and perpetuation of the colony was accomplished by mating them with male mites selected from another colony of L. (L.) akamushi that was proven not to be infected with R. tsutsugamushi. Strains of scrub typhus rickettsiae in successive generations were obtained in the same manner; i. e., by feeding of the sibling larvae individually on separate mice. The results of the study of the transovarial and filial infection rates in successive generations of the colony and the possible relationship of infection of the larvae and the sex of its adult stage have been reported elsewhere by Rapmund et al. (20).

The antigenic composition of the strain of R. tsutsugamushi transmitted by sibling larvae of successive generations was to be determined as follows. After the isolate from the individual mouse used for feeding had been established by 1 to 3 serial passages of spleen suspensions inoculated intraperitoneally into other normal mice, smears of peritoneum were to be prepared for direct immunofluorescent staining with fluorescein-labeled antibody specific for each of the 3 prototype and the 5 candidate prototype strains under study at WRAIR. These reagents were to be produced by the Department of Rickettsial Diseases and provided to USAMRU, Malaysia.

The numbers of rickettsiae in the smears were to be ascertained by microscopic examination of duplicate smears stained with Giemsa stain. Each of 3 guinea pigs was to be inoculated intracerebrally with a suspension of the spleens obtained from the same mice used for preparation of the peritoneal smears. Sera collected from the guinea pigs 28 days after inoculation were to be tested at WRAIR with strain-specific complement-fixing antigens prepared with the prototype and candidate prototype strains.

Although it has not yet been possible to prepare all of the immunofluorescent conjugates and complement-fixing antigens included in the plan, the results of tests that have been carried out with available reagents are sufficiently important to be reported.

Several years ago, USAMRU, Malaysia, kindly provided the Department of Rickettsial Disease, WRAIR, the parent strain, strains from 6 sibling larvae in the first generation (F-1) and strains from 32 second generation (F-2) larvae. During the current year, antigenic analysis of the parent strain, all F-1 strains and 12 of the F-2 strains was carried out as described above using anti-Karp, -Kato

and -Gilliam fluorescein conjugates for direct immunofluorescent staining of rickettsiae, and Karp, Kato and Gilliam complement-fixing antigens for testing the guinea pig sera. Table 10 summarizes the results of these tests and presents information about the family history of the strains and their virulence in mice. Based upon the effect of intraperitoneal inoculation of a 10 to 20% suspension of infected spleen into mice after at least two consecutive, uninterrupted serial passages; 4+ denotes that all mice were dead by the 14th day; 3+, all mice died between the 14th and 21st days; 2+, one or more mice survived the infection; and 1+ signifies that none of the infected mice died. The complement fixation tests with the 3 prototype antigens provided information about the major antigenic components of the larval strains. The titers presented indicate the range of antibody levels found in the serum from each of the 3 guinea pigs inoculated with the same strain. These data indicated that the parent strain, MF-1854, was Karp-like, whereas the F-1 and F-2 generation strains were Kato-like.

Inspection of the results of the immunofluorescent staining of the rickettsiae in mouse peritoneal smears indicated that the antigenic composition of the mite strains were different from the corresponding prototype strains (Table 10). One F-1 generation strain and seven F-2 generation strains that were classified as Kato-like on the basis of complement fixation tests were omitted from the table because rickettsiae were not seen in smears of mouse peritoneum either after immunofluorescent staining or in Giemsa-stained smears. The intensity of fluorescence of the mite strains was significantly less than that observed with the related prototype. Furthermore, 4 of the mite strains, except MF-2328 and MF-2651, were stained with two or more of the conjugates indicating interrelationships that were not found among the prototype strains themselves. Prior tests with anti-prototype conjugates diluted in normal yolk sac diluent showed the conjugates to be strain-specific. However, the results presented in Table 10 were obtained with 0.005% Evans' blue added to the diluent. Background staining and autofluorescence were decreased revealing a minor degree of staining of the Kato strain with the Karp conjugate that was not evident previously. It is apparent from these data that the antigenic composition of strains from sibling larvae from the same progenitor was different. Changes in the pattern of reactivity of the larval strains with the anti-prototype conjugates were unrelated to changes in mouse virulence.

Table 11 summarizes the results of the immunofluorescent tests of mite colony strains through the fifth generation performed at USAMRU, Malaysia, with the same anti-prototype fluorescein conjugates used at WRAIR. Information about the mouse virulence of these strains was not available. The complement fixation tests were performed at

TABLE 10

ANTIGENIC ANALYSIS OF R. TSUTSUGAMUSHI IN A NATURALLY INFECTED COLONY OF LEPTOTROMBIDIUM (L.) AKAMUSHI

Larval Strains				Immunofluorescent Staining with Anti-Prototype Conjugates			Complement-Fixing Antibody of Immune Guinea Pig Sera Prototype Antigens		
Generation	Progenitor	Designation	Mouse Virulence	Karp	Kato	Gilliam	Karp	Kato	Gilliam
Parent	Unknown	MF-1854	4+	1	±	±	320-640	----*	
F-1	MF-1854	MF-2327	4+	1	2	0	----	640-1280	----
		MF-2332	4+	±	1	0	----	1280	----
		MF-2328	2+	0	2	0	----	640-1280	----
		MF-2330	4+	±	±	0	----	640-1280	----
		MF-2334	2+	2	1	±	----	640-1280	----
F-2	MF-2327	MF-2567	2+	2	1	0	----	640	----
		MF-2651	4+	0	1	0	----	640-1280	----
		MF-2749	4+	1	1	0	----	320-1280	----
	MF-2332	MF-2569	4+	0	1	0	----	40-320	----
		MF-2624	2+	1	1	0	----	1280	----
Prototype Strains		Karp	4+	3	0	0	640	----	----
		Kato	4+	±	3	0	----	640	----
		Gilliam	4+	0	0	3	----	----	>1280

* Negative at initial 1:10 dilution

TABLE 11

ANTIGENIC ANALYSIS OF R. TSUTSUGAMUSHI IN A NATURALLY INFECTED COLONY OF LEPTOTROMBIDIUM (L.) AKAMUSHI

Larval Strains			Immunofluorescent Staining Reaction with Anti-Prototype Conjugates*		Kato CF Antibody Titers of Immune Guinea Pigs**
Generation	Progenitor	Designation	Karp	Kato	
Parent	Unknown	MF-1854	4	2	<10, <10
F-1	MF-185[illegible]	MF-2332	?	0	80, 160, 1280
F-2	MF-2332	MF-2569	0	0	10, 320, 1280
F-3	MF-2569	MF-2882	0	2	10, 10, 160
		MF-2883	1	1	<10, 40, 40
		MF-2885	1	1	20, 40, 40
		MF-2920	0	1	<10, <10, 40
		MF-2950	0	2	80, 160
F-4	MF-2883	MF-4100	0	2	40, 160
		MF-4101	0	0	40, 40, 80
		MF-4102	1	1	40, 80, 80
		MF-4103	0	2	80, 160
		MF-4104	0	2	80, 160, 320
F-5	MF-4102	MF-5546	2	[illegible]	80, 160, 160
		MF-5575	0	3	80 320, 320
		MF-5576	1	[illegible]	10, 40, 80
		MF-5577	0	3	80, 320, 320
		MF-5578	?	4	20, 40, 80
Prototype		Kato	0	4	640

* Reaction with anti-Gilliam conjugate was absent

** CF tests with Gilliam and Karp antigens were negative except with sera from guinea pigs infected with MF-1854 (see text)

WRAIR with serum from guinea pigs inoculated in Malaysia. For brevity, only the results of the complement fixation tests with the Kato antigen are included. All tests with the Gilliam antigen were negative and Karp antibodies were found only in serum from animals inoculated with the parent strain MF-1854. The Karp titers were essentially the same as found in sera from animals inoculated at WRAIR.

One or more of the guinea pigs infected with the F-1 through F-5 larval strains developed Kato complement-fixing antibody. Greater variation in the response of animals receiving the same inoculum, and generally lower titers were induced with mouse spleen suspensions of the F-3 through F-5 strains than with earlier generation strains. Explanation of this observation in the absence of results of tests with other prototype complement-fixing antigens and anti-prototype conjugates would be purely speculative.

Similar variations in the pattern of reactivity of strains from sibling larvae with the anti-Karp and anti-Kato conjugates were found in Malaysia. The negative reactivity with both conjugates, whenever present, was due to absence of rickettsiae in the peritoneal smears. Attempts are being made to develop methods for producing peritoneal smears from mice that uniformly contain heavy concentrations of rickettsiae. Until this is accomplished, it will not be possib'e to compare the results obtained by the two laboratories. Indeed, difference in concentrations of organisms in the smears may explain the apparent discrepancies between the results obtained in the respective laboratories with the parent strain and the F-1 strain MF-2332.

The changes in antigenic composition of the mite strains may be attributed to alteration of the relative concentrations of integral antigens in a single strain, or to variations in the relative population of two or more strains, if the mite colony is infected with a mixture of agents. Previously, it was observed that when a wild strain of R. tsutsugamushi was suspected of containing two or more types, two additional serial passages in mice provided an opportunity for changes in the relative populations of the strains to occur. This event could be detected when the serologic response of guinea pigs inoculated with higher passage material exhibited different relationships with the prototypes than was found previously. Guinea pigs inoculated with spleen suspensions from third passage mice infected with MF-1854 developed only Karp antibodies. After two additional passages, immune guinea pig sera fixed complement with the Kato antigen as well (Table 12). Comparison of the fifth and seventh passage levels of three F-2 generation strains, MF-2749, MF-2768 and MF-2677 showed the responses of infected guinea pigs to be the same. These data suggest that the parent strain was a mixture of

TABLE 12

ANTIGENIC ANALYSIS OF R. TSUTSUGAMUSHI IN A NATURALLY INFECTED COLONY OF LEPTOTROMBIDIUM (L.) AKAMUSHI

Strain Designation	Inoculum Passage Level*	Guinea Pig Number	Complement-Fixing Antibody Titers of Immune Guinea Pig Sera With Prototype Antigens		
			Karp	Kato	Gilliam
MF-1854	M-3	665	640	--**	---
		684	640	--	---
		686	640	--	---
	M-5	822	320	40	---
		823	320	40	---
		824	320	20	---
		835	>1280	80	---
MF-2749	M-5	756	--	320	---
		771	--	1280	---
		774	--	640	---
	M-7	855	--	80	---
		856	--	320	---
		862	--	80	---
MF-2768	M-5	779	--	640	---
		783	--	1280	---
		785	--	320	---
	M-7	862	--	320	---
		868	--	160	---
MF-2677	M-5	782	--	160	---
		789	--	160	---
		799	--	160	---
	M-7	857	--	640	---
		866	--	160	---
		869	--	640	---
Prototype	Karp		640	---	---
	Kato		---	640	---
	Gilliam		---	---	1280

* Number of serial passages in mice

** Negative at initial 1:10 dilution

two or more antigenic types and the F-2 generation strains were single types. When a method for plaque assay of R. tsutsugamushi is developed, it will be used to confirm these preliminary findings and evaluate the purity of larval strains in succeeding generations.

2. Evaluation of Existing and Potential Military Importance of R. canada

a. Investigation of the Etiology of Tick-Borne Rickettsial Disease in the Fort Bragg-Fayetteville Region of North Carolina.

The coordinated clinical and field study initiated in 1969 in collaboration with Womack Army Hospital and the Preventive Medicine Activity, Fort Bragg, was continued during the current year. Three of four patients suspected of having experienced a severe febrile illnes caused by R. canada contracted their disease at Fort Bragg (21). The aim of the project was: (a) to establish a causal relation between R. canada, or other unknown agents, with human disease; (b) to characterize the clinical features and pathophysiologic changes of the illness; and (c) to identify the tick vectors and vertebrate hosts involved in the infection cycle in nature.

Although patients with Rocky Mountain spotted fever or with R. canada infection were treated at Womack Army Hospital in previous years, no illness was seen during 1970 that was suspected of being rickettsial etiology. Surveillence for possible rickettsial infections will be continued this year and a concerted effort made to isolate the etiologic agents causing disease.

The emphasis of the field studies previously had concentrated on the live-trapping of small- and medium-sized mammals in the areas where patients with R. canada infection most likely acquired the infected tick. Because cases were not recognized during 1969, the program was changed to include random trapping throughout the area of the post with special attention to places where the risk of tick exposure for military personnel and their dependents, at work or at play, was greatest. Blood was obtained for serum from all animals trapped and all ectoparasites removed for identification. Complement fixation tests with spotted fever and typhus group, R. canada and Q fever antigens were performed on 157 specimens submitted during the current reporting period (see Table 13). The collection consisted of 102 sera from 9 different animal species and 55 from animals whose identity had not yet been established. Ten of the specimens were anticomplementary and unsuitable for evaluation. Serologic evidence of prior spotted fever infection was found in approximately 7% of the sera; i. e., in 5 of 66 cotton rats, in 3 of 5 rabbits, in 1 of 4 foxes and in 1 unidentified animal. No

TABLE 13

FORT BRAGG ZOONOSES SURVEY

FALL 1970 - SPRING 1971

Animals	Number Tested	Number Specimens AC++	Number Specimens SF Group Positive	CF++ Antibody Titers
Cotton Rat	70	4	5	4, 4, 8, 8, >16
Field Mouse	6	0	0	
Opossum	6	0	0	
Raccoon	6	0	0	
Rabbit	5	0	3	16, >16, >32
Fox	4	0	1	8
Norway Rat	2	0	0	
Squirrel	2	0	0	
White-Footed Mouse	1	1	0	
Undesignated	55	5	1	8
TOTALS	157	10	10	

+ Anticomplementary

++ Complement-fixing

evidence of murine typhus, R. canada or Q fever infection was found. Most of the seropositive animals were trapped in areas used by military personnel and their dependents for out-of-doors leisure activity. The Preventive Medicine Officer has been appraised of the risk of exposure to Rocky Mountain spotted fever during the summer season when man-biting vector ticks are active and abundant.

b. Investigation of the Etiology of Typhus and Spotted Fever Group Infections Occurring in South Vietnam.

To date, a total of six patients are suspected of having been infected with R. canada on the basis of the results of complement fixation tests with soluble group-reactive and species-specific antigens prepared from members of the Typhus and Spotted Fever Groups of rickettsiae. One patient was a soldier who became ill in Michigan 1 week after returning from South Vietnam. Until tests with species-specific complement-fixing antigens had been carried out, he was presumed to have been infected with R. mooseri. In addition to high levels of R. canada antibody, four of the patients also developed spotted fever group antibodies. The cross-reactivity of sera from patients infected with Typhus and Spotted Fever Group rickettsiae found in tests with antigens representative of the agents in both groups were reported last year (22). Briefly, the study showed that after infection with either R. rickettsi or R. mooseri, the patient does not develop antibodies that will fix complement with the heterologous group-reactive antigen unless he has been immunized previously with a vaccine prepared with a member of the other group of rickettsiae.

Review of the Infectious Disease Confirmation Reports from the 9th Medical Laboratory (Med Lab) over the past several years has shown rickettsial disease to be an important cause of febrile illness among the military in South Vietnam. During 1969, of 1,266 cases studied by the 9th Med Lab, 228 (18%) were diagnosed as scrub typhus, 195 (15%) as murine typhus and 16 (1.3%) as tick typhus (23). Thus, more than 33% of all the illnesses investigated were due to rickettsial infection. Serologic diagnosis was established by indirect immuno-fluorescence using reagents supplied by the Department of Rickettsial Diseases (DRD). Smears of R. mooseri or R. prowazeki were provided for recognition of Typhus Group infections, R. akari for detection of Spotted Fever Group infections, and a pool of the Karp, Gilliam and Kato strains of R. tsutsugamushi for the diagnosis of scrub typhus.

In order to assess the performance of the indirect fluorescent antibody test under field conditions, and to attempt to identify the etiology of the Typhus and Spotted Fever Group infections in South Vietnam, serum specimens from patients with rickettsial diseases were requested from the 9th Med Lab. They kindly provided two or more

specimens from 117 cases of murine typhus, 97 cases of scrub typhus and 9 cases of tick typhus. The results of the tests performed at WRAIR and the 9th Med Lab, using the same rickettsial strains as antigens are compared in Table 14. Of 80 murine typhus cases diagnosed by the 9th Med Lab, 95% were confirmed with the tests at WRAIR. Specimens from 3 of the cases were diagnosed as scrub typhus at WRAIR and did not contain typhus group antibody. It was not possible to establish a rickettsial etiology in one case because the specimens did not react with any of the antigens. Only 80% of the scrub typhus diagnoses were confirmed at WRAIR. This low rate of confirmation probably was due to the relatively small number of cases sampled, since in previous years it was possible to confirm almost 100% of their scrub typhus diagnoses. The results of tests on specimens from nine cases diagnosed as tick typhus were less impressive. Specimens from three of the cases were negative in all tests. Two of three other cases that were diagnosed as murine typhus at WRAIR did have spotted fever antibodies, but at significantly lower titers than the typhus group antibody. Sera from the other case reacted only with the murine typhus antigen. In two of the three remaining cases in which Spotted Fever Group infection was confirmed, the only reactivity found was a persistent titer of 1:40 in all of the specimens. This finding is indicative of a past rather than a current infection. The last patient's sera reacted only with the rickettsialpox antigen and this is probably a bona fide case of tick typhus.

These data indicated that difficulties were being experienced with the rickettsialpox antigen in South Vietnam. Although tests with complement-fixing antigens do not show heterologous cross-reactivity in sera from patients with Typhus and Spotted Fever Group infections under ordinary circumstances, the more sensitive indirect immunofluorescent test frequently detects antigenic relationships between members of these two groups of rickettsiae. Inspection of the results obtained by the 9th Med Lab showed that 58% of the specimens from cases diagnosed as murine typhus exhibited reactivity with the rickettsialpox antigen. However, the spotted fever group antibodies were significantly lower in titer than the typhus group antibody. The extent of the problem with the rickettsialpox antigen became evident when the results of tests performed by the two laboratories with convalescent sera from patients with Typhus Group infections and the rickettsialpox spotted fever group antigen were compared (Table 15). Tests at WRAIR confirmed the presence of spotted fever antibody in 51 (78%) of the 65 specimens found positive by the 9th Med Lab. However, 10 (83%) of the 12 specimens reported negative by the 9th Med Lab were found to have spotted fever antibody in tests at WRAIR. Analysis of the data showed that the 9th Med Lab's findings were confirmed at WRAIR in only 69% of the cases. At WRAIR,

TABLE 14

COMPARISON OF RESULTS OF INDIRECT IMMUNOFLUORESCENT TESTS WITH SERA FROM CASES OF SUSPECTED RICKETTSIAL DISEASES IN SOUTH VIETNAM

Serologic Diagnosis 9th Med Lab	Serologic Diagnosis Dept Rick Dis, WRAIR				
	Murine Typhus	Scrub Typhus	Tick Typhus	Unknown	Total
Murine Typhus	76	3		1	80
Scrub Typhus	1	15		1	17
Tick Typhus	3		3+	3	9
TOTAL	80	18	3	5	106

+ All specimens from 2 cases titered 1:40

TABLE 15

COMPARISON OF RESULTS OF INDIRECT IMMUNOFLUORESCENT TEST WITH SERA FROM PATIENTS WITH TYPHUS GROUP INFECTIONS WITH SPOTTED FEVER GROUP ANTIGEN

	Test Results DRD, WRAIR		
Test Results 9th Med Lab	SF Group+ Positive	SF Group Negative	Total
SF Group Positive	51	14	65
SF Group Negative	10	2	12
TOTAL	61	16	77

+ Rickettsialpox

80% of patients with Typhus Group infection developed antibodies that reacted with the rickettsialpox antigen.

When it was decided several years ago to provide the 9th Med Lab with antigen smears for the diagnosis of all rickettsial diseases by indirect immunofluorescene, R. akari (the agent of rickettsialpox) was the only Spotted Feve · Group organism that grew in the yolk sac of embryonated eggs in sufficient numbers for the preparation of antigen smears. It was known then that the rickettsialpox antigen tended to deteriorate more rapidly than the others under ambient conditions of high temperature and humidity. Nonspecific reactivity of low dilutions of certain human sera with particulate yolk sac components had been observed with this antigen at WRAIR. Similar findings may have been interpreted erroneously at the 9th Med Lab as being positive. Improved methods for cultivation of all species of Spotted Fever Group rickettsiae were developed last year, and in the future, smears of R. rickettsi which exhibit none of the unsatisfactory features of R. akari will be provided.

Details of the cross-reactivity observed in tests at WRAIR on convalescent sera from 77 patients who had Typhus Group infections in South Vietnam are presented in Table 16. Specimens were tested with R. mooseri, R. akari and R. canada antigens. R. canada is the most recently recognized member of the Typhus Group, and there is little known about its antigenic relationship with other Typhus Group species or members of the Spotted Fever Group. In 33 (43%) of the specimens, the murine typhus titers were 4- to 16-fold or greater than R. canada. The titers were equal in 41 (53%), and in 3 cases, 4-fold higher R. canada titers were found. The last-mentioned patients may have been infected with R. canada. It was not possible to establish the etiology as either a Typhus Group or Spotted Fever Group infection in only one patient. His serum titer was the same in tests with all three antigens.

The reactivity of convalescent sera from 27 Typhus Group patients with antigen smears of rickettsialpox and the Bitterroot strain of R. rickettsi are compared in Table 17. Antibody titers with Bitterroot antigen were 4-fold or greater than with rickettsialpox antigen in 5 (18%) of the specimens; the titers were the same with both antigens in 8 (30%), and significantly lower titers were obtained with the Bitterroot antigen in 11 (41%) of the patients. It is expected that the 9th Med Lab will find the Bitterroot antigen more suitable for the diagnosis of tick typhus than the rickettsialpox antigen.

Complement fixation tests with group-reactive and species-specific antigens are in progress. The results will be the subject of future reports.

TABLE 16
CROSS-REACTIVITY OF CONVALESCENT SERA FROM PATIENTS WITH TYPHUS GROUP INFECTIONS WITH TYPHUS GROUP AND SPOTTED FEVER GROUP ANTIGENS

Relative *R. canada* and Spotted Fever Group Titers	Relative Typhus Group Antibody Titers				
	RC > MT (4x)	MT = RC	MT > RC (4x)	MT > RC (≥16x)	Total
RC > SF (≥16x)	1	22	6	3	32
RC > SF (4x)		10	7	6	23
RC = SF	1	1	1	3	6
SF Negative	1	8	3	4	16
TOTAL	3	41	17	16	77

RC = *R. canada*
MT = Murine Typhus
SF = Rickettsialpox

TABLE 17

COMPARISON OF REACTIVITY OF CONVALESCENT SERA FROM PATIENTS WITH TYPHUS GROUP INFECTIONS WITH DIFFERENT SPOTTED FEVER GROUP ANTIGENS

Relative *R. canada* and Rickettsialpox Titers	Relative Spotted Fever Group Antibody Titers					
	BT > RP (16x)	BT >RP (4x)	BT = RP	RP > BT (4x)	BT Negative	Total
RC > RP (≥16x)	2	1	4	3	2	12
RC > RP (4x)		1	4	2	3	10
RC = RP				1		1
RP Negative		1			3	4
TOTAL	2	3	8	6	8	27

BT = Bitterroot strain of *R. rickettsi*

RP = Rickettsialpox

RC = *R. canada*

3. Canine Q Fever in Thailand

Results of a seroepidemiologic survey of rickettsial diseases in Thailand carried out during 1963-1964 showed that Coxiella burneti was widely distributed throughout the small wild animal populations in all of the major continental physiogeographic provinces. The incidence of enzootic infection was very low and infection of man was rare (24). Subsequent studies of cattle, pigs and water buffalo by the Thai Component, SEATO Medical Research Laboratory, Bangkok, found little evidence of infection of domestic animals. In contrast, when complement fixation tests were carried out with sera collected from 783 dogs in the municipal pound in Bangkok, Q fever antibodies ranging in titer from 1:10 to 1:160 were found in 386 (49.3%) of the specimens. All attempts to recover C. burneti from ticks infesting the dogs were negative (25).

A serologic epidemiologic study of viral respiratory diseases in Holland showed that serum from 30% of children convalescent from severe infections with adenovirus types 7 and 21 fixed complement in the presence of Q fever antigen. These findings were ascribed to a nonspecific reaction for the following reasons: (a) Q fever was rarely recognized in Holland and C. burneti was not enzootic in Dutch dairy herds; (b) the children had no known possible source of exposure to the disease; and (c) in complement fixation tests, 4- to 8-fold more antigen was required for optimal reactivity and heating the positive serum to 60 C destroyed the antibody activity (26).

The Department of Rickettsial Diseases was interested in confirming the results reported by the SEATO Medical Laboratory and determining if the presence of Q fever antibody in dogs was related to infection with human or canine adenovirus. Upon request, the Thai Component kindly provided 15 canine sera that titered 1:32 or greater and 15 negative sera. The LBCF 50% end point method of complement fixation adapted to the microtiter system was used for all tests. Known positive and negative sera, as well as normal tissue antigens corresponding to each of the diagnostic antigens, were included in each test. Dog sera were heat-inactivated at 53.5 C for 45 min.

The results of complement fixation tests performed at WRAIR with the same commercial antigen provided the Thai Component are compared with their findings in Table 18. It was not possible to confirm the presence of Q fever antibody in the sera found positive by the Thai Component because 13 of the specimens reacted nonspecifically with the normal yolk sac control antigen, as well as the Q fever antigen, and the other two were anticomplementary (AC). Titers of the nonspecific reactivity ranged from 1:10 to 1:80 or greater. Sera were classified as unsatisfactory because of nonspecific reactivity when the titer with

TABLE 18

COMPARISON OF RESULTS OF Q FEVER COMPLEMENT

FIXATION TESTS WITH CANINE SERA FROM BANGKOK, THAILAND

CF Test Results Thai Component SEATO LAB	CF Test Results Dept Rick Dis, WRAIR Number of Sera				
	Q Fever Positive (≥1:10)	Q Fever Negative	Specimens Unsatisfactory Reactivity with Control Antigen	Specimens Unsatisfactory Anti-Complementary	Total
Q Fever Positive (≥1:32)	0	0	13	2	15
Q Fever Negative	0	11	2	2	15
Total	0	11	15	4	30

the normal control antigen was 2-fold or greater than the AC activity. When both titers were equal, or higher levels of AC activity were found, the sera were considered AC. Attempts to remove the nonspecific reactivity and AC effect by treatment with canine sera and periodate (27) and with dry-ice (28) were unsuccessful.

Canine sera is notorious for the ease with which it becomes AC when subjected to heat treatment for inactivation of complement. Consequently, the usefulness of a procedure for decomplementation with sensitized sheep red blood cell stromata developed by the Department of Serology, WRAIR, is being evaluated.

Microagglutination tests with highly purified Henzerling Phase I and Nine Mile Phase I antigens were negative, as were indirect immunofluorescent tests. It should be noted that a canine serum known to contain Q fever antibody was not available for use as a control in these tests.

The possible relationship between Q fever antibody and adenovirus infection was evaluated by examining the Bangkok canine sera for adenovirus antibodies, and by testing sera from dogs experimentally infected with adenovirus for Q fever antibody. Human adenovirus complement-fixing antigen and the control antigen were obtained from a commercial source. Dr. L. N. Binn, Department of Veterinary Microbiology, Division of Veterinary Medicine, kindly supplied the following materials: (a) complement-fixing antigens prepared in dog kidney cells with the Utrecht strain of infectious canine hepatitis and the C955L strain of Toronto canine adenovirus, as well as normal control antigens; (b) 10 serum specimens from two dogs collected before and at regular intervals until the 91st day after infection with the Toronto strain; and (c) 15 sera collected from four dogs before infection or vaccination with infectious canine hepatitis, and at regular intervals thereafter up until the 91st day.

The results of the adenovirus complement fixation tests of the Bangkok canine sera are presented in Table 19. None of the sera fixed complement in the presence of the human adenovirus antigen. Sera considered positive for canine adenovirus reacted with either the Toronto or infectious canine hepatitis antigen, or both. Titers ranged from 1:10 to 1:80 or greater. There was no obvious relationship between the presence or absence of adenovirus antibody and the results of the Thai Component Q fever complement fixation tests. Adenovirus antibody was found in 6 sera, 3 in the Q fever positive group, and 3 in the Q fever negative group.

None of the six dogs experimentally infected or immunized with canine adenovirus developed Q fever antibodies (Table 20).

TABLE 19

PREVALENCE OF CANINE ADENOVIRUS COMPLEMENT-FIXING

ANTIBODY IN DOGS FROM BANGKOK, THAILAND

CF Test Results Dept Rick Dis, WRAIR

Number of Sera

CF Test Results Thai Component SEATO LAB	Canine Adenovirus Positive (≥1:10)	Canine Adenovirus Negative	Specimens Unsatisfactory: Reactivity with Control Antigen	Specimens Unsatisfactory: Anti-Complementary	Total
Q Fever Positive (≥1:32)	3	2	2	8	15
Q Fever Negative	3	11	0	1	15
Total	6	13	2	9	30

TABLE 20

PREVALENCE OF Q FEVER COMPLEMENT-FIXING ANTIBODIES

IN SERA FROM DOGS EXPERIMENTALLY INFECTED OR IMMUNIZED

WITH CANINE ADENOVIRUS

Canine Adenovirus CF Test Results	Q Fever CF Test Results — Number of Sera				
	Positive (≥1:10)	Negative	Specimens Unsatisfactory: Reactivity with Control Antigen	Specimens Unsatisfactory: Anti-Complementary	Total
Positive (≥1:10)	0	15	4	0	19
Negative	0	6	0	0	6
Total	0	21	4	0	25

Microagglutination tests with the Henzerling and Nine Mile strains were also negative. The adenovirus negative sera were the pre-inoculation specimens from the animals. Reactivity with the human adenovirus antigen was found only in one dog. The pre-inoculation specimen and the serum collected 21 days after infection with infectious canine hepatitis titered 1:80 or greater. Subsequent specimens collected on the 42nd, 56th and 91st days, which contained high levels of antibody detected by both the Utrecht and Toronto antigens, were negative in tests with the human adenovirus antigen.

Summary and Conclusions.

1. Scrub Typhus Vaccine Development

a. Antigenic Analysis of Prototype Strains

(1) Scrub Typhus Complement-Fixing Antigens from Infected Yolk Sacs. Definition of the geographic distribution of established prototype and candidate prototype strains of Rickettsia tsutsugamushi and the recognition of different antigenic types is dependent upon the production of strain-specific complement-fixing antigens. Karp, Gilliam and Kato antigens can be prepared regularly. Continued passage of the 5 candidate prototype strains, TA678, TA686, TA716, TA763 and TH1817, in embryonated eggs has not yet resulted in the emergence of variants more suitable for growth in the yolk sac. Numerous attempts to produce antigens in order to complete their antigenic analysis have failed because of insufficient concentrations of rickettsiae in infected yolk sacs.

(2) Scrub Typhus Complement-Fixing Antigens from Cell Cultures. Large scale tissue culture production methods employing roller bottles were used for the growth of scrub typhus rickettsiae. Antigens prepared from Vero-infected cells generally were anti-complementary (AC) and unsatisfactory. However, antigens prepared from BS-C-1 and 14pf cells were less AC and most of this undesirable activity due to host cellular components could be removed by differential centrifugation through 20% w/w sucrose solution. Differences were observed in the reactivity of antigens produced by infection of different cell lines with the same seed suspensions in tests with heterologous immune sera. These findings may have been due to variations in concentrations of major and minor component antigens of a single strain, or to the presence of two or more antigenically different agents in the inoculum.

(3) Antigenic Interrelationships Among Prototype Scrub Typhus Strains. The antigenic interrelationship among the 3 prototype strains, Karp, Gilliam and Kato and the 5 candidate prototype strains, TA678, TA686, TA716, TA763 and TH1817 was characterized by indirect immunofluorescent tests with immune rabbit sera. The pattern of

[illegible] infection with the agents revealed similarities [illegible] strains. Although TC586 was considered to [illegible] because of cross-reactivity [illegible] complement [illegible], the present studies showed its antigenic composition [illegible] different from the Gilliam strain. [illegible] is considered [illegible] prototype strain. Based upon the relative [illegible] heterologous reactivity found, it was postulated that the [illegible] P1817, TC586, TA67[illegible] possibly [illegible] would be expected to [illegible] protection [illegible] all 9 antigenic types.

[illegible] System for R. tsutsugamushi

Attempts have been made to develop a method for cloning [illegible] candidate [illegible] strains to insure purity. A concerted [illegible] to reproduce exactly the plaque system for rickettsiae using chick embryo fibroblasts reported by other workers has been only partially successful. Difficulties attributed to inadequate wetability of the growth surface of plastic culture vessels were identified and corrected. Moderate success was achieved with R. rickettsi which produced plaques 5 to 6 days after inoculation and to a lesser extent with R. mooseri where plaques were formed after 10 to 11 days. Comparison of the titers of seed suspensions of these organisms showed the tissue culture plaque system to be greater than 10-fold more sensitive than embryonated hens' eggs for assaying infectivity. A problem that yet has to be resolved is concerned with maintaining the cell monolayers for the 16 to 18 days required for production of plaques of R. tsutsugamushi.

c. Characterization of the Immune Response of Mice after Infection and Challenge with Scrub Typhus

The immune response of mice treated with chloramphenicol after inoculation with 10^2 LD_{50} of the Karp strain and subsequently challenged with $10^{5.5}$ LD_{50} of the Kato strain was characterized. Antibody levels in pools of serum collected from the animals and in sucrose density gradient fractions of the specimens were determined by indirect immunofluorescent (IF) and complement fixation (CF) tests with Karp, Kato and Gilliam antigens. The anti-mouse whole serum fluorescein conjugate reacted with antibodies belonging to both the IgM and IgG classes of immunoglobulins. The CF test detected only IgG antibodies. The evidence accumulated thus far indicated that the antibody response after primary infection and after challenge were both of the primary type. Thus, the postulate that after initial infection the animal develops homologous immunity under the protection of antibiotic treatment, and becomes primed for a secondary booster response to antigens shared with the heterologous strain after challenge,

could not be substantiated. Alternate mechanisms to explain the protection afforded mice by infection against subsequent lethal challenge will be investigated.

d. Development of a Tissue Culture Scrub Typhus Neutralization Test

Antibodies detected by indirect immunofluorescence and complement fixation, the only serologic tests currently available for study of scrub typhus immunity, are not related to the ability of experimental animals to resist lethal challenge. The only method that will provide valid information about the immune status of man or experimental animals is to determine the response of the host to infectious challenge. Consequently, attempts are in progress to develop a means of measuring neutralizing antibody that will be indicative of immunity against scrub typhus that develops after infections or immunization with experimental vaccines. Thus far, it has been possible to enumerate R. tsutsugamushi by counting, microscopically, the number of BS-C-1 cells infected with rickettsiae after staining with antirickettsia fluorescein conjugates. Titers expressed as tissue culture cell infectious units calculated from the mean values obtained were of the same magnitude as 50% mouse infectious doses (ID_{50}). However, because of problems encountered in differentiating rickettsiae from normal intracellular particles, it has not been possible to enumerate the rickettsiae reliably when the concentration of organisms in the inoculum was less than 2,240 mouse ID_{50}. With the substitution of 14pf cells and improvements in the procedure for immunofluorescent staining, it is hoped to increase the sensitivity of the system. Parallel studies have been initiated with guinea pig peritoneal macrophages. Titers of suspensions of scrub typhus rickettsiae calculated from mean values expressed as tissue culture cell infectious units were slightly lower than those obtained in BS-C-1 cells. When the sensitivity of the two cell systems is defined and enumeration of rickettsiae can be accomplished accurately, attempts to demonstrate neutralization with rickettsial antibody per se, and in combination with the species anti-IgG immunoglobulin, will be initiated. Neutralization in both systems will be indicated by a significant reduction in numbers of infected cells.

e. Study of Genetic Stability of R. tsutsugamushi in Vector Mites

(1) Preparation and Performance of Type-Specific Antirickettsial Serum Fluorescein Conjugates. Methods for rapid, specific identification of antigenically distinctive strains of scrub typhus were required to determine if changes in antigenic composition of R. tsutsugamushi occurred during transmission from generation to generation of vector mites. Attempts are in progress to prepare fluorescein-and rhodamine-conjugated antisera for each of the 3 prototype and 5 candidate

prototype strains for this purpose, as well as to [illegible] the detection of mixtures of strains. Initial efforts to use IM sera containing antibodies principally of the IgM class were limited [illegible] because of insufficient titers. Of the four strain-specific conjugates prepared this far, only the Karp, Gilliam and TA686 reagent [illegible] differentiate the homologous agent from the others. Heterologous reactivity of the Kato, TA716 and TA763 conjugates with as few as two [illegible] as many as five other strains precludes their use for specific identification. A Gilliam rhodamine conjugate was highly specific [illegible] reactivity only with itself and the Gilliam homotype, TA686. Efforts are continuing to prepare specific fluorescent antibody reagents for the scrub typhus strains and also for the other species belonging to the Typhus and Spotted Fever Groups of rickettsiae.

(2) Influence of Transovarial Transmission of L. (L.) akamushi upon the Antigenic Composition of Scrub Typhus Rickettsiae. A collaborative study with the U. S. Army Medical Research Unit in Malaysia was initiated to determine if the antigenic composition of a strain of R. tsutsugamushi in a naturally-infected colony of Leptotrombidium (L.) akamushi remains constant through developmental stages and transovarial transmission in this species of vector mite. The evidence accumulated thus far suggests that the parent strain, recovered from a mouse used to feed 100 larvae from which the colony was established, contained at least two antigenic types which are related to the Karp and Kato prototype strains. Analysis of strains from the first through the fifth generation larvae by complement fixation tests with Karp, Kato and Gilliam antigens showed all of them to be related to the Kato strain. Immunofluorescent staining of the larval strains with anti-Karp, -Kato and -Gilliam fluorescein conjugates showed them to be different from the corresponding prototype strain. Futhermore, differences were observed in the antigenic composition of strains from sibling larvae from the same progenitor in all 5 generations. It is not yet known if the changes in antigenic composition were due to alteration of the relative concentrations of integral antigens in a single strain, or to variation in respective populations in a mixture of two or more strains.

2. Evaluation of Existing and Potential Military Importance of R. canada

a. Investigation of the Etiology of Tick-Borne Rickettsial Disease in the Fort Bragg-Fayetteville Region of North Carolina

No cases with suspected rickettsial disease were admitted to Womack Army Hospital, Fort Bragg, North Carolina last year. Serologic tests on sera from small- and medium-sized mammals trapped at Fort Bragg showed that some of the cotton rats, rabbits and foxes had been infected with R. rickettsi. Enzootic foci of R. canada infection have not yet been identified.

b. Investigation of the Etiology of Typhus and Spotted Fever Group Infections Occurring in South Vietnam

Indirect immunofluorescent tests performed by the 9th Medical Laboratory over the past several years with reagents supplied by the Department of Rickettsial Diseases, WRAIR, have shown rickettsial infections to be an important cause of febrile disease in military personnel in South Vietnam. In 1969, when 1,266 cases were studied, 18% were diagnosed as scrub typhus, 15% as murine typhus and 1% was considered to be tick typhus. In response to a request, the 9th Med Lab kindly provided the serum specimens from the majority of the cases of rickettsial diseases. The Department of Rickettsial Diseases was interested in: (a) assessing the performance of the indirect fluorescent antibody test under field conditions; (b) attempting to identify the etiology of the Typhus and Spotted Fever Group infections to determine if R. canada was causing disease in South Vietnam; and (c) accumulating more information about antigenic interrelationship among Typhus and Spotted Fever Group rickettsiae. Using the same rickettsial strains as antigens, as was supplied to the 9th Med Lab, 95% of the cases diagnosed as murine typhus, and 89% of the scrub typhus diagnoses were confirmed at WRAIR. The indirect fluorescent antibody test provided information only about the group of rickettsiae to which the etiologic agent belonged. Since murine typhus is the only member of the Typhus Group known to be present in South Vietnam, all illnesses caused by a Typhus Group agent were presumed to be murine typhus. The relatively poor performance of the test with respect to scrub typhus may be related to the small number of cases sampled; i. e., only 17 of 97 submitted. It was possible to confirm current or past spotted fever (tick typhus) infection in only 3 of the 9 cases diagnosed by the 9th Med Lab. A comparison of the results obtained by each laboratory showed that the greatest discrepancies were in tests with sera from patients with Typhus Group infection and the rickettsialpox spotted fever group antigen. In the future, R. rickettsi will be supplied for use in the detection of Spotted Fever Group infections.

Complement fixation tests with heterologous antigens rarely show cross-reactivity with antibodies in the serum of patients with Typhus Group or Spotted Fever Group infections. However, the more sensitive indirect immunofluorescent test clearly demonstrates intergroup antigenic relationship. Tests performed at WRAIR with antigen smears of R. mooseri, R. canada and R. akari showed that 80% of 77 patients with Typhus Group infection developed antibodies that reacted with the rickettsialpox antigen. Three of these cases had significantly higher R. canada titers, and their illness may have been caused by infection with that agent. Complement fixation tests

with group-reactive and species-specific antigens to establish the etiology of the Typhus Group infections are in progress.

5. Canine Q Fever in Thailand

Prior serologic evidence indicated that (a) Q fever infection of man in Thailand seldom occurred; (b) the level of enzootic infection in small wild animals was low; and (c) infection of domestic animals was rare. In contrast to this, serum from over 40% of the dogs in the Bangkok municipal pound were positive in Q fever complement fixation tests performed at the SEATO Medical Research Laboratory. Tests were carried out by the Department of Rickettsial Diseases, WRAIR, to confirm the findings and determine if there was a relationship between Q fever and adenovirus infection as had been observed in human disease.

It was not possible to interpret the results of the complement fixation tests because all of the sera found positive in Thailand were unsatisfactory in tests at WRAIR because of nonspecific reactivity with a normal yolk sac antigen or they were anticomplementary. Attempts to remove these undesirable qualities of the specimens failed. Microagglutination and indirect immunofluorescent tests with Q fever antigens were entirely negative. There was no apparent relationship between the presence of canine adenovirus antibody in the specimen and the results of the complement fixation tests in Thailand. Dogs experimentally infected or vaccinated with canine adenovirus did not develop Q fever complement-fixing or agglutinating antibodies.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 167, Rickettsial diseases in military personnel

Literature Cited.

References:

1. Kawamura, A., Shimojo, H., Yamani, K., Hara, H., and Aikawa, K.: Preparation of Complement-Fixing Antigen of Rickettsiae. Jap. J. Exper. Med. 28: 317-327, 1958.

2. Shishido, A., Kohno, S., Hikita, M., Iida, T., Kawashima, H., and Kawamura, A.: Complement Fixation and Direct Immunofluorescence for Typing of Tsutsugamushi Disease Rickettsia. Acta Med. Biol. 15: (Suppl) 87-95, 1967.

3. Weinberg, E. H., Stakebake, J. R., and Gerone, P. J.: Plaque Assay for Rickettsia rickettsii. J. Bact. 98: 398-402, 1969.

4. McDade, J. E., Stakebake, J. R., and Gerone, P. J.: Plaque Assay System for Several Species of Rickettsia. J. Bact. 99: 910-912, 1969.

5. McDade, J. E.: Determination of Antibiotic Susceptibility of Rickettsia by the Plaque Assay Technique. Appl. Microbiol. 18: 133-135, 1969.

6. McDade, J. E., and Gerone, P. J.: Plaque Assay for Q Fever and Scrub Typhus Rickettsiae. Appl. Microbiol. 19: 963-965, 1970.

7. Rights, F. L., and Smadel, J. E.: Studies on Scrub Typhus (Tsutsugamushi Disease). III. Heterogeneity of Strains of R. tsutsugamushi as Demonstrated by Cross-Vaccination Studies. J. Exper. Med. 87: 339-351, 1948.

8. Rickettsial Infections, in: Annual Progress Report Walter Reed Army Institute of Research, 1 July 1967 - 30 June 1968.

9. Kunkel, H. G.: Macroglobulins and High Molecular Weight Antibodies, Chapter in: The Plasma Proteins, edited by F. W. Putnam. Academic Press, Inc., New York, p. 294, 1960.

10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193: 265-275, 1951.

11. Ouchterlony, O.: Antigen-Antibody Reactions in Gels. IV. Types of Reactions in Coordinated Systems of Diffusion. Acta Path. Microbiol. Scand. 32: 231-240, 1953.

12. Crowle, A. J.: Enhancement by Cadmium of Double-Diffusion Precipitin Reactions. J. Immunol. 81: 194-198, 1958.

13. Rickettsial Infections, in: Annual Progress Report Walter Reed Army Institute of Research, 1 July 1966 - 30 June 1967.

14. Bennett, B. L., Smadel, J. E. and Gould, R. L.: Studies on Scrub Typhus (Tsutsugamushi Disease). IV. Heterogeneity of Strains of R. tsutsugamushi as Demonstrated by Cross-Neutralization Tests. J. Immunol. 62: 453-461, 1949.

15. Barker, L. F., Patt, J. K., and Hopps, H. E.: Titration and Neutralization of Rickettsia tsutsugamushi in Tissue Culture. J. Immunol. 100: 825-830, 1968.

16. Hahon, N., and Nakamura, R. M.: Quantitative Assay of Psittacosis Virus by the Fluorescent Cell-Counting Technique. Virol. 23: 203-208, 1964.

17. Hahon, N., and Cooke, K. O.: Fluorescent Cell-Counting Neutralization Test for Psittacosis. J. Bact. 89: 1465-1471, 1965.

18. Williams, T. D., and Hahon, N.: Serum Neutralization Anti-IgG Test for Psittacosis. Inf. and Imm. 2: 7-10, 1970.

19. Wisseman, C. L., Jr.: personal communication.

20. Rapmund, G., Upham, R. W., Jr., Kundin, W. D., Manikumaran, C., and Chan, T. C.: Transovarial Development of Scrub Typhus Rickettsiae in a Colony of Vector Mites. Trans. Roy. Soc. Trop. Med. Hyg. 63: 251-258, 1969.

21. Bozeman, F. M., Elisberg, B. L., Humphries, J. W., Runcik, K., and Palmer, D. B., Jr.: Serologic Evidence of Rickettsia canada Infection of Man. J. Inf. Dis. 121: 367-371, 1970.

22. Rickettsial Diseases of Military Personnel, in: Annual Progress Report Walter Reed Army Institute of Research, 1 July 1968 - 30 June 1969.

23. Read, John K., LTC, MSC: personal communication.

24. Rickettsial Infections, in: Annual Progress Report Walter Reed Army Institute of Research, 1 July 1964 - 30 June 1965.

25. SEATO Medical Research Study on Rickettsial Diseases in Thailand, in: Annual Progress Report SEATO Medical Research Laboratory, 1 April 1968 - 31 March 1969.

26. Veen, J. Vander: Verschillen in het epidemiologische gedrag von respiratory virusinfecties. Nedrl. T. Geneesk. 111: 2099-2103, 1967. (Dutch).

27. Bucca, M. A., and Alder, A. O.: Method for Eliminating Anticomplementary Activity in Animal Sera. Appl. Microbiol. 18: 291-293, 1969.

28. Imam, I. Z. E., and Alfy, L.: The Elimination of the Anti-Complementary Reaction of the Sera by CO_2. J. Egypt. Publ. Health Assn. 41: 33-36, 1966.

Publications:

1. Elisberg, B. L.: Q Fever. Chapter in: Current Therapy, 1971 ed., W. B. Saunders Co., Philadelphia, pp. 39-40, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6443	71 07 01	CSGRD(AR)1A

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DOD'N INSTR'N	8b. SPECIAL DATA (CONTRACTOR ACCESS)	9. LEVEL OF SUM
70 07 01	D. Change	U	U	NA	NI	YES / NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3A061102B710	00	168
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Include with Security Classification Code)
(U) Bacterial Diseases (09)

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:
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 71	10	250
CURRENT FY 72	11	275

19a. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
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RESPONSIBLE INDIVIDUAL
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TELEPHONE: 202-576-3551

20. GENERAL USE
Foreign Intelligence Not Considered

19b. PERFORMING ORGANIZATION
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ASSOCIATE INVESTIGATORS
NAME: DA
NAME:

PII Redacted

21. KEYWORDS (Precede Each with One Asterisk)
(U) N. meningitidis; (U) Bacteria: (U) Mycoplasmas; (U) L-Forms; (U) Immunology; (U) Endotoxin; (U) Air Sampling; (U) Antibiotics; (U) Viral Pneumonia; (U) Adenovirus

22. TECHNICAL OBJECTIVE, 23. APPROACH, 24. PROGRESS (Numbered paragraphs identified by same numbers used in blocks 22, 23, and 24.)

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 and mycoplasma infections in military forces.

24 (U) - Development of bacteriologic techniques - isolation, identification, antibiotic sensitivity tests, etc. - for study of various infectious diseases. Field studies on prophylactic regimens, spread and persistence of organisms in various military populations.

25 (U) - 70 07 - 71 06 A bactericidal serotyping technique has been developed which has identified a specific epidemic strain of serogroup C as the cause of most of the meningococcal disease recorded in the past 5 years. Three new serologic tests have been perfected; a radioactive polysaccharide binding assay has proven extremely sensitive, quantitative and group specific; a C^{14} labelled bacterial lysis test has compared favorably with the standard bactericidal assay; a latex fixation test has been found to be sensitive, simple, and group specific. Serotype factor II antigen of group C meningococci has been extracted, partially purified and characterized as a protein. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

25. Available to contractors under contractual agreement

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 168, Bacterial diseases

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Description.

Studies of meningococcal disease explored microbiological and host factors. By means of a bactericidal serotyping technique a specific serotype of group C meningococcus has been incriminated as the epidemic strain prevalent since 1966. The type specific antigen has been extracted from the bacterial cell wall and has been shown to be a protein.

New serological tests have utilized radioactive antigens to provide highly sensitive and quantitative assays. A latex fixation test may provide the simplicity and stability to become adapted as a routine serologic test of infection or vaccination.

Penicillin sensitivity of meningococci has not changed significantly. A haptene inhibition test for serogrouping of meningococcal isolates has been developed which should significantly conserve time and reagents as well as improve the accuracy of the identification of isolates.

Special diagnostic bacteriological studies on clinical specimens have provided valuable data useful in management of patients. Wall-defective variants of Treponema pallidum have been created by treatment

with penicillin and/or lysozyme but could not be propagated serially. A taxonomic scheme for Mollicutes (mycoplasmas) has been devised and minimum standards for definition of species have been recommended.

Progress.

1. Meningococcal disease.

a. Epidemiology - Identification of an epidemic strain of group C Neisseria meningitidis.

During the seven year period, 1964 through 1970, a marked change occurred in the pattern of serogroups of Neisseria meningitidis associated with systemic disease in Army recruits. Although group B strains accounted for over 85 percent of the 200-300 cases per year before 1966, in 1966 at Fort Dix and in 1967 at other training posts group C strains began to increase in prevalence (Table 1).

Table 1. Number of group B and group C strains isolated from cases of meningococcal disease at Fort Dix and other recruit training centers - 1964 through 30 July 1970.

	Fort Dix			Other Posts		
Year	No. of B strains	No. of C strains	% of C strains resistant	No. of B strains	No. of C strains	% of C strains resistant
1969	22	0	0	237	29	4
1965	14	3	0	139	19	11
1966	33	18	83	193	22	45
1967	6	49	96	47	33	72
1968	5	150	99	32	125	93
1969	5	65	98	32	243	98
1970	0	75	100	4	122	96

The percentage of cases caused by group C meningococci has continued to rise, reaching 96 percent in 1970. Even though mass chemoprophylaxis with sulfonamides had not been used at Army posts since 1964, the strains responsible for the rising frequency of group C meningococcal disease were almost all sulfadiazine resistant. A similar change, though occurring more gradually, has been observed in the civilian population.

A method of identifying antigenically distinct serotypes among group C strains has recently been described. This report presents the results of applying the bactericidal serotyping technique to 143 strains isolated from cases of group C meningococcal disease in military and civilian populations. The distribution of serotypes found in this sample suggests that a single serotype was responsible for the changing patterns of meningococcal disease.

Materials and methods:

Selection of strains: Five groups of strains were studied (Table 2).

Table 2. Strains studied by serotyping.

	Number	Source	Years
1.	47	Fort Dix - cases	1965-1970
2.	23	Fort Lewis - cases	1968-1970
3.	32	Other posts - cases	1964-1970
4.	21	Army dependents	1964-1970
5.	20	Civilians (CDC)	1969-1970

Bactericidal assay: A significant modification of the original procedure concerned the normal rabbit serum used as a source of complement. Sera obtained from the rabbits available to us were found to kill most strains of meningococci in the absence of specific antiserum. Such nonspecific killing could be eliminated by absorption of the sera with a group C strain (1381 or 321) before use as a complement source. Absorption was performed by transferring the organisms from an overnight growth on one BYE agar plate to 5 ml. of normal rabbit serum and incubating for one hr. in an ice bath at 0°C. The organisms were removed by centrifugation followed by filtration through a 0.45 mμ millipore filter. The absorbed serum was stored in 1 or 2 ml. aliquots at -70°C and was thawed immediately before use. Because of some loss of complement activity during absorption the concentration of normal rabbit serum was increased to 20 percent instead of the 10 percent originally employed.

Anti-factor sera were prepared as previously described. In the course of this study several strains were found which were not killed by antisera to factors I through IV. Antisera were prepared against these strains and after appropriate cross absorptions factors V and VI were identified. The anti-factor sera were used in a final dilution of 1:20, a dilution which resulted in 90 percent killing of the homologous strains but no killing of heterologous group C strains.

Results:

Definition of serotypes: The 143 strains were grouped into six serotypes which were defined by the presence of one factor common to all strains of a given serotype. Strains of serotypes 3, 5 and 6 contained only one factor: III, V and VI respectively. Serotypes 1, 2 and 4 were more complex in that many strains contained factors in addition to the one defining the serotype. Of the 23 strains in serotype 1, 10 had only factor I while 11 had factors I and IV. Eighty-eight strains were grouped together in serotype 2: 15 had only factor II, 32 had factors II and III, 10 had factors II and IV, and 31 had factors II, III and IV. Finally, 20 strains were assigned to serotype 4: 9 had factor IV alone, 11 had factors III and IV, and 1 had factors IV and V.

The consequence of this particular serotype scheme is that cross reactions occur between certain strains of serotypes 1, 2 and 4 as a result of the sharing of factors III and IV. However, as will be shown below, the separation of these serotypes appears to be justified since strains containing factor II have been responsible for the epidemics of group C disease in military recruits.

Fort Dix. The serotypes identified in the strains isolated at Fort Dix between 1965 and 1970 are presented in Table 3.

Table 3. Serotypes identified from cases of group C meningococcal disease at Fort Dix between 1965 and 1970.

Year	No. of strains studied	Serotype			
		1	2	4	6
1965	2	1	1	0	0
1966	11	0	10	1	0
1967	10	0	10	0	0
1968	6	0	5	0	1
1969	8	1	7	0	0
1970	10	1	9	0	0
	47	3	42	1	1

Although 4 different serotypes were found, 42 of the 47 strains were serotype 2.

Fort Lewis. The serotypes found among the 23 strains isolated from cases in basic trainees at Fort Lewis between October 1968 and May 1970 are indicated in Table 4.

Table 4. Serotypes identified at Fort Lewis.

Date of isolation	No. of strains	Serotype		
		2	4	NT*
Oct 68 - Feb 69	4	0	4	0
Mar 69 - May 69	14	10	3	1
Jun 69 - Jun 70	5	5	0	0
	23			

*Nontypable

An epidemic of group C disease occurred at Fort Lewis between February and May 1969. Serotype 2 strains accounted for 10 of 14 isolated during March-May 1969.

Other posts. The distribution of serotypes among the 32 strains isolated between 1964 and 1970 at posts other than Fort Dix is given in Table 5.

Table 5. Serotypes identified from cases of group C meningococcal disease at posts other than Fort Dix and Fort Lewis between 1964 and 1970.

Year	No. of strains studied	Serotype			
		1	2	4	NT*
1964	5	2	1	2	0
1965	4	1	1	1	1
1966	8	1	4	3	0
1967	4	0	4	0	0
1968	2	0	2	0	0
1969	4	0	4	0	0
1970	5	1	3	0	1
	32	5	19	6	2

*Nontypable

Three serotypes were found: 1, 2 and 4. Two strains were not typable with the available anti-factor sera. Of the 17 strains recovered before 1967, only 6 were serotype 2. However, 13 of the 15 strains isolated after 1967 were serotype 2.

Civilian strains. Five serotypes were found among the 12 strains recovered from military dependents. No serotype 2 strains were found in 11 cases occurring before 1967; 8 of 10 strains recovered after 1967 were serotype 2. Three of the 20 strains isolated from civilians not related to military personnel were nontypable. Four serotypes were found in the 17 typable strains as indicated in Table 6.

Table 6. Serotypes identified from cases of group C meningococcal disease in civilians.

Source of strains	No. studied	Serotype					
		1	2	3	4	5	NT
Military Dependents	21	8	8	1	2	2	0
CDC	20	7	5	1	4	0	3

Relationship between serotype and sulfadiazine susceptibility. Twenty of 23 serotype 1 strains had MIC's of 1.0 mcg/ml. or less. Eighty-three of the 88 serotype strains were resistant to greater than 1.0 mcg/ml. Between these two extremes were the 21 serotype 4 strains, 10 of which were inhibited by 1.0 mcg/ml. Eight of the 11 strains of serotypes 3, 5 and 6 and NT were sensitive.

Discussion:

These results suggest the existence of an epidemic strain of group C meningococci (serotype 2) which also is resistant to sulfadiazine. Recent studies by other workers have shown a similar phenomenon based upon a meningocin typing scheme. As yet, the two techniques have not been compared on the same set of strains. The next section of this report will describe the serotype specific antigen which has been extracted from the whole organism. These new tools should provide considerable new insights into the epidemiology of and immunity to the meningococcus.

b. Meningococcal antigens - Serotype antigens of N. meningitidis.

N. meningitidis has been the subject of immunochemical analysis for over 35 years. During this time, definitive evidence has been obtained which shows that group specificity resides in the capsular

polysaccharide. However, investigation of other cell surface antigens has been almost totally neglected. That they exist and are of some significance can be deduced from the many reports in the literature of intergroup cross-reactivity and intra-group heterogeneity.

As described above by using a bactericidal assay it has been possible to identify distinct serotypes within the meningococcal serogroup C.

Recent work has succeeded in isolating and identifying the antigens responsible for this serotype specificity.

Methods:

The organisms for the antigen extraction were grown in 10 liters of Mueller-Hinton broth in a microferm fermentor for 18 hrs. at 37°C. They were removed from the media by continuous flow centrifugation, washed three times and suspended in saline to a 10% w/v concentration. Using a modified Ribi ether extract, two volumes of cold ether were added to the saline suspension. The ether-saline-organisms suspension was stirred for 18 hrs. in the cold and then transferred to a separatory funnel to stand overnight. The mixture separated into essentially two phases: the upper ether phase and the lower aqueous phase. The lower phase was carefully drained off and organisms were removed by centrifugation and discarded. The saline supernatant was concentrated 5X by ultrafiltration over an Amicon PM 30 membrane and dialyzed against Tris-HCl buffer over the same membrane. This dialyzed retentate was the crude antigen (Fig. 1).

The bactericidal inhibition test was used to assay for the presence of the type specific antigen.

Fig. 2 illustrates bactericidal inhibition obtained with varying dilutions of crude antigen. It can be seen that a linear response in inhibition is obtained with increasing concentrations of protein antigen.

The three prototype meningococcal strains for this investigation were chosen on the basis of their serologic patterns: two group C strains that were of different serotypes (60E = type I; 1381 = type II). One serogroup B strain whose serotype was identical to one of the other serogroup C strains was also chosen (99M - type II).

In Table 7 is shown the serotype specific inhibition with the crude antigens.

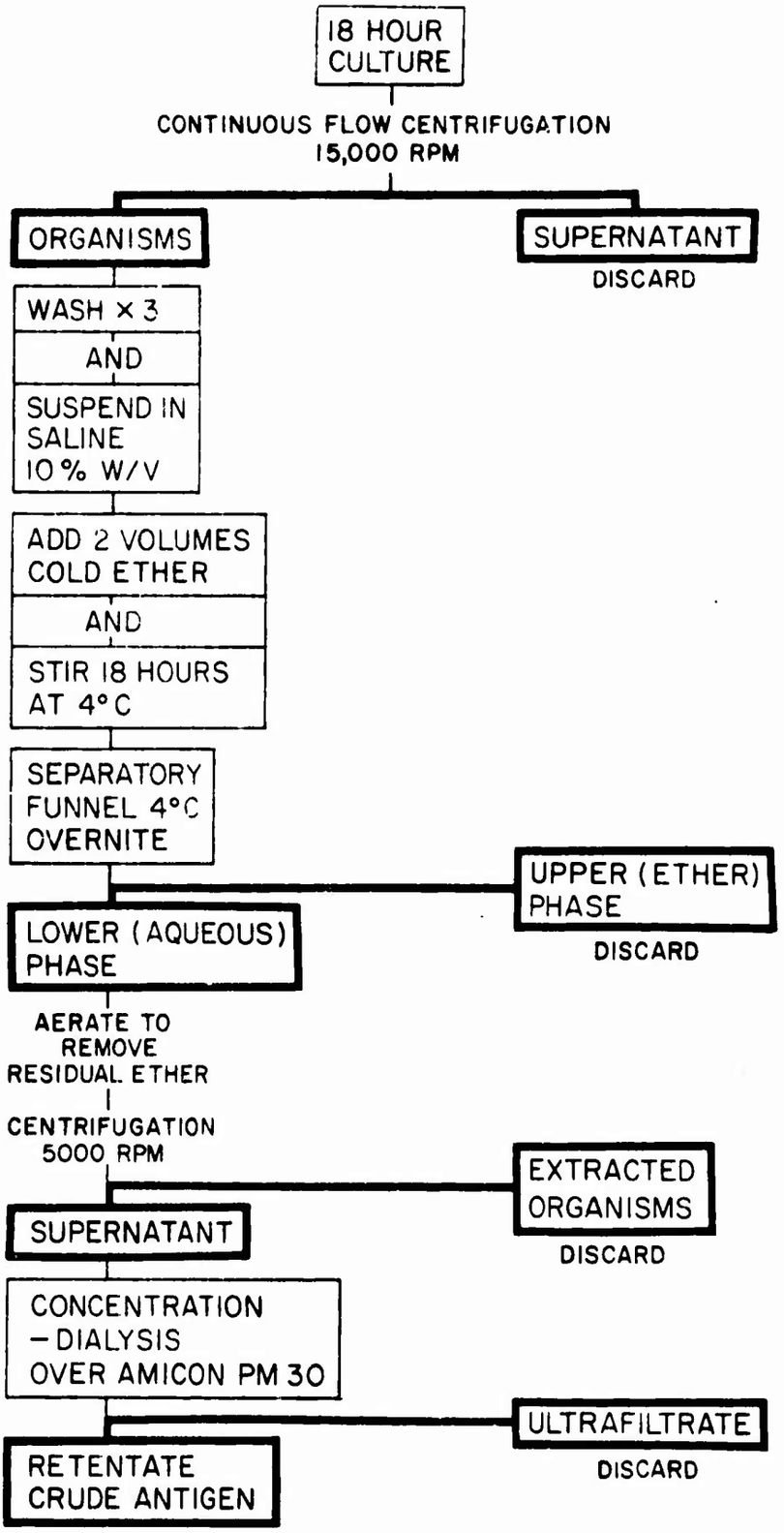


Fig. 1. Procedure for extraction of serotype antigen (crude).

Fig. 2. Bactericidal inhibition of serotype specific antibody with crude antigen extract.

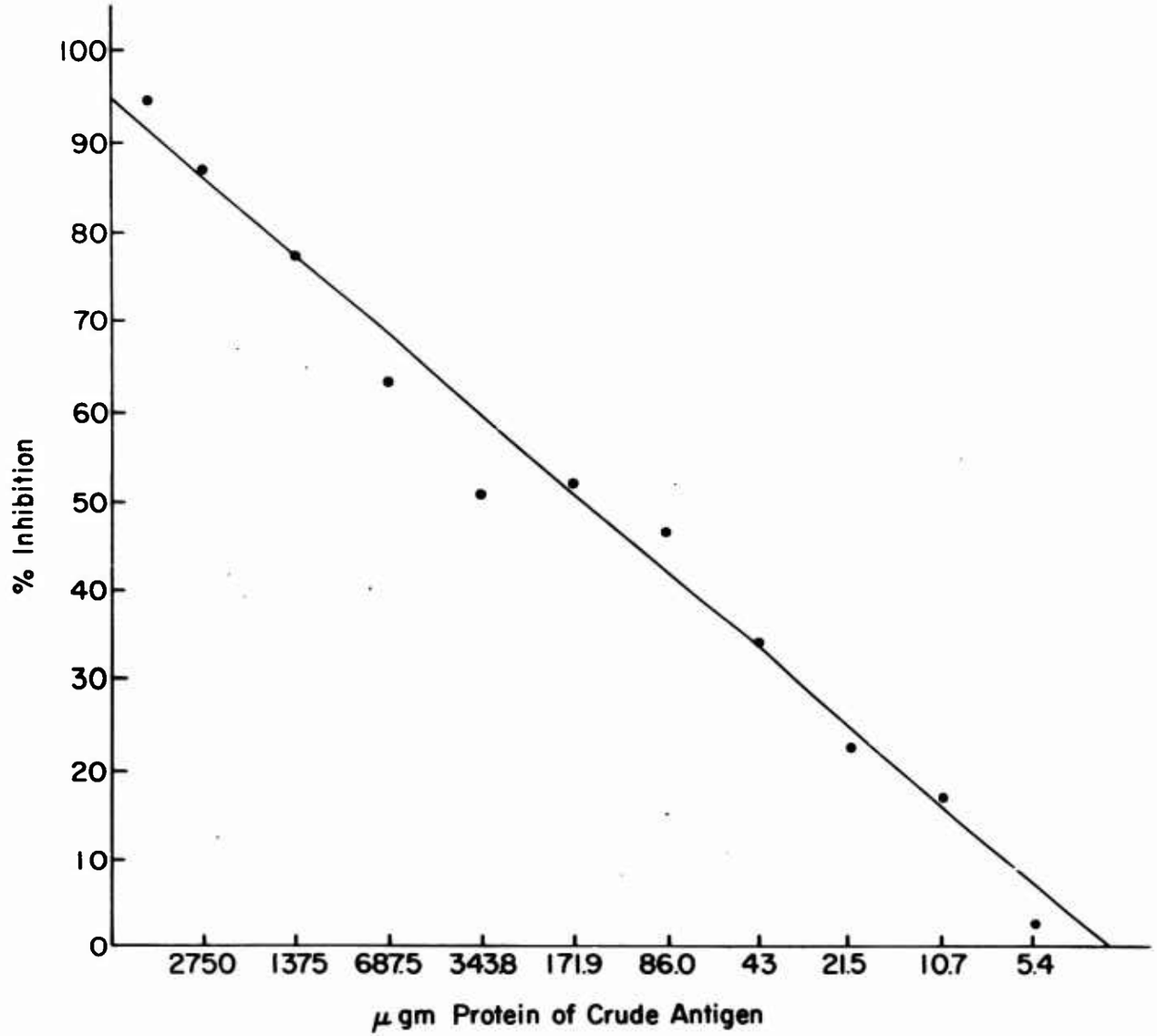


Table 7. Bactericidal inhibition by crude antigens.

	Antigens extracted from		
Serotype	60E (I)	1381 (II)	99M (II)
I_C	100%	0%	0%
II_C	5%	100%	94%
II_B	3%	98%	100%

The crude antigens extracted from strains 1381, 60E and 99M gave inhibition patterns that demonstrated their serotype specificity. The antigen extracted from strain 1381, a group C serotype II organism, inhibited only the type II system. Strain 99, a group B serotype II organism and its extracted antigen inhibited only the type II system. Strain 60E is a group C, serotype II organism and its antigen inhibited only the type I system.

Preliminary purification of the serotype antigen was accomplished using Sepharose 4B gel filtration chromatography (Fig. 3). Pretreatment of the crude antigen with RNASE eluted the antigen activity almost free from nucleic acids.

To further test the relation between protein and inhibitory activity, trypsin digestion was studied (Table 8).

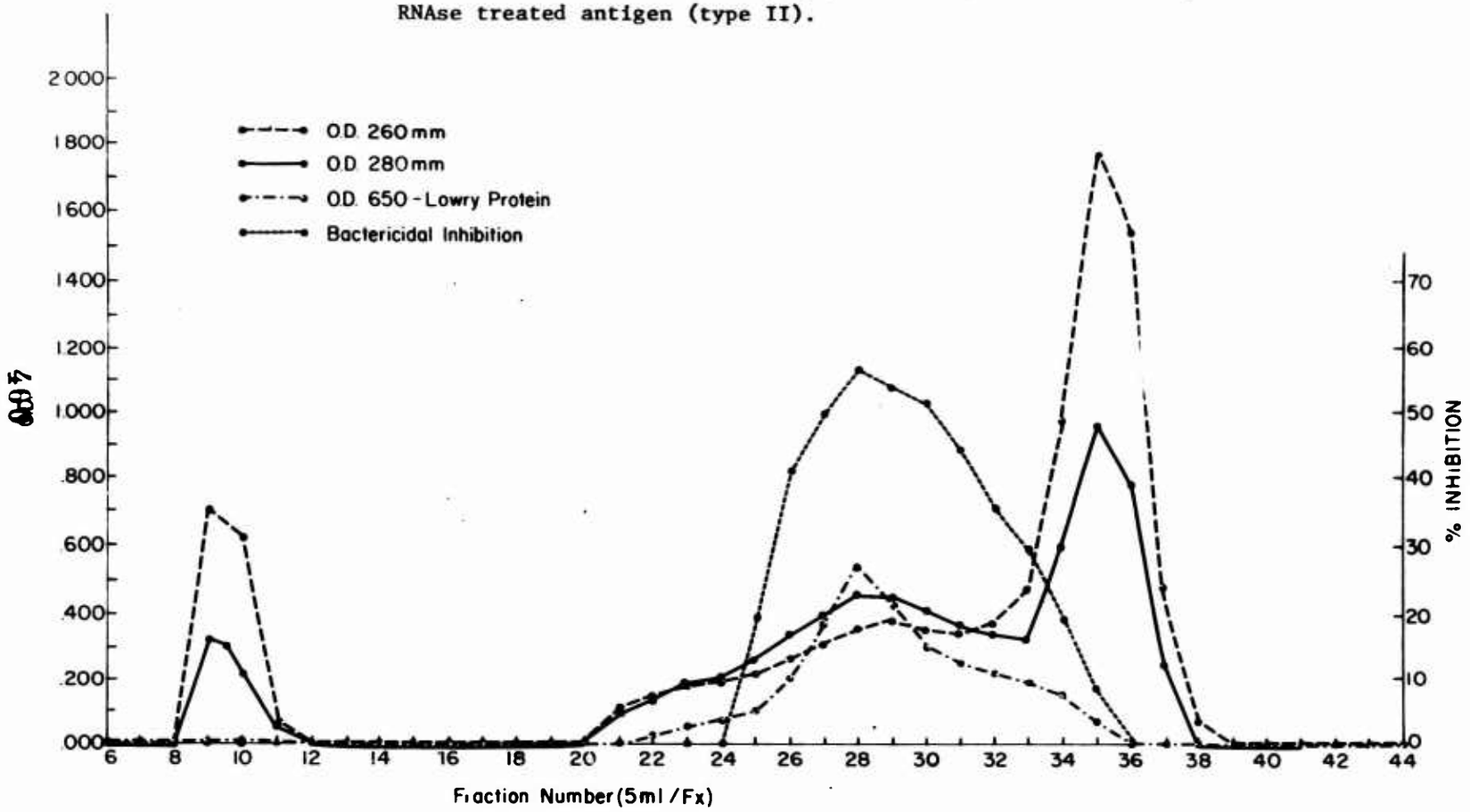
Table 8. Effect of trypsin on crude antigen.

Inhibitors	% Inhibition
Untreated antigen	100%
Trypsin treated antigen	3%
Trypsin alone	0%

The results showed 100% inhibition of bactericidal activity by the untreated antigen and elimination of almost all this inhibitory activity after trypsin treatment.

To test the immunogenicity of crude antigens, rabbits were immunized with the antigens and the sera were tested in the bactericidal assay. Not only was high titered antisera produced

Fig. 3. Sepharose 4B column chromatography of crude, ether extracted, RNAse treated antigen (type II).



but the sera demonstrated the same serotype specificity obtained in the inhibition tests (Table 9).

Table 9. Bactericidal activity of rabbit antisera produced against [illegible] antigens.

Rabbit antisera against crude antigen	Titer* against homologous organisms in bactericidal test	
	99M (II)	60E (I)
99M	1:320	0
1481	1:320	0

*50% release endpoint of the complete bactericidal assay.

Preliminary toxicity studies in embryonated eggs have shown that while the partially purified antigen from Sepharose 4B chromatography has some toxicity, it was less so in the fractions eluted earlier from the column. Whether this means that the antigen is inherently toxic or that it is contaminated with endotoxin has yet to be determined.

While the structural significance of the meningococcal serotype antigen is not yet known, it is believed to have a cell surface location for two reasons: first, in the intact viable organism, the antigen is readily available to bind with antibody as demonstrated by the bactericidal reaction. Second, other workers have reported that ether extraction removes the outer layer of the meningococcal cell wall leaving the murein layer and the rest of the cell intact.

The answer as to whether this antigen is part of the classical protein-lipopolysaccharide complex of gram negative organisms or a separate cell wall protein must await the results of further purification and analysis. Studies are now in progress.

c. Serological tests.

(1) Radioactive antigen binding assay for antipolysaccharide N. meningitidis antibody

With the recent development of methods for preparing highly purified meningococcal polysaccharide antigens and vaccines the need for a highly sensitive and quantitative antibody assay has become apparent. None of the presently available techniques fulfill both of these requirements. Therefore, a radioactive antigen binding assay modified from the technique described by Kerr was studied. This type

of test is very sensitive since it measures the primary interaction between antigen and antibody.

The present report describes the development and use of an antigen binding assay (ABC) for the detection of antipolysaccharide antibodies for group B and C meningococci.

Methods:

Intrinsically labeled group specific meningococcal polysaccharide antigens were prepared by growing the organisms in the presence of C^{14} labeled sodium acetate in modified Frantz medium. After the cultures had grown, the organisms were removed by centrifugation and the supernatant fluid was sterilized by millipore filtration. The filtrate was both concentrated and dialyzed over an Amicon-PM-30 membrane.

Gel permeation chromatography was used to purify the crude antigens. Antigen was applied to a Sepharose 4B column and was eluted with Tris-HCl sodium chloride buffer. Fractions were collected and assayed for both radioactivity and sialic acid content, since the group B and C polysaccharides are polymers of sialic acid.

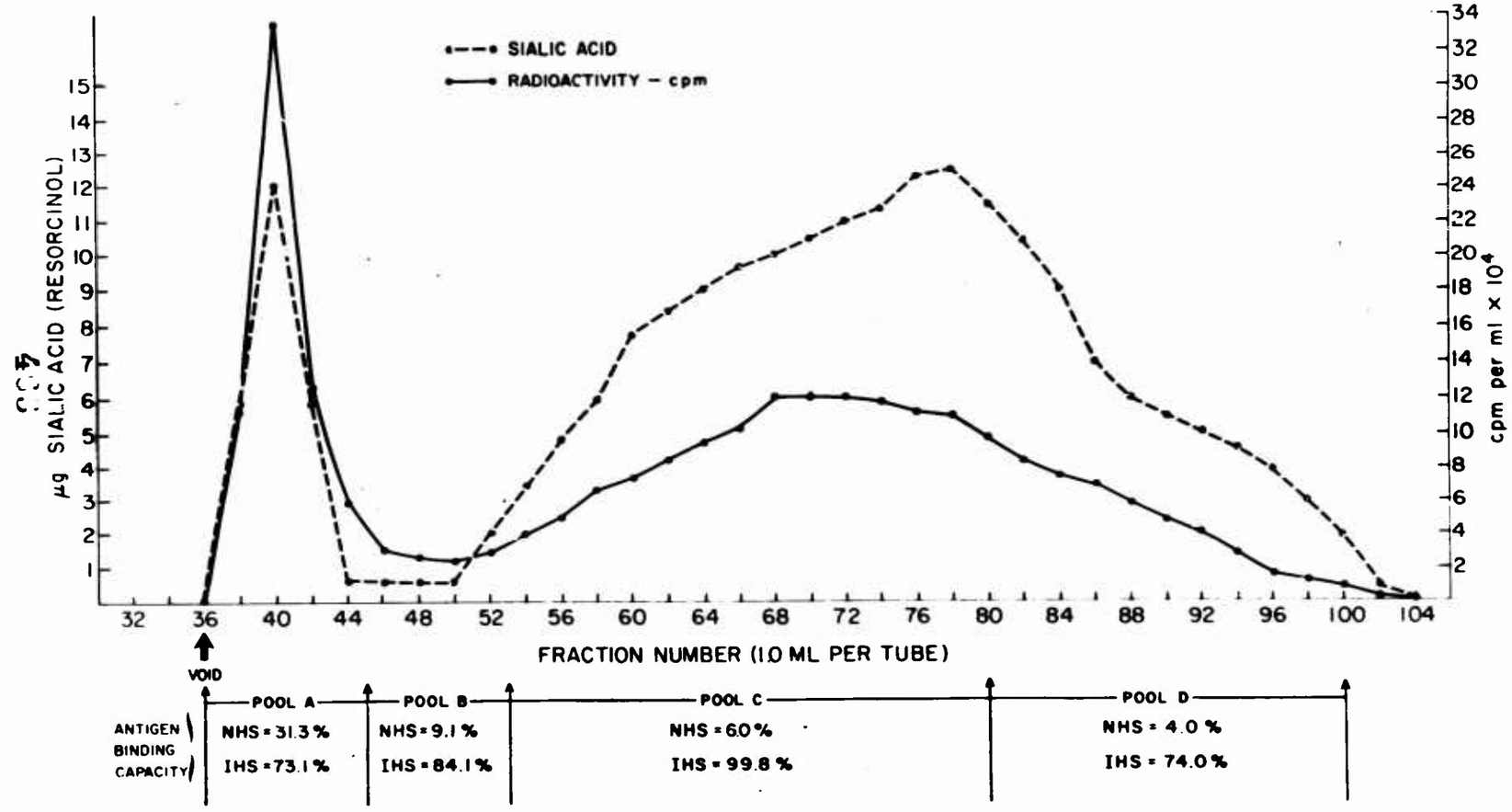
Fig. 4 illustrates a typical elution pattern for both the group B and C polysaccharides. The solid line shows the radioactivity in counts per minute, whereas the broken line gives the sialic acid content as measured by the resorcinol test. The horizontal lines at the bottom of the graph illustrate the pools made from the fractions collected.

The elution pattern demonstrates two peaks which contained both sialic acid and radioactivity. The first peak (pool A) while containing a significant amount of sialic acid, also contained another labeled substance which gave a high (21.3%) binding with normal human sera. Pool C, the second peak, was the antigen of choice for further studies since it showed a 6% or lower reactivity with normal human sera and the highest reactivity (99% precipitation) with the immune sera.

The specific activity of the B polysaccharide antigen was 2,500 cpm per microgram of sialic acid; the C polysaccharide, 5,100 cpm per microgram of sialic acid. These radioactive antigens have been found to be very stable at 4°C, giving similar results with control sera over a period of six months.

The reaction mixture for the radioactive antigen binding assay consists of undiluted serum, borate buffer and C^{14} labeled polysaccharide antigen. After an incubation of 16-18 hrs. at 4°C, one volume of saturated ammonium sulfate was added. A 50 percent ammonium sulfate concentration precipitates antibody and in this case the antibody-antigen complex. Antigen alone was not precipitated with 50 percent

Fig. 4. Sepharose 4B elution pattern of radioactive group B polysaccharide antigen.



ammonium sulfate. The reactants were incubated for 30 minutes at 4°C, followed by centrifugation. The collected precipitate was then washed once with 50 percent ammonium sulfate and air dried. It was then dissolved in solubilizer and washed into a counting vial with scintillation fluid. Samples were counted in a Packard Tri-Carb liquid scintillation counter. Controls consisted of a normal human serum, three positive sera, a background and a vial containing antigen alone.

The antibody level in the test serum was calculated by dividing the counts per minute in the test serum by the counts per minute in the antigen added control. The results are expressed as the percent of the total antigen added that was precipitated.

Results and Discussion:

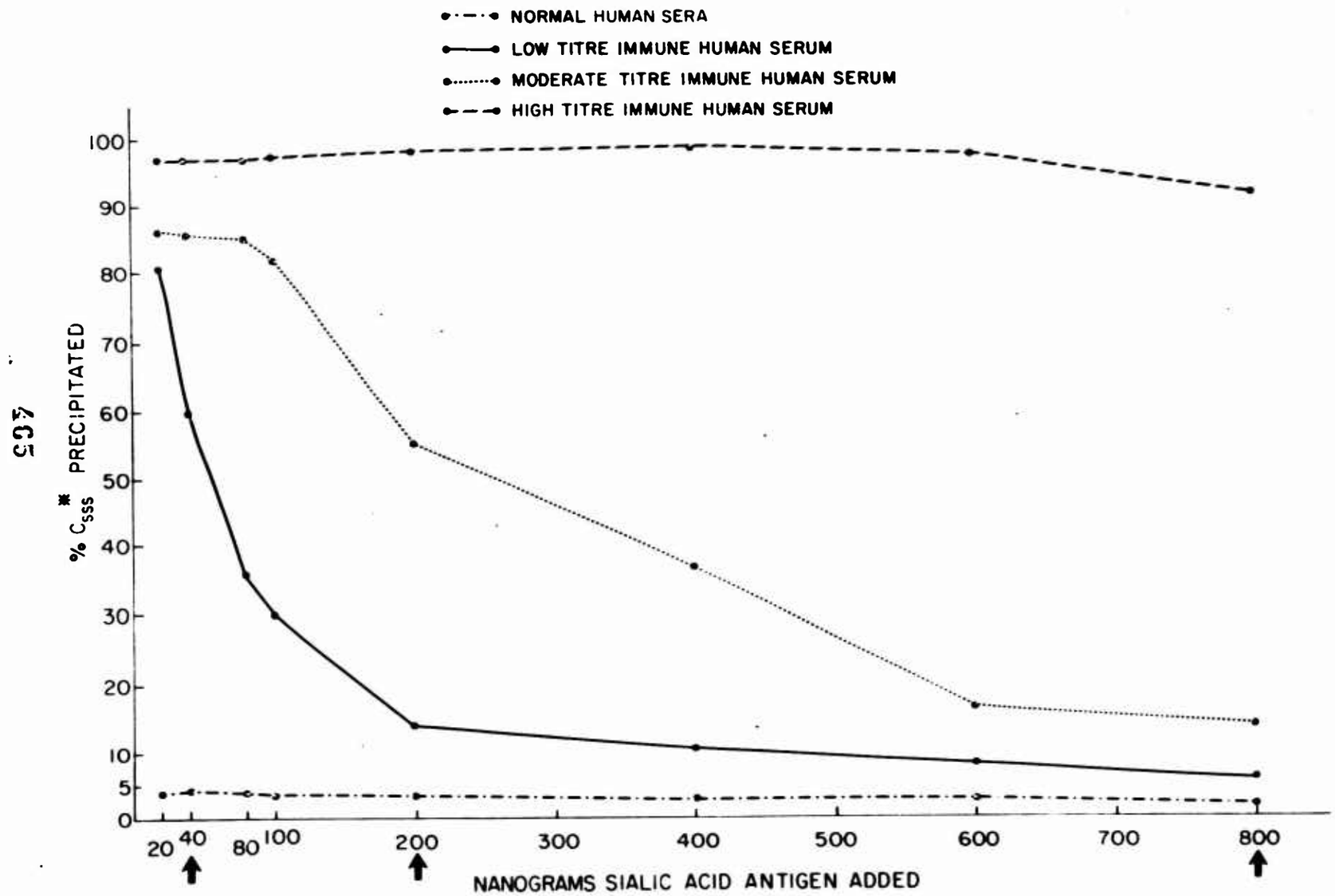
Fig. 5 shows a titration of the labeled group C antigen with sera from three adult volunteers immunized with the group C polysaccharide vaccine as well as a normal human serum. These sera were chosen because they represented a high, intermediate and low antibody response. The ordinate indicates the amount of C polysaccharide which was precipitated; the abscissa represents the amount of antigen added in nanograms of sialic acid. As can be seen with the serum of intermediate titer, the antigen titration results in a sigmoid type curve with plateaus in the regions of both antigen and antibody excess, and with a relatively linear response in the region between the extremes. The high titered serum demonstrates only the plateau in the region of antibody excess; the low titered serum shows the antigen excess plateau and part of the linear portion.

This figure also illustrates that at high antigen concentration, as is shown by the arrow at the right, only very small differences in the percent antigen precipitated could be demonstrated between the normal, low and medium sera. By decreasing the antigen concentration the percent antigen bound was mathematically increased, and this enabled greater discimination between the lower titered sera. The antigen concentration can be reduced to such a small quantity that almost all sera tested show an 80 percent or greater binding. The low antigen concentrations were useful for demonstrating differences in the percent of antigen precipitated with very low titered sera.

The passive hemagglutination test, because of its simplicity and specificity, has been used extensively in our laboratory to measure antipolysaccharide antibodies. Compairson of HA titers and ABC of sera from a large number of adult volunteers who received group C vaccine showed a very good correlation between the two tests. A correlation coefficient of +0.8936 was calculated with p less than 0.001.

The real need for an antigen binding assay was demonstrated when

Fig. 5. Titration of radioactive group C polysaccharide antigen against human immune sera.



the group C polysaccharide vaccine was administered to children. Many of the immunized children showed no detectable change or only a low level 1-. tube change in group C hemagglutination titers. Even the one or two tube change was not significant, since the hemagglutination test varies plus or minus one tube in reproducibility. By modifying the antigen binding assay, that is, using less antigen, it was possible to obtain greater discrimination with the low titered sera. The antigen binding assay demonstrated significant changes between the prevaccination and post-vaccination sera in 93 percent of the children whereas only 55 percent of these same children showed antibody increase when measured by the passive hemagglutination test.

Table 10 demonstrates the serologic specificity of the group B and C antigen binding assays using hyperimmune rabbit sera to eight of the meningococcal serogroups. Except for a one way cross reaction between the C antigen and the B sera the test was serogroup specific.

Table 10. Specificity of A.B.C. assay.

	% precipitated antigen	
Rabbit Antiserum	B* Test	C* Test
Normal	3.1	5.7
Group A Mgc	2.8	3.3
Group B Mgc	92.6	23.8
Group C Mgc	5.4	100.0
Groups X, Y, Z, 29E, 135 Mgc	3.7	5.4

*C^{14} labeled polysaccharide

The same specificity was found with sera from humans immunized with the group A and C polysaccharide vaccines and individuals with systemic disease caused by the group B and C meningococcus (Table 11).

Repeated studies performed over a period of four months have shown that the binding assay has a reproducibility of ±5 percent.

Conclusions:

The radioactive assays for groups B and C polysaccharide antibodies have proven to be extremely sensitive and highly quantitative. It will be used with increasing frequency in studies of immunity and vaccination.

Table 11. Specificity of A.B.C. assay.

Human sera	% precipitated antigen	
	B* Test	C* Test
Group A Vaccine		
Prebleed	5.3	3.0
4 weeks	5.2	4.3
Group C Vaccine		
Prebleed	2.6	6.6
4 weeks	2.1	92.9
Group B Case		
Acute	3.5	4.8
7 days	84.0	17.4
10 days	83.0	16.6
Group C Case		
Acute	6.2	6.9
7 days	7.6	97.0
30 days	7.7	80.8

*C^{14} labeled polysaccharide

(2) Serum bactericidal assay using C^{14} labeled *N. meningitidis*.

The serum bactericidal assay is an important tool in the study of immunity to disease caused by bacteria. In the meningococcal research field the bactericidal assay has been instrumental in the study of natural and vaccine induced antibodies. In addition, this assay system has been important in defining certain specific antigens in the bacteria, as evidenced by the serotyping scheme which has been described above.

In order to devise a more simplified and efficient method of measuring bactericidal activity a series of studies were undertaken which utilized lysis of radioactive organisms.

Methods:

Organisms were grown in Mueller-Hinton broth containing 4 µc/ml of C^{14} acetate. Maximal uptake of radioactivity occurred at about the mid-log phase of growth and the resulting specific activity approximated 5000 cpm/10^6 organisms. Then, mid-log phase organisms were centrifuged, washed and suspended in cold Gey's salt solution at 4°C. Preliminary experiments showed that release of radioactivity from stored organisms was minimal under these conditions for at least two hours. Since normal rabbit serum used as a source of complement was in itself bactericidal all such serum was absorbed with a standard meningococcal strain for one hour at 4°C. The bactericidal reaction mixture consisted of antiserum, complement, radioactive organisms and Gey's salt solution.

In the experiments controls used were heat inactivated complement plus serum and active complement without serum.

The experiments started with the addition of radioactive organisms to the reaction mixture. Aliquots were removed for testing at various time intervals. Millipore filtration was used to separate the radioactivity released from killed organisms from that remaining in live organisms.

At the time of each sampling, 0.1 ml of the reaction mixture was placed directly into scintillation vials as an unfiltered control. Another portion of the reaction mixture was filtered through a millipore filter and 0.1 ml duplicate aliquots of the filtrate were placed into scintillation vials.

Upon completion of the sampling, solubilizer and scintillation fluid were added to each vial, which was then placed in a scintillation counter for determination of radioactivity. We have found that with the level of specific activity of the organisms accurate counts can be

obtained in one minute.

Results:

The efficiency of the filtration method for recovery of released radioactivity compared very well with high speed centrifugation to remove the bacteria from the reaction mixture containing hyperimmune serum. Both methods recovered approximately 45 percent of the total label. A large series of controls (no immune sera) resulted in no greater than 10 percent release in a 60 minute incubation period.

To study the correlation of the standard bactericidal test with the radioactive bactericidal test the two methods were tested simultaneiously on the same reaction mixture.

As can be seen in Fig. 6 the initial rate of killing exceeded that of release; however, by 60 min. these rates were equal. A possible explanation of the lag of net percent release behind percent kill is that the standard bactericidal test measures the viability of an organism after interaction with antibody and complement, whereas the new test requires that some disruption of cellular integrity (lysis) occurs in order to release radioactivity. There appears to be an interval in which viability is lost but there is not as yet enough structural damage to permit gross release of radioactive substances.

The radioactivity released and the killing approximated each other at 60 min., therefore, this was the time period chosen for further experiments.

Titration of an immune serum against the homologous organisms is shown in Fig. 7. At each dilution both the radioactive counts and colony counts were done. It can be seen that the curves of the percent release and percent kill approximate each other very closely. There is a statistically significant relationship with a correlation coefficient of 0.9933 and a p value of $<.001$.

Conclusions:

The radioactive bactericidal assay has been found to be highly reproducible and to correlate extremely well with the standard bactericidal test. The chief advantages of the new test are its freedom from multiple plating and counting of colonies of surviving bacteria, procedures with inherent errors and with opportunities for contamination. In addition, results are available within a few hours of beginning the test. The radioactive assay has been used already in studies of the specificity of cell wall protein antigens (described above) with highly satisfactory results.

Fig. 6. Comparison of kinetics of bactericidal test (% killing) and radioactive assay (% release).

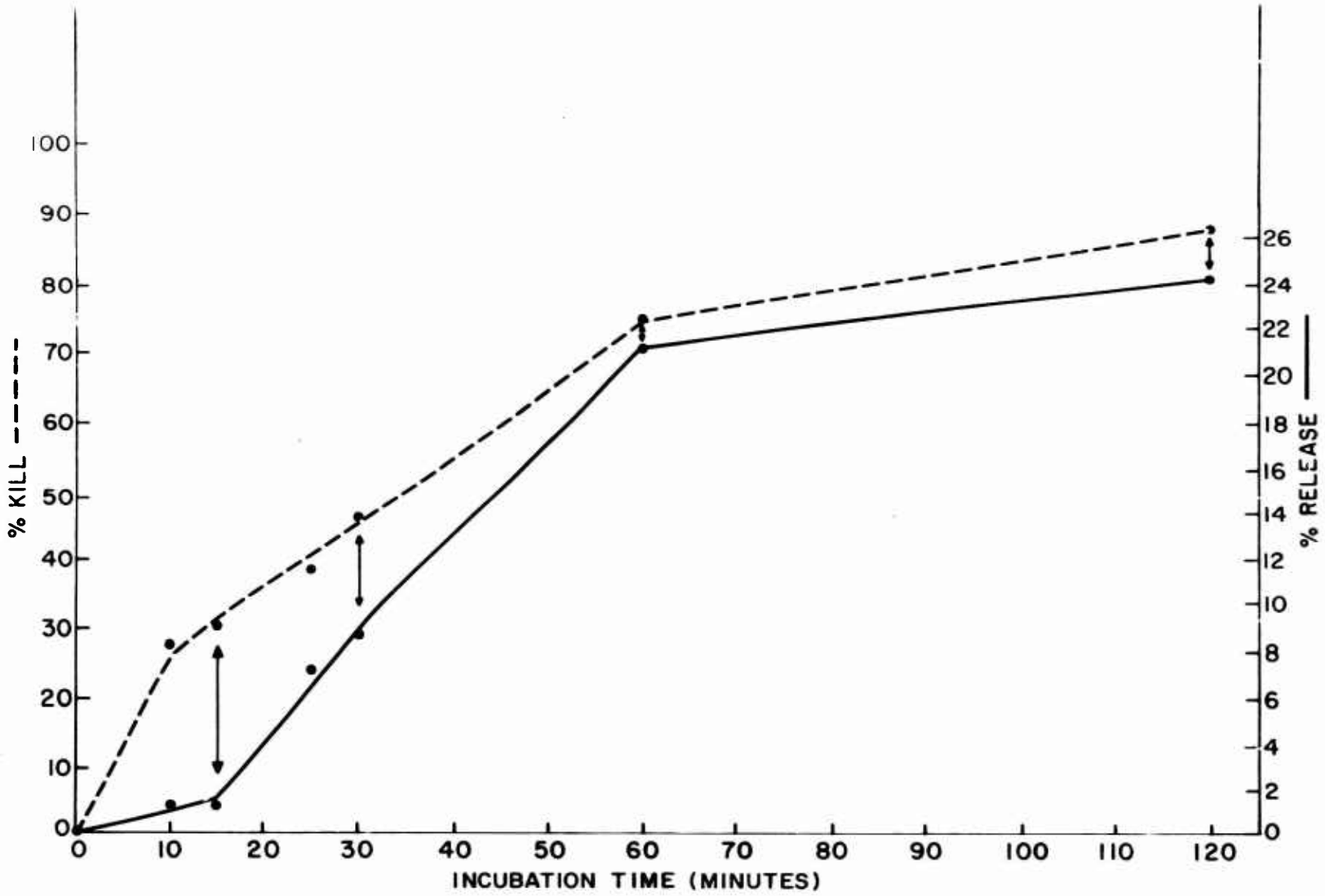
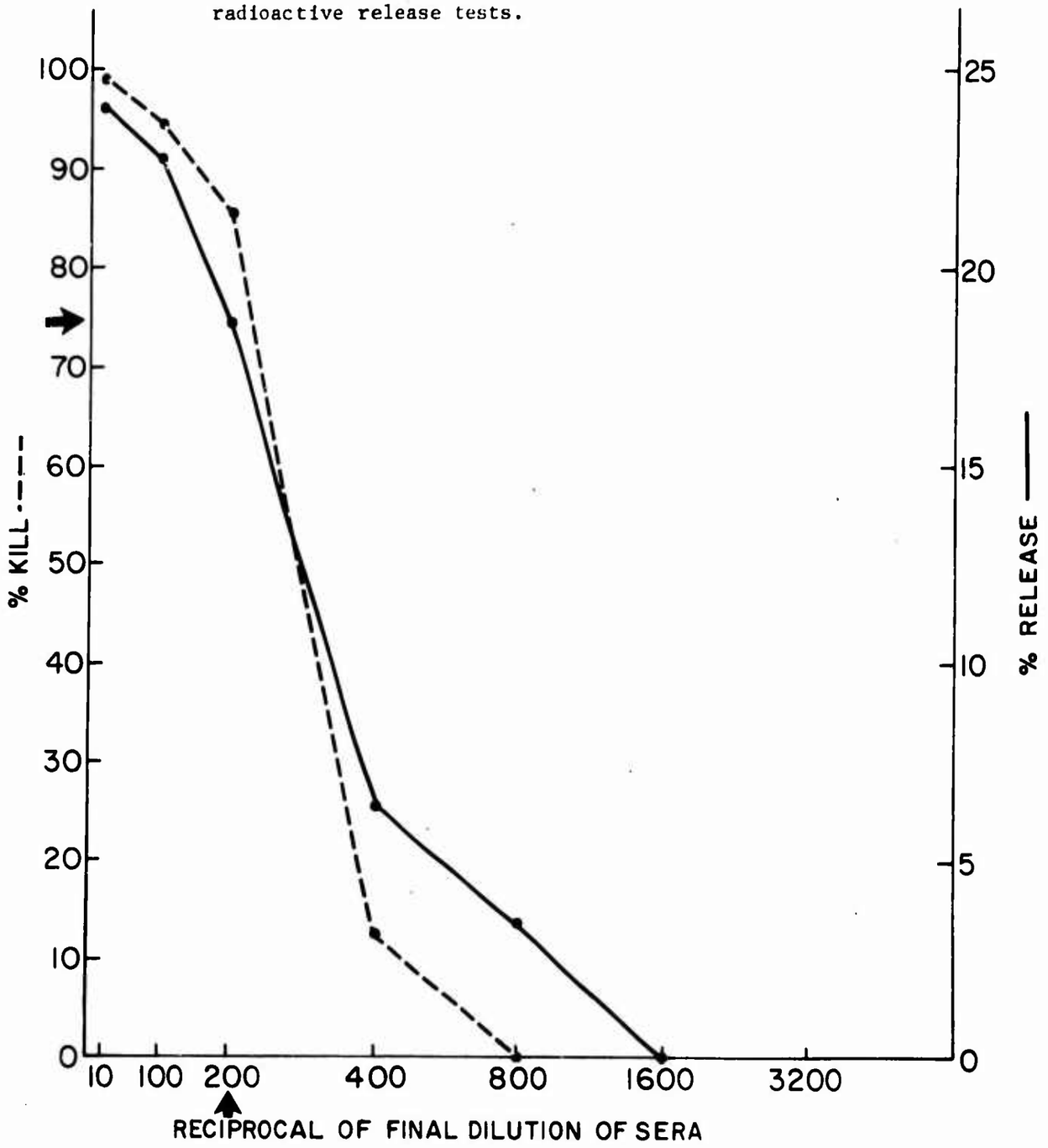


Fig. 7. Titration of immune serum to compare bactericidal and radioactive release tests.



(3) A latex agglutination test for measurement of antibodies to meningococcal polysaccharides.

The use of inert nonbiolgical particles as carriers of various antigens offers as its primary advantage a stable, uniform and easily obtainable vehicle. Latex particles have been used for the detection and measurement of antibodies in a wide variety of diseases. The present report describes a test in which group specific meningococcal polysaccharide is absorbed onto latex particles for use in an indirect agglutination test to measure serum antibodies.

Materials:

Antigens. Meningococcal strains of group A (A-1), group B (B-11), and group C (C-11) were previously described. The group Y (Boshard, WRAIR 6524) strain was isolated from the cerebrospinal fluid of a patient who developed meningococcal meningitis. Purified meningococcal polysaccharides A, B and C were prepared by the method of Gotschlich. Antigens were stored as lyophilized powder in a dessicator jar in the cold or as stock solutions at a concentration of 100 or 250 micrograms/ml which were held frozen until used.

Crude polysaccharide antigens were also used. These were prepared by suspending a six hour culture of meningococci grown on chocolate agar in Phosphate Buffered Saline (PBS) pH 7.2 ±0.1 for 30 minutes (MacFarland $BaSO_4$ Standard #8). The suspension was then centrifuged (2000 rpm) and the supernatant was filter sterilized (millipore .045μ) for use as the antigen.

Latex particles. A commercial preparation of polystyrene latex (Lx) suspension of uniform particle size 0.81μ (Difco Bacto Latex 0.81) was used as the antigen carrier.

Methods:

Sensitization of latex particles. One volume of latex particles (3% v/v) was added to an equal volume of polysaccharide and mixed gently for 30 min. at 37°C. The suspension was pelleted by centrifugation at 2000 rpm for 15 min., the supernatant discarded, and the latex particles diluted 1:3X the original volume in PBS, pH 7.2 (±0.1). A grid titration of antigen vs. a human serum of high antibody content and one of low antibody content was performed on each new batch of polysaccharide to determine the optimal concentration for that antigen. Purified polysaccharide antigens (serogroups B and C) had optimal concentrations ranging from 50 to 250 micrograms/ml.

The latex agglutination test. Serial two-fold dilutions of inactivated serum (56°C for 30 min.) are made in PBS (pH 7.2 ±1) using disposable microtiter V plates (Limbro Chemical Co., Inc., New Haven,

Conn.) and 0.025 ml. diluters (Cooke Engineering Co., Inc., Alexandria, Va.). To each well is added 0.025 ml. of sensitized latex particles. The plates are sealed, gently rotated to mix the reagents and then incubated on a vibration dampening block. The latex A, B and C tests are incubated for 4 hrs. at 37°C or 16 hrs. (overnight) at room temperature. The Lx-Y test is incubated for 16 hrs. at room temperature (overnight). Agglutination patterns are read against a dark background on a 1+ to 4+ scale, 1+ being considered positive. Positive and negative control sera are included in each test.

Immunoglobulin characterization. To determine the immunoglobulins responsible for the Lx activity sera were treated with 2-mercaptoethanol or fractionated by sucrose gradient ultracentrifugation. The immunoglobulin content of the fractions was determined in a micro-Ouchterlony assay using monospecific goat antihuman globulins (Hyland Laboratories, Los Angeles, Calif.).

Results:

Standardization of Lx test. In order to standardize the test a number of variables were studied for at least one of the antigens.

Effect of pH. The following buffered saline preparations were studied: glycine 0.1M pH 1.4-3.6, phosphate 0.15M pH 5.1-8.2, borate buffer 0.1M pH 8.2-9.0. The highest agglutination titers for all four serogroup antigens occurred between pH 7.0 and 7.3. Thereafter, phosphate buffered saline pH 7.2 ±0.1 was utilized as the standard.

Washing the sensitized particles. Experiments were performed which showed that agglutination of group C sensitized particles by immune sera was unaffected by two buffered saline washes. Thus, the sensitized latex particles were used without the washing steps.

Incubation time. In comparative studies it was found that the patterns of agglutination with the A, B and C test were identical after incubation for 4-5 hrs. at 37°C or 16-20 hrs. (overnight) at room temperature. The Y test, however, gave consistent results only when incubated overnight.

Stability of sensitized latex particles. Once sensitized, latex particles could be stored (4°C) for many months without effect on serum titers: Group A-Lx, 2 months only; groups B and C-Lx, 8 months or more; and group Y-Lx, 2 or more months.

Effect of heating sera. The effect of heat treatment of antisera was found to be an important variable only with the Lx-A test. When freshly drawn serum from vaccinated volunteers was used, high prevaccination titers were frequently observed which masked antibody rises. Two subjects had high prevaccination titers which decreased

following heat treatment whereas one showed no change with heating. The two week post-vaccination antibody titers were essentially unaffected. The results of similar experiments with early and late sera from patients with B, C or Y infections or C vaccination showed no consistent effects of heat although occasional paired sera demonstrated identical titer changes. Freeze-thawing of sera three or more times accomplished the same effect as heating at 56°C.

Antigen standardization. Standardization of the antigens must be done using both a serum with a high and one with a low antibody content since an occasional preparation causes a nonspecific agglutination at higher dilutions which may obliterate the end point of the high antibody serum.

Properties of antigens used to sensitize latex particles. As different batches of antigens were tested in the latex system it became apparent that some were much more satisfactory than others. The amount of polysaccharide absorbed to the latex was considered a probable factor. In order to quantitate the binding of antigen to latex a radioactive group A polysaccharide was prepared using C^{14} acetate. A "crude" preparation containing 240 mcg/protein/ml. and a more highly purified product contained only 25mcg/protein/ml. The latex particles were sensitized with the labeled polysaccharide in the usual manner. The particles were then collected on a 0.45μ millipore filter, washed and dried. The activity of the latex particles plus filter paper, filtrate and a filter paper control were measured in a Packard Tricarb Scintillation counter. The percent polysaccharide bound to latex was then determined.

It was found that 16 times more crude antigen remained bound to the latex than purified antigen (0.839% vs. 0.053%). When these antigens were titrated against an immune serum the cruder antigen was active when diluted as much as 1:64 compared to 1:8 for the purified antigen.

Similarly, when different lots of serogroup A antigen taken at various stages of purity were tested in the same manner, the cruder antigens were more active than the purified antigens.

Specificity. The latex agglutination test using meningococcal polysaccharides as antigens is a highly specific test for detecting antipolysaccharide antibodies. Of 21 cases of meningococcal septicemia or meningitis caused by serogroups B (5), C (11), or Y (5) organisms, 19 showed antibody rises only with those latex particles coated with their homologous serogroup antigens. One case in which Y organisms were isolated from CSF showed no antibody rise to any of the standard antigens, nor in tests using latex particles sensitized with a crude polysaccharide preparation made from the infecting organism. Another case, in which a group B organism was isolated from the blood, showed

antibody increase to both the B and C polysaccharides. In Table 12 are listed results of a battery of latex tests performed on sera from representative cases of group C, group B and group Y disease.

Table 12. Specificity of latex test in patients with meningococcal meningitis.

Patient	Meningococcus isolated	Day of disease	Latex antigen A	B	C	Y
GL	Y	1	<1*	2	3	<1
		10	<1	2	3	5
SU	B	1	<1	<1	<1	<1
		7	<1	5	<1	<1
		14	<1	5	<1	<1
WO	C	1	<1	<1	<1	<1
		7	<1	<1	7	<1
		36	<1	1	5	<1

*Number of reactive tubes

The latex test was also found to be group specific in patients receiving the group A or C polysaccharide vaccines. In Table 13 are shown results from four representative volunteers who received 50 microgram injections of group C vaccine or 50 micrograms of group A vaccine. All of the individuals showed an increase in antibody titer within two weeks to the corresponding homologous polysaccharide antigen.

Sensitivity. The latex test is quite sensitive as a measure of group specific meningococcal infection. In a series of 27 proven cases of group C disease, 24 showed antibody rises. In the three remaining cases a high unchanging titer was found. Since the date of onset of illness was not known for these three patients it is possible that the first serum tested may have been obtained after the acute stage and thus the antibody increase would have been missed. In a series of 12 group B cases, 11 showed at least a 4-fold rise (range 4 to 256 fold) within 10 days following hospitalization. One group B patient had a high unchanging titer. Four out of five cases of group Y disease developed antibody rises. The remaining case of Y disease had no detectable Lx antibody against any of the serogroup polysaccharides.

Persons who had no clinical evidence of disease but who developed positive nasopharyngeal cultures (carriers) were also tested for development of antibodies. Seventeen of 18 persons who had become

Table 13. Specificity of latex agglutination test in volunteers receiving meningococcal polysaccharide vaccines.

Subject	Vaccine	Days post vaccine	Latex antigen			
			A	B	C	Y
GI	A	0	2*	<1	<1	<1
		14	5	<1	<1	<1
IM	A	0	<1	<1	<1	<1
		14	8	<1	<1	<1
SC	C	0	<1	<1	<1	<1
		14	<1	<1	7	<1
HC	C	0	1	<1	<1	<1
		14	1	<1	5	<1

*Number of reactive tubes.

group C carriers developed group specific antibodies as did 8 out of 10 persons who had become group Y carriers. However, only 2 of 24 individuals who had become carriers of group B organisms developed an increase in antibody titer.

Comparison of the Lx and FAB tests. The indirect fluorescent antibody test which uses the whole organism fixed onto a glass slide as the antigen is a very sensitive assay for detecting antibodies in patients receiving the meningococcal polysaccharide vaccines. When the two tests were compared in 23 volunteers who received meningococcal polysaccharide vaccines (16 group C vaccine; 7 group A vaccine) both tests detected antibody increases in the same 17 individuals and no antibody rise in the remaining six individuals. Similar results were obtained in five cases (two serogroup B, three serogroup C) of meningococcal disease.

Comparison of Lx and HA tests. The Lx and HA tests using meningococcal polysaccharides as antigens showed 100 percent correlation in patients with disease, carriers and vaccinated subjects (Table 14).

Table 14. Comparison of Lx and HA tests.

Serogroup and category	No. of subjects	Lx pos. HA pos.	Lx neg. HA neg.	Lx pos. HA neg.	Lx neg. HA pos.
C cases	18	15	3*	0	0
C carrier	22	20	2	0	0
C vaccine	90	87	3	0	0
B cases	9	8	1*	0	0
A vaccine	50	46	4	0	0

*High unchanging titers

When the same batch of polysaccharide antigen was used in both assays mean titers were higher in the HA test although the mean change in titer was essentially identical (Table 15).

Table 15. Comparison of mean titers of Lx-A and HA-A in volunteers receiving group A polysaccharide vaccine.

Days after vaccination	Mean titer (Log 2) vs. indicated antigen	
	Lx-A	HA-A
0	0.18	3.11
14	2.79	6.00
Mean antibody rise	2.61	2.89

Immunoglobulins active in the Lx test. Sera from a patient with group C meningococcal disease was examined for immunoglobulins active in the Lx system. Results of sucrose density centrifugation and 2 ME treatment showed all the activity to reside in the IgM moiety. A second patient tested by 2 ME treatment only showed IgM activity exclusively also.

Discussion:

The latex agglutination test as herein described has a number of advantages over HA and FAB tests. Latex particles of known size can be purchased and sensitized without further treatment. The use of such particles should avoid the variables found in human or animal erythrocytes which change surface characteristics upon storage, often settle unpredictably and may require preabsorption of the sera to be tested. Polysaccharide sensitized latex particles retain their properties when stored for two months or longer. Thus, large batches may be prepared and used for long periods of time.

In terms of sensitivity and specificity the latex test is quite similar to the HA test for meningococcal antibodies. The group A and B HA tests and the Latex A and B tests have shown differences in antibody titers of normal human sera even when the same lot of polysaccharide was used for both assays. However, mean titer increases are approximately equal in both assay systems. The FAB test measures antibody against other antigens as well as the polysaccharide of the meningococcus and, therefore, this test is cross reactive among the various meningococcal serogroups. In sensitivity the Lx test is equal to the FAB.

The immunoglobulin responsible for Lx agglutination appears to be IgM in the present meningococcal assay as it has been in a number of other disease states.

A major factor in the Lx system appears to be the nature of the antigen preparation used. The data presented above suggest that crude polysaccharide preparations bind to latex particles more firmly and/or in a greater quantity than purified antigens and thus provided a more sensitive indicator of antibody that the highly purified materials.

In a recent experiment using sera from children who had received group C vaccine, seven individuals failed to show antibody response by HA test and Lx-C test using purified C antigen. All seven showed 4-fold or greater Lx-C responses when a crude C antigen (saline extract) was used. These results were confirmed by the very sensitive radioimmunoprecipitation test.

Furthermore, in a series of 57 adult volunteers who received the group C vaccine, the mean antibody increase between the prevaccination and two week post-vaccination sera using the pure Lx-C antigen was 4.08 tubes. Whereas, the same samples tested against the crude polysaccharide resulted in a 5.86 tube mean difference.

As with the more purified antigens the crude preparations gave no cross reactions among the serogroups.

Preliminary studies have not implicated inorganic cations as important constituents of the Lx reaction.

Other preliminary experiments have shown the feasibility of sensitizing latex particles with multiple antigens (B, C and Y simultaneously). For this test the three antigens were mixed together at their optimum sensitizing doses and incubated with the latex. Homologous serum antibody titers were identical with mono- or multiple sensitized particles. The A antigen, however, showed loss of sensitivity when added to the other polysaccharides.

d. Penicillin susceptibility of serological group C Neisseria meningitidis isolated from 1964 to 1970.

In recent months an isolate of Neisseria meningitidis suspected of being penicillin-resistant was referred from an outlying Army hospital to the Department of Bacterial Diseases, WRAIR for study. Subsequent testing proved the isolate to be penicillin susceptible but prompted a study to determine if, indeed, there has been any decrease in penicillin susceptibility among a number of isolates collected since 1964 by this laboratory.

Thirty-nine isolates of serological group C N. meningitidis were selected at random from lyophilized stocks as follows (Table 16).

Table 16. Strains used for penicillin sensitivity studies.

Year	No. of strains	Laboratory No.
1964	1	35E
1965	6	32I, 38IV, 60E, 95E, 34I, 41I
1966	9	70I, 85I, 88I, 89I, 92I, 94I, 98I, 126E, 62I
1967	1	79II
1968	2	190I, 253I
1969	6	690219, 690236, 690286, 690403, 690644, 690937
1970	14	5571, 5629, 5663, 5677, 5736, 5835, 5843, 5928, 6150, 6192, 6197, 6387, 6390, 705664

Methods:

The agar-dilution technique was used throughout. Potassium penicillin G (Wyeth, Control No. W653904) of known potency was dissolved in distilled water and immediately frozen in aliquots at -15°C. For use an aliquot was thawed and added to sterile, cooled (48°C) Mueller-Hinton agar (Difco, Control No. 525687), pH adjusted to 7.0 before autoclaving, to give final concentrations of penicillin of 0.01 unit/ml and 0.02-0.18 units/ml in 0.02 unit steps. The penicillin agar was then dispensed into petri dishes, allowed to solidify, and incubated overnight at 37°C to test sterility. Plates were stored at 8°C and were used within 72 hours of preparation.

Lyophilized cultures of isolates to be tested were opened, suspended in sterile Mueller-Hinton (M-H) broth and one plate of M-H agar streaked for isolation (purity) and one for confluent growth. After overnight incubation at 37°C in a candle jar, pure cultures were further prepared for testing. A swab moistened with M-H broth

was rubbed over the plate of confluent growth and transferred to M-H broth in a 16x125 mm screw cap culture tube. The optical density at 650 nm was adjusted to 1.0, using a M-H broth blank in a test tube of the same type. Two ml. of this suspension was used to inoculate a nephelometer flask containing 18 ml. of M-H broth. This flask was shaken at approximately 165 rpm in a rotary shaker water bath at 37°C for 2-3 hrs. or until an optical density of approximately 0.5 was obtained, using a screw cap tube of M-H broth as the blank as above. The culture suspension was then adjusted to an optical density of exactly 0.5, diluted 1:100, and an aliquot pipetted into a template well in a Lidwell inoculum replicating apparatus. Nine isolates and a control, the Oxford strain of Staphylococcus aureus, were tested per template. The control was grown and diluted in exactly the same manner as the test cultures.

Using the Lidwell apparatus, the nine test cultures and the control were transferred from the template to duplicate sets of plates of the varying concentrations of penicillin. Following delivery, each drop was then streaked with a glass rod assembly into a single line of inoculum approximately 2.5 cm long. When completed, each plate had two rows of five parallel lines of inoculum. Growth was nearly confluent in most cases and more closely approximated the density usually attained by a flooding technique, for example, in which one strives for near confluence.

All plates were incubated at 37°C in a candle jar for 18-24 hrs. before reading.

Results:

The lowest concentration of penicillin producing complete inhibition of growth was recorded as the minimum inhibitory concentration (MIC) for each isolate tested. The values given represent, in most instances, the average of at least two separate determinations. In only one instance did the results of two determinations differ by more than 0.02 unit. The values obtained in any one experiment by duplication were always in complete agreement within the limits of the technique. The control strain consistently had an MIC of 0.02 units/ml penicillin.

MIC's ranged from 0.03 units/ml to 0.16 units/ml and were randomly distributed by the year-categories shown over the range between these values with skewness toward the lower MIC's. Of the 39 isolates tested, 82% had MIC's of 0.08 units/ml or less, of which approximately half were obtained in 1969-1970. The geometric mean was 0.07 units/ml.

The relationship of the MIC to the year of isolation showed what appears to be a slight tendency toward an increase in MIC's from 1964-1970. The average of the geometric means for the years 1964-1968

(19 isolates) was 0.06 units/ml whereas the average of the geometric means for the years 1969-1970 was 0.09 units/ml. However, the precision of the technique, as evidenced by repeating the test on different days, would suggest that a 0.02 unit variation was to be anticipated.

Conclusions:

The distribution of MIC values for 39 randomly selected isolates of serological group C N. meningitidis collected from 1964 to 1970 was such that there is no clear-cut evidence of a progression toward decreased penicillin susceptibility. However, there is an indication for monitoring penicillin susceptibility in meningococci periodically to detect a potential decrease.

e. Hemagglutination-inhibition for serogrouping of N. meningitidis.

Serogroups within the species N. meningitidis are identified on the basis of bacterial agglutination (BA) with specific antisera. Although other methods, such as precipitation, have been used for identifying new serogroups or studying relationships among the serogroups the BA test has been the standard procedure used in most laboratories for the examination of clinical specimens..

The BA test has a number of disadvantages, among the most prominent being the large volume of antisera needed and the long time required when survey studies provide hundreds of isolates for testing. Also, there is often considerable cross agglutination among the various antisera, probably due to common antigens unrelated to the serogroup specific polysaccharides.

With the recent development of improved methods to isolate and characterize meningococcal polysaccharide antigens and antibodies it has been possible to develop a haptene inhibition test (H.I.) to identify the serogroup of meningococci isolated from clinical materials.

Methods:

The test is essentially the inhibition by the unknown of a battery of standard passive hemagglutination tests.

Antigens. The following strains of meningococci from the Walter Reed Army Institute of Research collection were used to prepare antigens: Group A (A-4); group B (99M); group C (9M); group Y (135M); group 29E (60M); group 135 (135III) and Slaterus' X and Z. Crude polysaccharide antigens were prepared by the method of Edwards and Driscoll. This method utilizes milk alkali treatment of cells, followed by ethanol precipitation. The portion of the precipitate which is soluble in

saline acts as the polysaccharide antigen. Optimal sensitizing concentrations of each batch of antigen are determined by checkerboard titration against serogroup homologous immune serum. Specificity is determined using homologous and heterologous antisera. Although purified polysaccharides prepared by the method of Gotschlich were satisfactory such preparations were only available for serogroups A, B and C. Therefore, the less purified antigens were prepared for all serogroups studied.

Erythrocytes. Fresh, formaldehyde fixed, pyruvic aldehyde fixed, and formaldehyde-pyruvic aldehyde fixed erythrocytes were compared. Pyruvic aldehyde fixed sheep red blood cells (SRBC's) were selected for use because of their superior stability, sensitizing capacity and clarity of reaction.

Sensitization of RBC's. SRBC's were washed three times in phosphate buffered saline, pH 7.2 (PBS), and suspended to 4% v/v in PBS. The cells were sensitized by mixing equal volumes of washed 4% cells with antigen diluted to its predetermined optimal concentration in PBS. The mixture was incubated at 37°C for one hr. and washed five times in PBS to remove excess antigen. The sensitized cells were then diluted to 0.5% in PBS containing 0.5% w/v bovine serum albumin (BSA). Sensitized cells were stable for at least one week when stored at 4°C.

Antisera. Group specific rabbit antisera against whole organism were prepared by the method of Evans et al. Hemagglutination titer of each serum was determined using methods previously described. Four units of antisera were used for the H.I. test.

Cultures. Meningococcal carrier surveys were performed as previously described in Army basic trainees and laboratory personnel. The selective growth medium consisted of Mueller-Hinton agar containing 5% v/v chocolated, defibrinated sheep blood and 6 mg/ml Lincocin and 25 units/ml polymixin B sulfate.

Bacterial suspensions were prepared from positive cultures, either from the original plate or after one or two transfers.

Live, formalinized, β-propiolactone inactivated and heat killed cells as suspensions were investigated. Live cell suspensions were unsatisfactory because of the obvious hazards involved; formalin killed suspensions resulted in all SRBC's settling (complete inhibition) and β-propiolactone killed suspensions resulted in all SRBC's agglutinating (no inhibition). Therefore, heat killing was used. Extraction of group specific antigen from unknown cultures was carried out at 56°C using various times and various suspending media including physiological saline, PBS, PBS-BSA, and 0.3% w/v trypticase in physiological saline. Best results were obtained with PBS-BSA as suspending fluid and heating at 56°C for two hrs.

The killed suspensions were centrifuged for 10 min. at 3000 rpm in an International PR-6 centrifuge and the clear supernatants were used. Such supernatants can be used immediately or can be stored frozen (-20°C) for at least six months.

The H.I. test. 0.05 ml. bacterial supernatant is mixed with 0.05 serum (4 units) in "U" bottom microtiter plates (Linbro Chemical Co.). Each unknown is tested against eight different antisera in separate wells and incubated at 37°C for 30 min. Then 0.05 ml. of sensitized cells (0.5% v/v) is added, the plates are sealed with transparent tape, gently mixed and incubated at room temperature on a vibration damping platform for two hrs. Hemagglutination patterns which are difficult to read at two hrs. can be improved by further incubation overnight in the cold.

A positive test is indicated by complete inhibition of agglutination as shown by a clear small button of cells in the bottom of the well surrounded by a clear supernatant. Negative reactions are indicated by any pattern of agglutination.

Bacterial agglutination. For the standard bacterial agglutination (BA) tests moderately heavy suspensions of cells in normal saline were made from the same cultures used for preparing suspensions for the H.I. test. BA was carried out in plastic trays using one drop of suspension and one drop of rabbit antiserum containing four agglutinating units. The mixtures were shaken at room temperature for three min. and agglutination was read. Agglutination was graded from ± to 4+, but any agglutination was considered positive.

Results:

A summary of the results of BA and H.I. serogrouping on 476 carrier strains of meningococci are given in Table 17.

Table 17. Comparison of H.I. and BA tests on 476 meningococcal isolates.

Results	No. of strains	% of total
BA and HI agree	381	80.0
HI groupable BA nongroupable	82	17.2
BA and HI disagree	13	2.8

There was agreement between the two tests for 381 (80%) of the strains. Of these, 218 strains were identified as a specific serogroup; 163 strains were nongroupable by both methods. The nongroupables were either smooth nonagglutinable, roughly agglutinated or rough by the BA scheme and noninhibitory in the H.I. assay. Another set of 82 strains was identified as to serogroup by H.I. test but could not be identified by BA. Strains of all serogroups were found in this category.

Disagreement between the results of the two tests were observed in only 13 (2.8%) of the cultures. Of these strains six were nongroupable by BA and gave double reactions by H.I. Six other strains were typable by BA and not groupable in H.I. tests. Finally, only one strain was grouped differently in both assays. Tests on this strain were repeated three times. Each time the H.I. test gave the same result (serogroup B). In one test BA was recorded as serogroup Y; in the other two trials the culture was nongroupable.

H.I. and BA tests were performed on 24 meningococcal cultures derived from blood or cerebrospinal fluid. Each of these strains had been serogrouped by BA prior to lyophilization but at the time of reculture four strains were multiply agglutinated in the BA test. All cultures were serogroupable by the H.I. test.

Discussion:

Haptene inhibition is a well known serological tool for demonstrating similarities between antigens and has been particularly valuable in studies of polysaccharide antigens derived from bacteria. A limited H.I. system was used several years ago in this laboratory to identify group C meningococci in a large field study with good results. With the use of eight different polysaccharide hemagglutinating systems described herein it has been feasible to develop a rapid, precise method for serogrouping meningococci. This system has a number of practical advantages. Grouping antisera are used at high dilution (HA titers are 100 fold greater than BA titers) thus conserving this reagent. Sensitized red cells are stable for many months and experiments are in progress to test lyophilized red cells. Greater specificity and sensitivity of the H.I. test over the BA assay is indicated by the current results. Finally, many cultures were grouped by H.I. from the initial plate whereas BA grouping often required several transfers in the laboratory.

f. In vitro studies of meningococcal polysaccharides.

(1) Reactions of group B and C polysaccharides with influenza viruses.

Background. It is known that the group B polysaccharide

is a sialic acid (neuraminic acid) polymer which is susceptible to enzymatic cleavage by neuraminidases from Vibrio cholera and Clostridium perfringens. Since influenza viruses contain neuraminidase an attempt was made to determine if an interference by B polysaccharide with influenza virus hemagglutination could be detected.

Two influenza viruses were used, A2/Jap/305/57 and B/Mass/3/66; both consisted of chick allantoic fluid harvests. Hemagglutination (HA) of human erythrocytes was determined by standard titrations. Inhibition with B-1 or C-pool polysaccharides, 250 mcg/ml, was carried out by 30 min., 37°C incubation of virus + polysaccharide prior to HA titration. Results are shown in Table 18.

Table 18. Effect of polysaccharides on influenza virus hemagglutination.

Virus	Polysaccharide	Viral HA titer
A2	-	1:160
-	B-1	0
A2	B-1	1:160
B	-	1:160
B	B-1	1:160
A2	C	1:160
-	C	0
B	C	1:160

Under the conditions of the experiment there was no inhibition of influenza HA by either the group B or C polysaccharides. These data suggests that the meningococcal polysaccharides are different from the influenza virus HA inhibitors found in many body fluids (which are destroyed by neuraminidase) and different from the erythrocyte receptor sites to which influenza virus binds.

(2) Relation of group B and C polysaccharides with blood group substances M, N and Rh(D).

Background. Human M, N and Rh(D) blood group antigens contain sialic acid. An attempt to detect antigenic cross reaction with meningococcal polysaccharides was carried out by inhibition of erythrocyte agglutination by M, N and Rh antisera.

M and N blood group donors were identified by standard techniques using human anti-M and anti-N antisera. Polysaccharides B-4 and C-10,

250/ml, were incubated with antiserum overnight at 4°C and then the mixtures were titrated.

Results:

Table 19 shows that there was no inhibition of either the M or N agglutination system by either the group B or C polysaccharides.

Table 19. Effect of polysaccharides on M-N blood group agglutination.

		Agglutination titer				
Red cells	Polysaccharide	1:2	1:4	1:8	1:16	1:32
M	Saline	+	+	-	-	-
M	B	+	+	-	-	-
M	C	+	+	-	-	-
N	Saline	+	+	±	-	-
N	B	+	+	+	±	-
N	C	+	+	+	-	-

A similar experiement performed with an Rh positive donor and a saline anti-D antiserum showed no inhibition of the Rh system with meningococcal polysaccharides B-2 and C-10.

The experiment was performed a second time using four times the concentration of each polysaccharide and a one hr. 37°C incubation followed by overnight in the cold. Again, no inhibition of M, N or Rh(D) agglutination was observed.

g. Prevalence of the various serogroups among case strains of N. meningitidis submitted to WRAIR.

The data presented below reflects a change in presentation from fiscal year tabulation to annual year tabulation. This change was made so that the data from the U. S. Army could be more easily examined in the context of annual reporting years utilized by other governmental agencies. This report, therefore, encompasses the years 1969 and 1970 and the first five months of 1971.

During 1969 a total of 354 strains of N. meningitidis were submitted by other laboratories for confirmatory and sulfadiazine resistance studies. Table 20 describes the geographical source, serogroup and sulfadiazine resistance patterns seen among the 354 strains.

Table 20. Source, serogroups and sulfadiazine resistance of case strains of N. meningitidis submitted to WRAIR in 1969.

	Serogroup								
	A		B		C		Y		
Army Area	S*	R+	S	R	S	R	S	R	Total
I	0	0	3	2	1	65	2	0	73
II	0	0	1	1	0	25	0	0	27
III	1	0	4	7	2	61	2	0	77
IV	0	0	2	5	2	25	0	0	34
V	0	0	0	0	0	11	2	0	13
VI	0	0	2	3	0	110	1	0	116
Eur	0	0	7	0	1	6	0	0	14
Totals	1	0	19	18	6	303	7	0	354
% serogroup sulfa resist.	0%		49%		98%		0%		

* S denotes sensitivity to 1 µg/ml sulfadiazine or less.

+ R denotes resistance to more than 1 µg/ml sulfadiazine.

Most of these strains were derived from cases of clinical meningitis in military recruits and a small number from military dependents and military personnel beyond recruit training. Of these 354 strains, 309 (87%) were of serogroup C and the remaining 13 percent were primarily of serogroups B and Y (WRAIR "Boshard"). One serogroup A strain, which was encountered early in 1969, was described last year. In 1969, the distribution of sulfadiazine resistance among serogroups was not markedly different from that found in 1968; among group C strains sulfadiazine resistance increased from 96 percent in 1968 to 98 percent in 1969; among group B strains sulfadiazine resistance decreased from 54 percent in 1968 to 49 percent in 1969.

During 1970 the routine testing of all strains for their sulfadiazine resistance levels was discontinued. A significant factor in this decision was that group C strains comprise the greatest part of all strains submitted to this laboratory since 1967 and among these group C strains the vast majority (95 to 98%) were sulfadiazine resistant. It is apparent, therefore, that sulfadiazine

resistance has lost its value as a routine clinical measurement. However, sulfadiazine resistance testing will be resumed if spot checks reveal a shift from resistance to sensitivity among group C strains or if an epidemiologic shift occurs from group C to other meningococcal serogroups.

As seen in Table 21, in group C strains of meningococci again predominated as the cause of meningococcal disease in the U. S. Army. Of a total of 281 strains submitted, 257 (91.5%) were group C and the remaining 8.5 percent comprised serogroups B, Y, 135F and a single group A strain. The group A strain was isolated in Europe.

Table 21. Source and serogroups of case strains of N. meningitidis submitted to WRAIR in 1970.

Army Area	A	B	C	Y	135	
I	0	6	99	2	0	107 (38.1%)
II	0	1	13	0	0	14 (5.0%)
III	0	1	34	0	1	36 (12.8%)
IV	0	2	15	1	0	18 (6.4%)
V	0	1	50	4	0	55 (19.6%)
VI	0	2	44	1	1	48 (17.1%)
Eur	1	0	2	0	0	3 (1.1%)
Total	1 (0.4%)	13 (4.6%)	257 (91.5%)	8 (2.8%)	2 (0.7%)	281 (100%)

Table 22 shows that the proportion of group C cases has increased steadily, from 13 percent in 1965 to its 1970 level of 91.5 percent. In this same period group B cases have steadily declined from 86 percent to its present level of 5 percent.

The data on strains submitted during the first five months of 1971, which are presented in Table 23, continue the trend established over the past few years. Despite the use of group C vaccine in six training posts during the epidemic season, no change in prevalence of serogroups was obvious.

Table 22. Changes in prevalence of serogroups B and C N. meningitidis among case strains submitted to WRAIR from 1964 to 1970.

	1964		1965		1966		1967		1968		1969		1970	
Serogroup	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
B	295	85.8	157	84.0	231	80.5	54	35.3	35	17.1	37	10.5	13	4.6
C	33	9.6	24	12.8	41	14.3	86	56.2	268	84.5	309	87.2	257	91.5
Other*	16	4.6	6	3.2	15	5.2	13	8.5	14	4.4	8	2.3	11	3.9
Totals	344		187		287		153		317		354		281	

*Includes serogroup A, Y and WRAIR serogroups 29E and 135 III.

Table 23. Source and serogroups of case strains of N. meningitidis submitted to WRAIR from 1 January to 30 May 1971.

Army Area	Serogroup B	C	Y	To be identified*	Total
I	1	14	2	4	21
II	0	0	1	0	1
III	0	8	0	5	13
IV	2	3	3	0	8
V	0	22	1	2	25
VI	0	4	0	7	11
Europe	0	0	0	0	0
Total	3	51	7	18	79

*Strains received too late to complete identification prior to 30 May 1971.

2. Special diagnostic bacteriology.

A number of exceedingly complex clinical problems were studied by special methods to identify infecting microorganisms. The cases presented below illustrate how this type of specialized laboratory examination can contribute to clinical care of patients.

Case 1

Post-partum Corynebacterium infection (WRAIR #5622).

A 19 year old Negro female with sick cell trait and hemoglobin C developed septicemia after giving birth to twins. After initial improvement the patients course began to deteriorate. A Corynebacterium sp. was isolated from the patient's bone marrow. The patient made a good recovery on erythromycin therapy.

WRAIR laboratory results:

A culture of bone marrow in thioglycollate broth was forwarded to this laboratory. Growth of a Corynebacterium sp. occurred after seven days incubation at 37°C. No growth was obtained upon subculture to blood agar which was incubated aerobically and anaerobically. Very little growth occurred in a transfer to thioglycollate broth. The original broth was then subcultured to thioglycollate agar with inactivated horse serum and to another thioglycollate broth with serum. Both media were incubated at 37°C, the agar plate anaerobically. After several days, sufficient growth was obtained to do antibiotic sensitivities. The culture was sensitive to erythromycin by tube dilution technique.

Comment:

Recently more attention has been given to the presence of atypical or transitional forms of bacteria in clinical specimens. These variants may require osmotic protection and enriched media for cultivation in vitro.

In this laboratory, thioglycollate broth with 20% inactivated horse serum and L form media are being used in addition to other enrichment media. Use of these special media allowed more rapid growth of this anaerobic Corynebacterium thus allowing antibiotic sensitivity to be tested.

Case 2

Lymphadenitis due to Corynebacterium acnes (WRAIR #6367).

A 55 year old alcoholic male with recurrent thrombophlebitis of the right leg remained symptomatic over a three week period despite standard therapy. On examination, fever 101°, swollen, tender inguinal lymph nodes were noted. A biopsy of this lesion was submitted for special cultures. After the biopsy the patient made a spontaneous recovery without further therapy.

WRAIR laboratory results:

Tissue was minced on trypticase broth and was inoculated onto the following media:

Thioglycollate broth with horse serum - 37°C
Heart infusion broth with horse serum - 37°C
Blood agar aerobically, anaerobically with CO_2 at 37°C
Chocolate agar aerobically, anaerobically with CO_2 at 37°C
Chocolate cystine agar (37°C with 10% CO_2)

After five days incubation anaerobically, a gram positive rod was observed on the blood agar plate. Granular growth was apparent in the thioglycollate serum broth after 12 days at 37°C. A gram stain revealed gram positive short rods and filaments with singular branching. Both isolates were identified biochemically as Corynebacterium acnes.

Comments:

Isolation of the organism from the site of infection indicates a causal relation of this anaerobic organism with the disease. Recovery of the patient without specific antibiotic therapy is probably not unexpected in view of the saprophytic nature of the organism.

Case 3

Myeloproliferative disorder and skin ulcerations due to an atypical mycobacterium (WRAIR #6399 and #6421).

A 42 year old active duty Lt. Col. had a myeloproliferative disorder of two years duration manifested by anemia, extramedulary hematopoiesis and a fibrotic bone marrow picture. Over the past eight months he developed indolent, nonpainful ulcerations of both lower extremities. Biopsy of one of the skin lesions and bone marrow biopsy was cultured. Treatment with streptomycin and sulfamethazole, based upon sensitivity studies, resulted in initial improvement of the lesions. However, relapse occurred and combination therapy of rifampin, ethambutol and isoniazid was substituted.

#6399 - Leg lesion (Draddy) 2/8/71.- The same procedure and media as described for Case 4 below were used with the exception that Mycoplasma diphasic agar was omitted. One portion of the original suspension was refrigerated in tryptose broth at 4°C to be subcultured at a later time for Listeria. After six days incubation, aerobically at 37°C, tiny transparent colonies were observed on brain heart infusion agar and blood agar. In a gram stain of the growth, gram positive swollen rods and granulated filaments with singular branching were seen. Subcultures were made on brain heart infusion agar and incubated at 32°C and 27°C aerobically. After 14 days incubation an acid-fast stain was prepared using 1 percent sulfuric acid as the decolorizing agent and methylene blue as the counter stain. Pale staining acid-fast filaments with blue granules were seen as well as blue cross striations in large filaments. This organism is currently being studied as a possible atypical Mycobacterium.

The refrigerated portion was subcultured after 21 days storage. The same pleomorphic gram positive rod was isolated but in addition a slender gram negative bipolar staining rod grew on brain heart infusion agar. Both strains grew better if glycerol was added to the base medium. On glycerol agar, the gram negative rod was very mucoid and nonpigmented. In heart infusion broth, granular growth occurred with a nearly clear supernatant. When glycerol was added viscous growth concentrated at the bottom of the tube with very slight turbidity. The organism produced branching forms and short filaments in broth. It was biochemically inactive and nonmotile suggesting Actinobacillus but not glanders or melioidosis.

#6421 - Bone marrow (Draddy) 2/24/71. - After 23 days incubation in heart infusion broth with serum, granular growth occurred. This culture contained gram positive rods, beaded filaments and a few slender gram negative bipolar staining rods.

The pleomorphic gram positive rods from both specimens were inoculated onto Lowensteins Jensen slants and incubated at 37°C for 10 percent carbon dioxide. After 16 days the isolate from the bone marrow grew as smooth buff colored colonies. The organism isolated from the skin lesions produced the same type colonies after one month incubation. Both were strong catalase producers (68°C).

Comments:

These two bacterial isolates are undoubtedly the cause of his skin lesions and possibly responsible for the bone marrow picture. Further studies are underway to completely identify the organisms.

Case 4

Regional enteritis associated with Corynebacterium infection (WRAIR #6328).

A 12 year old boy had chronic fever and weight loss with no gastrointestinal symptoms. Low serum albumin and abnormal duodenal mucosa on X-ray study suggested the diagnosis of regional enteritis. Abdominal exploration revealed enlarged mesenteric lymph nodes which, on histology, showed multiple, noncaseating granulomas.

Laboratory studies - abdominal lymph node (Ostrovsky):

The specimen was minced in brain heart infusion broth. Thioglycollate broth with serum and Mycoplasma diphasic agar were inoculated. Blood and chocolate agar plates were streaked with the suspension. All media were incubated at 37°C under appropriate environmental conditions for the isolation of pathogenic organisms. After 20 days very small granules were seen in the thioglycollate broth. Four control tubes were negative. A gram stain revealed gram positive diplococci and coccobacilli. Upon subculture the organism grew as a short gram positive rod morphologically similar to a Corynebacterium. Biochemically it was the same as Corynebacterium acnes except for its failure to reduce nitrate.

Comment:

The relationship of the organism isolated to the patient's illness is, as yet, unclear.

3. Investigations on Mycoplasma (mollicutes and wall-defective variants of bacteria.

a. Wall-defective variants of Treponema pallidum.

Microbial variants possessing defective cell walls have attracted considerable interest in recent years because of the possibility of their having a role in the pathogenesis of disease. The characteristics of such microbial variants (consistent with damage to or deficiency of cell wall structure) are alterations in morphology, physiology, and cultural characters, including a gram-negative, non-rigid outer covering, resistance to penicillin, and colonial down-growth below the agar surface.

The nature of the polymorphous disease pattern in syphilis with its periodic course, frequent negative or doubtful results of microscopic examination, and resistance to treatment suggested the possible existence of altered microbial forms to early investigators.

Levaditi in 1941 using silver-impregnation methods observed granular material which he assumed represented involutional changes in Treponema pallidum in material from syphilitic lesions of man, rabbits and mice. Ustimenko (1963) more recently reported the production of "L-forms" of T. pallidum in vitro with the aid of penicillin or immune serum. A study was undertaken in this laboratory to determine whether cell-wall defective morphological variants of T. pallidum could be produced and whether such variant forms were viable.

The organisms employed were T. pallidum, the Nichol (non-cultivable) strain, pathogenic for rabbits and carried by passage in rabbit testes, and the non-pathogenic T. pallidum strains, Kazan 2, Kazan 5, Kazan 8 and Nichols (cultivable). The non-pathogenic strains were maintained in a fluid medium composed of Bacto heart infusion broth, Bacto yeast extract (1.0% w/v), sodium thioglycollate (0.076% w/v), magnesium sulfate (0.01% w/v), Bacto agar (0.1% w/v), sucrose (10% w/v), and inactivated horse serum (20% v/v). The pH was adjusted to 7.4 before the addition of sucrose or serum. Incubation was at 37°C in Brewer jars containing BBL Gaspak, hydrogen-carbon dioxide generators.

The pathogenic and non-pathogenic treponemes were indistinguishable by light microscopy, phase contrast and dark field microscopy, and by various stains. They displayed a delicate spiral morphology and measured 6-15 μm in length and 0.2 μm in width. Motility varied in the cultivable strains; young organisms were always actively motile, showing a rotation around the longitudinal axis, whereas in three to four week-old cultures the treponemes were either nonmotile or displayed merely a terminal quiver. The pathogenic treponemes were actively motile in fresh suspensions. Certain ultrastructural differences between pathogenic and non-pathogenic strains have been described. The present study confirmed these observations on shape of extremities and number and insertion of fibrils. The structure of the parent organism, examined in detail by electron microscopy, provided the basis for comparison of morphological changes in the variant forms.

Colonial growth of treponemes on solid media did not permit counting by the usual plating technique, due to lack of sufficient size and discreteness of the colonies. Estimation of the concentration of viable organisms of the Kazan 8 strain grown in the fluid medium was accomplished by the method of maximum likelihood estimation (most probable number) as described by Cochran (1950). The direct microscopic count of a 48 hr. fluid culture of the Kazan 8 strain was 5.2×10^6 organisms/ml, and the estimated concentration (Cochran method) of the same culture after centrifugation and dilution was 2.17×10^4 organisms/ml. Inoculation from these dilutions on to plates (which were then read for presence or absence of growth) showed an estimated concentration of 2.72×10^4 organisms/ml, indicating a close parallel to the results for viability in the fluid medium. The

disparity between the results of direct microscopic counts and the viable units per ml may have resulted from damage to some organisms by centrifugation.

Experiments were performed to determine whether penicillin could induce conversion of the parent treponemes to morphological variants. The morphology of treponemes and round bodies in fluid medium with concentrations of penicillin ranging from 0 to 10,000 units/ml (u/ml) was followed at varying time intervals by means of wet mounts, Giemsa stains, acridine orange, and electron microscopy. Treponemes of the Kazan 8 strain were grown for 24 hrs. in fluid medium containing osmotic stabilizers ($MgSO_4$, sucrose) and 20% (v/v) horse serum. At a penicillin concentration of 0.01 u/ml and incubation for 24 hr. there were 80 percent treponemes of which 10 percent were motile. About 20 percent of the culture consisted of round phase dense bodies measuring 0.2-0.4 μm in diameter. A few larger phase dense bodies measuring 0.4-0.6 μm were also seen. At 48 hr. no treponemes could be identified and occasional round bodies were present. Penicillin in a concentration of 0.1 u/ml had a more severe effect within 48 hr. no treponemes or round bodies could be identified. With a concentration of 10 u/ml of penicillin, there were only 20 percent motile treponemes and 75 percent round bodies after 24 hr. and none were detected after 48 hr. At penicillin concentrations of 100, 1000, and 10,000 u/ml no organisms were found by wet mounts at 24 and 48 hr. On Giemsa stains the round bodies were usually purple and varied in size from 0.2-0.4 μm in diameter, whereas treponemes were usually deep pink. Acridine orange vital stains and fixed smears revealed the bright reddish-brown fluorescence of RNA in younger treponemes and in some round bodies, whereas older treponemes and some of the variant forms displayed the greenish fluorescence of DNA.

Electron microscopy of cultures of the Kazan 8 strain treated with 0.01-0.1 u/ml of penicillin revealed the presence of round, oval or irregular structures and occasional treponemes. The round bodies were usually surrounded by a double unit membrane. The cytoplasm displayed a delicate fibrillar pattern with scattered dense ribosomes. More round bodies and fewer treponemes were observed in cultures containing higher concentrations of penicillin.

Negative stains for electron microscopy were done on a culture to which 0.1 u/ml of penicillin had been added. Preparations were studied hourly for 8 hr. after the addition of penicillin. Similar preparations were made of a control culture without penicillin. At 4 hr. the control appeared unaltered, whereas the penicillin-treated culture showed that the cell walls of some organisms appeared lacy, and there was disruption of numerous filaments. At 6 hr. some organisms appeared fragmented. At 8 hr. a few round bodies were detected, whereas treponemes were well preserved in the control cultures.

"Blind" passages were made to determine whether penicillin

treated treponemes. A total of 15 serial transfers were made from penicillin-treated cultures to fresh penicillin-containing and penicillin-free fluid medium. There was no evidence of growth in either of the serial transfers.

Penicillinase was tested for its ability to inhibit or reverse the effect of penicillin. It was found that penicillinase in concentrations of 0.1-10 μ/ml added to a culture of Kazan 8 in fluid medium one-half hr. after the addition of penicillin (0.01 μ/ml) resulted in partial inhibition of the effect of penicillin.

Penicillin-gradient plates with wells that contained penicillin in concentrations of 0.1-10,000 μ/ml and 20% (v/v) horse serum were investigated as means for producing variants on solid medium. The plates were inoculated with an actively growing 24 hr. culture of the Kazan 8 strain. Plates without penicillin were used as controls and were incubated under the same conditions. Colonies were noted in the control plates after 48 hr. The gradient plates with a penicillin concentration of 0.01 and 01. μ/ml showed a few scattered colonies in the area furthest from the trough and plates containing higher penicillin concentrations showed no growth. The colonies observed were very small or hardly visible, measuring 0.1-0.2 mm in diameter, rounded or irregular, cream-colored and glistening. Studied by Dienes' stained preparations, the colonies appeared to grow centrally into the agar for a depth of 4-6 μm. The colonies contained 70 percent round bodies and 30 percent treponemes, the latter usually at the periphery. The round bodies were smaller and denser at the center with a granular appearance and measured 0.1-0.2 μm in diameter. In the paracentral portion of the colony, the round bodies were both phase dense and phase pale, measuring 0.3-0.4 μm in diameter. Some treponemes at the periphery appeared relatively well preserved whereas other organisms were partly fragmented. The colonies were kept under observation for possible L-phase transformation. Dienes' stained preparations were made at weekly intervals of the colonies. Blocks excised from portions of penicillin-containing agar which had received broth inocular but failed to show L-form colony growth were passed after one week to agar plates containing the same concentration of penicillin and to similar medium without penicillin. Growth was not detected in the subsequent transfers. Agar blocks bearing colonies were also transferred directly into fresh penicillin-free fluid medium. Since growth was not detected, 12 "blind" serial transfers in penicillin-free fluid medium were made and each passage was subcultured on penicillin-containing agar. Growth was not detected in any of the serial transfers.

When a specified concentration of penicillin was incorporated in the serum agar medium before pouring plates, much the same results were obtained. In plates incorporating penicillin in concentrations of 0.01-10 μ/ml, a moderate number of very small colonies appeared after 5-7 days of incubation. The colonies were similar to those

found in the penicillin-gradient plates and were most abundant in plates containing 0.01 u/ml penicillin. They appeared to grow into the agar forming central cores up to 6-12 µm in depth. Colonies were not found in plates containing concentrations of penicillin higher than 10 u/ml.

Experiments were conducted to determine whether lysozyme could induce the formation of morphological variants of treponemes. Twenty-four hour cultures of the Kazan 5 strain were harvested by centrifugation and examined by phase contrast microscopy. The inoculum contained 10^6 organsism per ml of which 90 per cent were motile. Half of the suspension was treated with lysozyme in concentrations of 200 µg/ml and the other half used as a control for viable counts. The lysozyme-treated suspension was incubated for 48 hr. and then passed through at least five serial transfers to fresh fluid medium without lysozyme, before re-exposure to 100 µg/ml of lysozyme. Plates were inoculated from each transfer. The original suspension was treated with lysozyme three times (200 µg/ml initially and 100 µg/ml for the 2nd and 3rd exposures. The initial lysozyme-treated culture in fluid medium was examined by wet mounts at 24 and 48 hr. The suspensions contained 80 percent treponemes of which 70 percent were motile. Round bodies, mostly phase dense, comprised 20 percent of the total suspension. Examination of Dienes' stained colonies revealed a greater number of treponemes (75%) and fewer round bodies (25%). Most of the treponemes appeared well preserved. Electron microscopy of the lysozyme-treated broth suspension showed that some treponemes displayed mild to moderate damage of the cell wall with disruption of the filaments. Other organisms appeared intact. The few scattered variants appeared similar to those in penicillin-treated cultures and were surrounded by a single or double unit membrane. In some, portions of treponemes could be seen blending into the variant forms.

Preparations made from the serial transfers (lysozyme-free) showed almost the same growth as the control cultures. The second treatment with lysozyme (100 µg/ml) resulted in more severe damage to the organisms. Only a few intact treponemes were found. The variant forms were surrounded by a single or double unit membrane. Wet mounts and Dienes' stained preparations showed a greater number of round bodies (50%) and a lesser number of treponemes (50%). Following the third treatment with lysozyme (100 µg/ml), there was even greater damage to the treponemes. Wet mounts after 24 hr. showed 20 percent mostly non-motile treponemes and 80 percent round bodies. Plated on to solid medium colonies grew into the agar to a depth of 3-6 µm. Electron microscopy showed more numerous variant forms and fewer treponemes. Passage into lysozyme-free fluid medium did not result in growth. The plates were incubated for six weeks and examined at frequent intervals. L-form colonies were not found. Initial treatment with lysozyme was not lethal to the treponemes, but subsequent lysozyme exposure to the same culture resulted in irreversible damage to the organisms. Lysozyme-induced damage to the cell wall was not as

severe, however, as that of penicillin.

In conclusion, it appears to be relatively simple to produce certain cell-wall defective variants of T. pallidum by use of penicillin or lysozyme. These variants have characteristics suggestive of spheroplasts or transitional phase variants with poor ability to propagate as such. Self-propagating true L-phase variants were not produced in this study.

b. Type culture collections of Mollicutes and L-phase variants of bacteria.

The joint American Type Culture Collection (ATCC) and WRAIR collection of Mollicutes and L-phase variants has been in existence for 10 years. The policies set up for this collection are firmly established and operating effectively. Most depositors have cooperated with the ATCC by submitting their strains at a lower passage level than those previously acquisitioned, and by checking sample vials after preservation of their strains before the strains are released by ATCC. This offers greater assurance to both ATCC and other investigators regarding authenicity and purity of strains. All mycoplasma strains in the collection (with the possible exception of ATCC #25298, Mycoplasma orale, type 3) are now cultivable from the freeze-dried state; this has eliminated the expense of shipping frozen whole cultures in dry ice. The notable absence of complaints that cultures were contaminated at the time of receipt can probably be credited to constant vigilance at each procedural step and thorough sterility testing. There have been complaints that four cultures were nonviable out of 326 vials of cultures sent out from ATCC. Vials of these four cultures were re-checked, and the colony forming units were found to be still as high as at the time of initial processing. Due to the increased number of vials preserved at the time of submission, it has been necessary to replenish the seed stock of only eight strains of Mycoplasmatales and L-phase variants in the past year.

The recommended medium for 65 of the 78 strains now in the collection is Bacto heart infusion broth (pH 7.5) containing 20% (v/v) horse serum (inactivated) and 10% (v/v) of a 25% (w/v) fresh baker's yeast extract. With the exception of M. orale, type 3, the T-strain mycoplasma, and Thermoplasma acidophilum, the other 62 strains will adapt to the above medium. Three swine mycoplasma strains, M. hyopneumoniae, M. suipneumoniae, and M. hyosynoviae (all of which are considered very fastidious) have now been adapted, after a maximum of five passages from their recommended broth medium, to the above medium. Adaptation to this standard medium enables investigators to employ these strains readily in studies that require larger numbers of organisms with reliable growth habits. This medium is not recommended for the initial isolation of the more difficult-to-grow organisms.

The number of vials of Mollicutes and L-phase variants purchased

from the ATCC has increased by only one percent this year (317 vials in 1970 and 326 vials in 1971), reflecting the decrease of the funds to many of the universities and research institutions that ordinarily purchase cultures. Ninety-two vials of ATCC cultures were distributed free of charge to personnel at WRAIR and other military installations. Between 40 and 50 percent of the total requests including both Mollicutes and L-phase variants were made for strains derived from human origin, suggesting a continued active interest in the significance of these agents for man. At least 30 new strains including Mollicutes, L-phase variants, and selected parent or revertant bacterial forms have been received by WRAIR and will be accessioned by the ATCC in the near future.

The freeze-drying method used for preserving the mycoplasmas at ATCC was recommended to a WRAIR meningococcal research group. A strain of meningococcus propagated serially in the presence of penicillin was nonviable after freeze-drying in the routine suspending medium employed at WRAIR. By use of 12% (w/v) sucrose in the special mycoplasma suspending medium used by ATCC, the meningococcal strain was successfully preserved by freeze-drying.

The ATCC Virus Department routinely cultured for the presence of mycoplasmas in viral material received for preservation. Recommendations as to media, methods for cultivation, and microscopic examination for growth were supplied by the Mycoplasma Research Section at WRAIR. Although this is a recent collaborative project, the presence of mycoplasmas in tissue cultures of viral material has already been confirmed by investigators at both institutions. This project will be continued, and hopefully may be expanded to serve similar needs of virologists at WRAIR.

A recent publication by Darland described the isolation of a thermophilic, acidophilic, "mycoplasma-like" organism from a burning coal refuse pile. Because of the possible presence of viable thermophilic organisms in laboratory water baths, incubators, and heated biological material, recognition of and familiarity with such organisms was deemed valuable to the ATCC and WRAIR. Therefore, studies on the appearance, biological properties, and optimal methods for the preservation by freeze-drying on one such strain, Thermoplasma acidophilum, were undertaken.

The descriptions of the original investigators on the shape and size of the cells were confirmed in this laboratory by phase contrast examination of wet mounts of broth cultures and by Dienes stained preparations of the organism on agar blocks. The cells appeared as vacuolated round bodies, coccoid bodies, ring forms, lysed phase-pale bodies, and an occasional filamentous form. The size of the round forms varied from 0.2 to 3.0 µm in diameter and filamentous forms measured 4 to 10 µm in length.

The reported growth requirements of T. acidophilum were also confirmed. The strain was cultivated in a simple, serum-free broth at a pH of 2.0-3.0 and was incubated in a water bath at a temperature of 55-60°C. Growth was determined by opacity of the broth which occurred after 1-7 days of incubation. The organisms were still viable after 19 days of incubation.

Attempts to cultivate colonies on a solid medium had been thwarted by the need for a medium that would remain solid at 60°C and at a pH of 2.0 to 3.0. Sporadic colony formation has been reported by the original investigators on a medium suggested by our laboratory consisting of Darland's broth containing a final concentration of 1.2 percent Oxoid Ionagar No. 2 and with a final pH of 2.3.

Methods for demonstrating purity of these unusual thermophilic, acidophilic cultures have also been developed. The usual media (thioglycollate broth containing horse serum and sheep blood agar) employed to test for contamination in cultures of bacteria of medical importance are unsuitable for thermophilic organisms. Instead specific media for isolation of soil organisms, nutrient media, and T-strain mycoplasma media, covering a pH range of 2.0-7.8 and a temperature range of 37-60°C were employed to test for contaminating bacteria. The Thermoplasma strain did not survive in these media nor were any other organisms isolated.

This laboratory recommended to the original investigators various combinations of suspending media and various freeze-drying methods used by ATCC. Good preservation by both laboratories has been obtained by using a suspending medium of 12% (w/v) sucrose in double strength skim milk and the "batch" method of freeze-drying. A sample of the ATCC freeze-dried material tested for viability after storage at room temperature for one week resulted in growth of at least $1x10^5$ organisms per ml by the tube dilution method.

c. Taxonomy of the class Mollicutes.

The constantly expanding activity in the field of mycoplasmology necessitates a continual up-dating of the taxonomy of the class Mollicutes and a frequent reevaluation of the minimum standards for the valid description of new species. The tables that follow summarize the current thinking on these subjects.

As shown in Table 24, the class Mollicutes still contains a single order, Mycoplasmatales, defined in terms of morphologic characters, including structure of the outer covering and size and shape of the cells. There are two families defined in terms of physiologic requirements, the sterol-requiring Mycoplasmataceae, and the sterol nonrequiring Acholeplasmataceae. There are now three genera, Mycoplasma, Acholeplasma and Thermoplasma defined, for all

Table 24. Class Mollicutes.

Taxonomic Ranks		Characters used to Circumscribe Taxa
Order:	Mycoplasmatales	Morphologic Features: (1) Structure of outer covering. (2) Size and shape of cells.
Families:	Mycoplasmataceae Acholeplamataceae	Physiologic Requirements: (1) Special growth factors. (2) Factors inhibiting growth. (3) Temperature. (4) pH. (5) Atmospheric conditions.
Genera:	Mycoplasma Acholeplasma Thermoplasma	Genetic and Physiologic Factors: (1) Genome size. (2) G+C content of DNA. (3) Requirements for growth.
Species:	CA. 40 spp.	Biochemical Activities: (1) Metabolic products. (2) Respiratory pathways. (3) Enzyme systems.
Subspecies:		Chemical Composition: (1) Electrophoretic patterns of cell proteins. (2) Nucleic acid homologies.
(Infrasubspecific subdivisions)		(Not subject to rules of bacteriological code)
Serotypes:		Immunologic Patterns: (1) Various serologic tests.

practical purposes, in terms of the same physiologic requirements used to distinguish families. This is inadequate, and the inclusion of certain genetic criteria is indicated for determining generic constitution. Genetic characters such as genome size and G+C ratio, would have very real value for excluding inappropriate members from a genus. There are approximately 40 species recognized at present; these can be differentiated on the basis of biochemical activities such as formation of metabolic products and use of respiratory pathways and enzyme systems. Several subspecies are presently recognized by their differences in chemical composition. At the infrasubspecific level, beneath the taxonomic level regulated by the Bacteriological Code, are the serotypes.

When an investigator discovers what he believes to be a new species of Mollicutes, and when he wishes to propose a new name, it is his obligation to provide adequate evidence that his organism is indeed distinct from all known species. For this purpose the Subcommittee on the Taxonomy of Mycoplasmatales has been preparing the specifications or "minimum standards" for the description of new species and makes the recommendations that follow. As shown in Table 25, there should be a preliminary clonin of the strain by filtration of a broth culture through a 0.22 μm pore diameter membrane filter (or the smallest pore size the organism will pass). Then there should be serial dilution of the filtrate, plating of the dilutions, and cloning for colonies. The clones should be cultivated in broth, and the whole procedure repeated two more times. At least two separate clones should be examined in the subsequent tests.

To establish that the proposed new species belongs to the order Mycoplasmatales, it is necessary to show by electron microscopy that the organism lacks a rigid cell wall and that it is bounded by a single triple-layered membrane. As a consequence of the lack of a cell wall, there are other features demonstrable by routine techniques. Thus, it must be shown that the organism is gram-negative, forms the distinctive "cored" colonies on agar, and does not revert to a walled bacterium during five successive passages in the absence of penicillin, when examined macroscopically and microscopically.

By phase contrast or darkfield microscopy, the cells must appear as coccoid bodies, ring forms, or filaments some of which may branch. The cell size must be estimated, and at least some cells should pass a 0.45 μm pore diameter membrane filter. Some species may be motile, but tests for motility are optional at present.

To establish the family (Table 26) to which the proposed new species belongs, dependence of the organism on sterol must be determined. It is not sufficient to test for growth in serum-free media, because false positive and false negative results may occur. Therefore, specific methods employing cholesterol, Tween 80 and

Table 25. Minimum standards for description of new species of Mollicutes.

Morphologic Features used to Circumscribe Order Mycoplasmatales
Preliminary:
A. Filtration, dulution, plating, cloning of colonies. (Procedure repeated 3 times)
B. At least two clones examined in all subsequent tests.
Minimum required:
A. Structure of outer covering.
1. Absence of rigid cell wall.
2. Presence of single triple-layered membrane.
a. Gram negative.
b. Colonial "down-growth" into agar.
c. Absence of reversion to wall-containing organism.
B. Shape of cells.
1. Coccoid bodies, ring forms, filaments, some branching.
C. Size of cells.
1. Less than 0.5 µm, greater than 0.1 µm.
a. Filterable through membranes of 0.45-0.22 µm pore size.
Optional:
D. Motility

and albumin supplements, as described by Tully and Razin (1969) and Razin and Tully (1970), should be used. The optional characters may soon become mandatory since new thermophilic, acidophilic, or strictly anaerobic organisms have recently been described.

Table 26. Minimum standards for description of new species of Mollicutes.

Physiologic Requirements used to Circumscribe Family Mycoplasmataceae or Acholeplasmataceae	
Minimum required	**Optional**
A. Special growth factors.	B. Factors inhibiting growth.
1. Sterol.*	C. Temperature.
a. Fatty acids.	D. pH.
	E. Atmospheric conditions.

*By methods of:
Tully & Razin, 1969
Razin & Tully, 1970

As long as there was only one genus in each of the two families, there was no need to define specific criteria for genera. Now that a new genus, Thermoplasma, has appeared, and various proposals for splitting the genus Mycoplasma have been made, the Subcommittee will have to reconsider the criteria to be required for generic classification (Table 27). It is here that genetic criteria could be most helpful.

To determine that a proposed species is new and distinct from existing species (Table 28), the following tests are required: hydrolysis of urea and arginine, acid from glucose and whether by oxidative or fermentative attack (O-F test), and production of carotenoids. Although optional at present, other activities, many of which will probably appear in the new Bergey's Manual classification, are quite useful for differentiating species. These are acid from mannose and other carbohydrates, aesculin hydrolysis, phosphatase activity, film and spot production on selected media, digestion of coagulated horse serum, gelatin and casein, tetrazolium reduction, hemolysis and adsorption of specified erythrocytes. On the basis of all the foregoing characters a dichotomous key to the class Mollicutes with accompanying chart of characteristics of species has been prepared for use until a new edition of Bergey's Manual providing such information becomes available.

Table 27. Minimum standards for description of new species of Mollicutes.

Physiologic and Genetic Factors used to Circumscribe genus Mycoplasma, Acholeplasma, and Thermoplasma	
Minimum required	Optional
A. Physiologic (as for family)	B Physiologic (as for family).
1. Growth factors required.	1. Growth inhibitors.
	2. Temperature.
	3. pH.
	4. Atmospheric conditions.
	C. Genetic
	5. Genome size.
	6. G+C content of DNA.

Table 28. Minimum standards for description of new species of Mollicutes.

Biochemical Activities used to Circumscribe Species	
Minimum required	Optional
A. Urea hydrolysis.	F. Acid from mannose.
B. Arginine hydrolysis.	G. Acid from other carbohydrates.
C. Acid from glucose.	H. Aesculin hydrolysis.
D. O-F test (glucose).	I. Phosphatase activity.
E. Carotenoid production.	J. Film and spot production on horse serum and egg yolk media.
	K. Proteolysis of coagulated horse serum, casein, and gelatin.
	L. Tetrazolium reduction aerobically and anaerobically.
	M. Hemolysis of sheep and guinea pig RBC.
	N. Adsorption of guinea pig RBC.

Finer distinctions at the subspecies level are optional at present, and are based on more complicated tests, polyacrylamide gel patterns and nucleic acid homologies (Table 29).

Table 29. Minimum standards for description of new species of Mollicutes.

Chemical Composition used to Circumscribe Subspecies	
Minimum required	Optional
	A. Electrophoretic patterns of cell proteins.
	B. Nucleic acid homologies.

Whereas immunologic characters are too specific to be of use in classifying organisms into groups that constitute the higher taxonomic ranks covered by the Bacteriological Code, immunologic characters are indispensible for identification of individual organisms. As shown in Table 30, the minimum standards require serologic comparison, if not with all species, at least with all species that have the same habitat and biological characters. To do this at least two serologic tests must be used, one with high specificity and high sensitivity, such as growth inhibition, and another with broader specificity and lesser sensitivity, such as complement fixation.

Table 30. Minimum standards for description of new species of Mollicutes.

Tests for Antigenic Relatedness used to Circumscribe Serotype	
Minimum required	Optional
A. Growth inhibition or metabolic inhibition.	D. Fluroescent antibody (direct or indirect).
B. Complement fixation or double immunodiffusion.	E. Agglutination (direct or indirect).
C. Comparison with all named species having same habitat and same biological characters.	F. Comparison with all names species in Mycoplasmatales.

These guidelines, with modifications as needed, should prevent the proposal of new species names for new isolates that are, in fact, members of taxonomically recognized species.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 168, Bacterial diseases

Literature Cited.

Publications:

1. McGee, Z. A., Wittler, R. G., Gooder, N., and Carache, P.: Wall-defective microbial variants: terminology and experimental design. J. Infect. Dis. 123:433-438, 1971.

2. Williams, C. O. and Wittler, R. G. Hydrolysis of aesculin and phosphatase production by members of the order Mycoplasmatales which do not require sterol. Int. J. Syst. Bact. 21:73-77, 1971.

3. Rodrigues, M. Studies on wall-defective variants of Treponema pallidum. Ph.D. Thesis. George Washington University, Washington, D. C., 1971.

4. Norman, M. C., Franck, E. B., and Choate, R. V. Preservation of Mycoplasma strains by freezing in liquid nitrogen and lyophilization with sucrose. Appl. Microbiol. 20:69-71, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

3. AGENCY ACCESSION	4. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 644		

5. DATE PREV SUMRY	6. KIND OF SUMMARY	7. SUMMARY SCTY	8. WORK SECURITY	9. REGRADING	9A. DISSEMINATION	9B. SPECIFIC DATA CONTRACTOR ACCESS	9C. LEVEL OF SUM
70 07 01	D. Change	U	U	NA	NI	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
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PRIOR YEAR		
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CURRENT YEAR	12	1.5

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence not considered.

22. KEYWORDS (Precede each with Security Classification Code) (U) Zoonoses; (U) Epidemiology; (U) Leptospirosis; (U) Melioidosis; (U) Leptospiras; (U) Animal Viruses

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

23. (U) Biological characteristics of zoonotic agents of diseases of real or potential military importance are studied to develop more suitable diagnostic procedures, treatment and control measures. Current studies are in leptospirosis and melioidosis.

24. (U) Conventional microbiological and chemical technics are used. New procedures are developed as needed.

25. (U) 70 07 - 71 06 Genetic heterogeneity within and between pathogenic and saprophytic leptospiras was further defined by DNA annealing tests. It was demonstrated that salt concentrations in the annealing milieu can affect relative binding among DNA's of partial homology. This finding has significance for utilization of annealing technics with other organisms, and also served to bring out 2 different types of DNA in select strains of Leptospira - a possible reflection of occurrence of phage, episomes, or other extrachromosomal nucleic acids. The genetic disclosures may be relevant to cross-immunological relationships and pathogenicity of strains. Serological tests conducted on human and animal sera submitted by NAMRU 3 indicate that endemic foci of leptospirosis occur in Egypt. The practicability of maintaining the viability and virulence of leptospiras by storage in liquid nitrogen refrigerators has been now demonstrated over a 5 year observation period. New host - leptospiral serotypes have been established from test findings on specimens submitted from different parts of the world. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70-30 Jun 71.

* Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. AGO FORMS 627A AND 1498, 1 MAR 66, FOR ARMY USE ARE OBSOLETE

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 170, Militarily important diseases transmissible between animals and man

Investigators.
Principal: A. D. Alexander, Ph.D.
Associate: M. Rogul, Ph.D., L. Evans, B.S., J. Brendle, B.S.

Description.

Studies are conducted on basic biological characteristics (e.g., strain differences in genetic, chemical, physical and serologic properties, disease and immunity mechanisms) which bear on development of suitable diagnostic, treatment and control measures. Current efforts are on agents of leptospirosis.

Progress.

1. Genetic Characteristics of Leptospira.

In previous studies (Annual Report 1969-1970, ref. 1) leptospiras within and between the pathogenic and saprophytic "complexes" could be separated into distinct genetic groups on the basis of DNA composition and annealing tests. The genetic grouping appeared to be related to cross-immunity, pathogenicity and other biological characteristics of leptospiras. Additional studies were done to clarify the "partial" genetic relatedness observed within groups of both pathogenic and saprophytic complexes. When the results of agar column studies which were performed in 2 X SSC (SSC=0.15M NaCl and 0.015M Na citrate) were compared with the results from filter annealing tests (3 and 6 X SSC) it appeared that comparatively more DNA annealed with an increase in salt concentration. Tests were performed to determine whether this was caused by increased retention of the DNA on the filters or if the increase was directly related to the effect of salt on DNA annealing, secondly whether increases in DNA annealing were linear and thirdly to determine the characteristics of the additional DNA annealed.

The annealing of homologous DNA from pathogenic reference strain of bataviae was perofrmed in 2, 3 and 6 X SSC at 60, 65 and 70C. Figure 1 shows that 65C is the incubation temperature of choice. At this temperature the temperature midpoints (Tm) and total amounts of DNA considered to be specifically annealed were at their highest estimation. This incubation temperature was used for the rest of the study. This figure and TAble 1 show that high salt concentration does

increase the total amount of DNA annealed, that this DNA has to have some generic specificity in common with the reference DNA (Proteus mirabilis DNA does not) and that the high salt concentration obviously increases the binding of a species of DNA that is characterized by low thermal elution. Therefore it was shown that high salt concentration actually increased DNA annealing rather than a special effect of membrane filters. The comparative annealing was not linear.

The identity of the lower peak was unknown to us. It was hypothesized that it might be an artifact of our assay method, such as an accumulation of DNA that would have eluted at lower temperatures without the peaking effect. Perhaps these were small DNA fragments or some other kind of low stability binding capacity, such as high concentrations of adenine and thymine. There is the possibility of a phage or episome being demonstrated or a phylogenetic divergence of bases which is best demonstrated at high salt concentration. There is also the possibility of a direct effect of salt on the DNA such as unfolding or hydration which allows greater interaction among the strands. In order to obviate artifacts of the agar column annealing technique, elutions of DNA from bataviae*/icterohommorhageae (RGA) (* = radioactive, sheared and denatured DNA) were collected at 1 degree intervals starting at 34C. The initial DNA elutions occurred at 42C in 2 X SSC, 40C in 3 X SSC and 37C in 6 X SSC. This suggested that thermally labile bonds might be involved but since the shapes of the elution profile peaked at the same points it was assumed that adenine-thymine bonds had a minimal involvement. In this annealing of two strains which are serologically heterologous, yet apparently very similar genetically, the lower peak was still present. If this is a phage or episome, it is contained in both strains.

The annealing of DNA from heterologous strains did not increase our understanding of the lower peak. When radioactive bataviae* fragments were reacted with DNA from strains javanica and biflexa (CDC) (2 different genetic groups) only relatively heat labile peaks were demonstrable (Fig. 2). Their relatedness to the thermolabile bataviae peak has not been investigated as yet. Even though the total "specific" counts retained increased with high salt concentration, the Tm's at each concentration remained about the same. This suggested that either more potentially active DNA was made available for the reaction or else our estimation of Tm's is not accurate enough to reflect the differences involved.

When the biflexa group strain patoc DNA was investigated there was little if any thermolabile DNA demonstrated in the homologous annealing reaction (Fig. 3). We therefore concluded that the bataviae strain has at least two species of DNA within each cell. Figure 3 also showed that the amount of relatively thermostable DNA increased

with increased salt concentration. The Tm's remained about the same. In the heterologous reaction of patoc*/CDC the Tm's were quite similar at each salt concentration, but the relative homologies definitely increased. Figure 4 depicts an expanded thermal elution of patoc*/CDC. It is obvious that a specific reaction of low thermal stability occurred. As in the homologous bataviae*/bataviae profile, elutions at low temperatures showed a slight increase in the formation of low heat stable structures at high salt concentrations. However, it is obvious that overall DNA reactions were increased.

Our genetic grouping of the leptospiras is still the same as that reported in the previous annual report.

2. Preservation of Leptospiras.

Observations on the effect of storage in a liquid nitrogen refrigerator on the viability and virulence of leptospiras were projected for a 5 year observation period. These studies, now completed, were initiated to find a more economical and less time consuming method to maintain the large number of leptospiral serotype strains needed for operation of a leptospirosis reference laboratory. Heretofore, several hundred stock cultures were labouriously maintained by periodic transfers at 3 to 6 month intervals.

The preparation studied was an eight-day-old culture of serotype canicola, strain Moulton, to which glycerol was added to final 10% concentration. The preparation was distributed in "cryules" then rapidly frozen to -130C at the rate of 60C per minute. "Cryules" were stored in the vapor portion of liquid nitrogen refrigerator. Tests were done on thawed, pooled cultures from 12 "cryules." The pooled culture was serially diluted by half-log increments with Stuart's basal medium to 10^{-8}. Dilutions were inoculated intraperitoneally each into 10 weanling hamsters using a 0.5 ml dose. The concentration of viable organism was determined by direct microscopic counts with the use of Petroff-Hauser counting chamber and also by cultural tests in Fletcher's medium. A summary of findings of periodic examinations over the 5 year observation period is shown (Table 2). One day after freezing the viability and virulence of the culture decreased 1 to 2 logs but thereafter over 5 years of storage there were no further changes. The differences in counts and virulence titers at various test times may be attributable to one or more of the following factors: difference in relative disease susceptibility of hamsters used at different times; difference in susceptibility of hamsters used at different times; difference in growth-promoting properties of various lots of media, variations inherent in procedures used to thaw cultures, operator variations.

Two different groups of leptospiral cultures kept in liquid nitrogen refrigeration were examined for viability. Strains in both

groups were rapidly frozen in vapor portion of liquid nitrogen refrigerator and 10% glycerol was used as the cryoprotective agent. The first group comprised 61 strains which were grown in Stuart's medium and had been stored for 37 months at time of examination. One ampule was opened for each strain. Leptospiras were recovered from 53 strains; four were contaminated and 4 were nonviable. The nonviable strains initially had poor growth when processed. The second group of 27 strains were grown in a tween-80 albumin medium and had been frozen approximately 1 month previously. All except one strain were recovered. The exceptional strain was contaminated.

3. New Serological Leptospirosis Finding.

The following new information was obtained or affirmed from tests on cultures or sera submitted from various parts of the world.

1. The reactions of a total of 50 selected leptospirosis positive sera from human beings, livestock and dogs in Egypt were affirmed by conventional microscopic agglutination tests. The sera were obtained by NAMRU-3 in Cairo during the course of a sero-epidemiological study. The findings affirmed that leptospirosis is enzootic in Egypt.

2. Two strains were submitted from Razi Institute, Iran. One isolated from Apodemus sylvaticus was identified to be a member of the hebdomadis group; the second was a grippotyphosa serotype isolated from Mus musculus.

3. Five strains recovered from human patients in New Zealand were identified to be members of the hebdomadis (2 strains) ballum (2 strains) and pomona (1 strain) groups.

4. A strain isolated from cattle in Tasmania was identified to be serotype hardjo.

5. Serotype icterohaemorrhagiae was identified to be the etiologic agent of a case of human leptospirosis in Ecuador.

Summary and Conclusions.

1. Genetic heterogenicity within and between pathogenic and saprophytic leptospiras was further defined by DNA annealing tests. It was demonstrated that the total amounts of leptospiral DNA strands which annealed to each other increased with high salt concentration. This is especially evident with certain relatively heat labile homologous DNA's and in heterologous DNA duplexes. The results indicated that strain bataviae contained at least two species of DNA, whereas strain patoc seems to have only one. The use of high salt concentrations during DNA annealing and the resulting profiles

magnify homologies that are small but real. This method provides another reasonable approach to the search for phage and episomes among the spirochetes.

2. It has now been demonstrated that the viability and virulence of stock cultures of Leptospira can be maintained by storage in liquid nitrogen refrigerator. This would obviate the need for laborious maintenance of cultures by regular transfers.

3. Serologic check tests have served to affirm the existence of enzootic foci of leptospirosis in Egypt. New host-serotype relationships were established from test findings on specimens submitted from various countries.

Table 1
Comparative Homologies of Leptospiral DNA
in Different Salt Concentrations

Radioactive DNA from Reference Strain[a]	DNA Test Strain	Agar			Filters[b]	
		2 X SSC	3 X SSC	6 X SSC	3 X SSC	6 X SSC
bataviae*	bataviae	100.0	100.0	100.0	100.0	100.0
	RGA	92.4	102.0	98.7	92.6	80.0
	javanica	30.7	41.0	50.2	41.7	48.8
	CDC	4.9	6.6	9.4	4.6	8.9
	P. mirabilis	2.5	3.3	3.4	3.6	2.9
patoc*	patoc	100.0	100.0	100.0	100.0	100.0
	CDC	20.5	24.5	31.2	29.0	42.7
	A-183	15.3	26.8	28.4	35.9	32.8
	P. mirabilis			0.5	4.6	1.9

[a] Ratio of radioactive DNA to immobilized DNA was 1:30. All annealing reactions were carried out at 65 C.

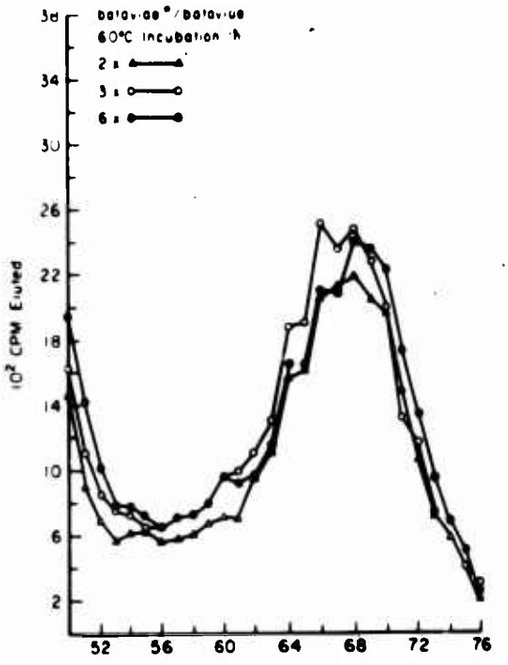
[b] Filter method of Denhardt.

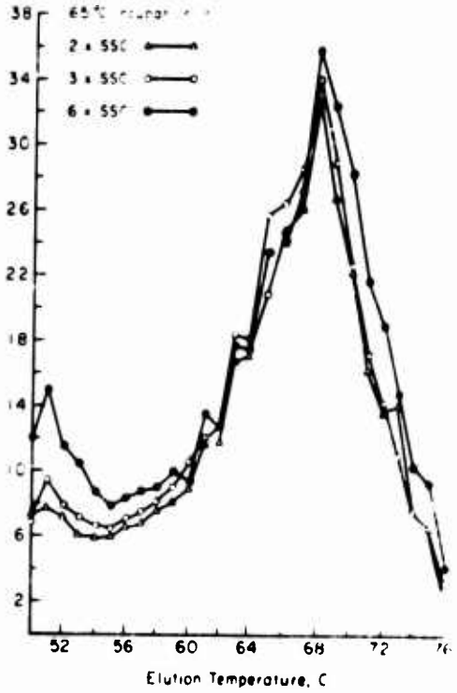
Table 2
Observations over a 5 year period on the viability and virulence of a culture of serotype canicola after freezing and storage in liquid nitrogen vapor

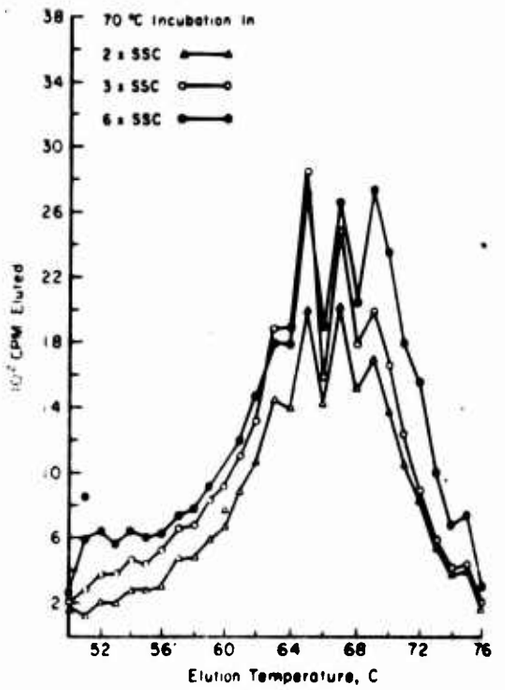
Time of Examination After Freezing and Storage	Microscopic count per ml	Viable Count per ml by Culture	LD 50 Hamsters	LD* Hamsters
Pre-treatment (control)	$1.25 \times 10^{8.8}$	$10^{7.8}$	$10^{-7.25}$	$10^{-7.5}$
1 day	$8.12 \times 10^{7.0}$	$10^{6.1}$	$10^{-5.9}$	$10^{-6.5}$
1 month	$1.12 \times 10^{7.0}$	$10^{6.0}$	$10^{-4.4}$	$10^{-6.0}$
7 months	$1.14 \times 10^{7.0}$	$10^{6.25}$	$10^{-5.3}$	$10^{-5.5}$
1 year	$1.5 \times 10^{7.0}$	$10^{4.6}$	$10^{-4.9}$	$10^{-5.5}$
2 years	$2.0 \times 10^{7.0}$	$10^{6.0}$	$10^{-6.25}$	$10^{-6.0}$
4 years	$8.6 \times 10^{6.0}$	$10^{5.9}$	$10^{-5.7}$	$10^{-6.0}$
5 years	$8.0 \times 10^{6.0}$	$10^{6.1}$	$10^{-6.0}$	$10^{-6.5}$

*Lowest dose producing death.

Figure 1


60°C incubation in
2 x
3 x
6 x
10^3 CPM Eluted
52
56
60
64
68
72
76


2 x SSC
3 x SSC
6 x SSC
Elution Temperature, C


70 °C incubation in
2 x SSC
3 x SSC
6 x SSC
10^3 CPM Eluted
Elution Temperature, C

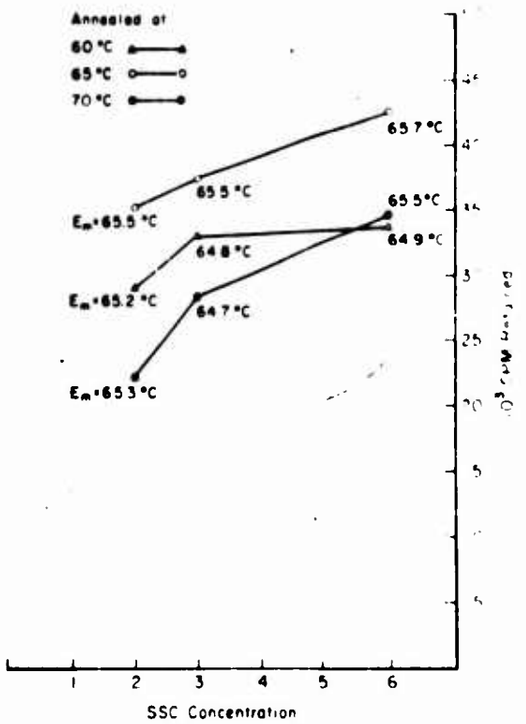

Annealed at
60 °C
65 °C
70 °C
65.7 °C
65.5 °C
E_m=65.5 °C
65.5°C
64.9°C
64.8 °C
E_m=65.2 °C
64.7 °C
E_m=65.3 °C
SSC Concentration

Figure 2

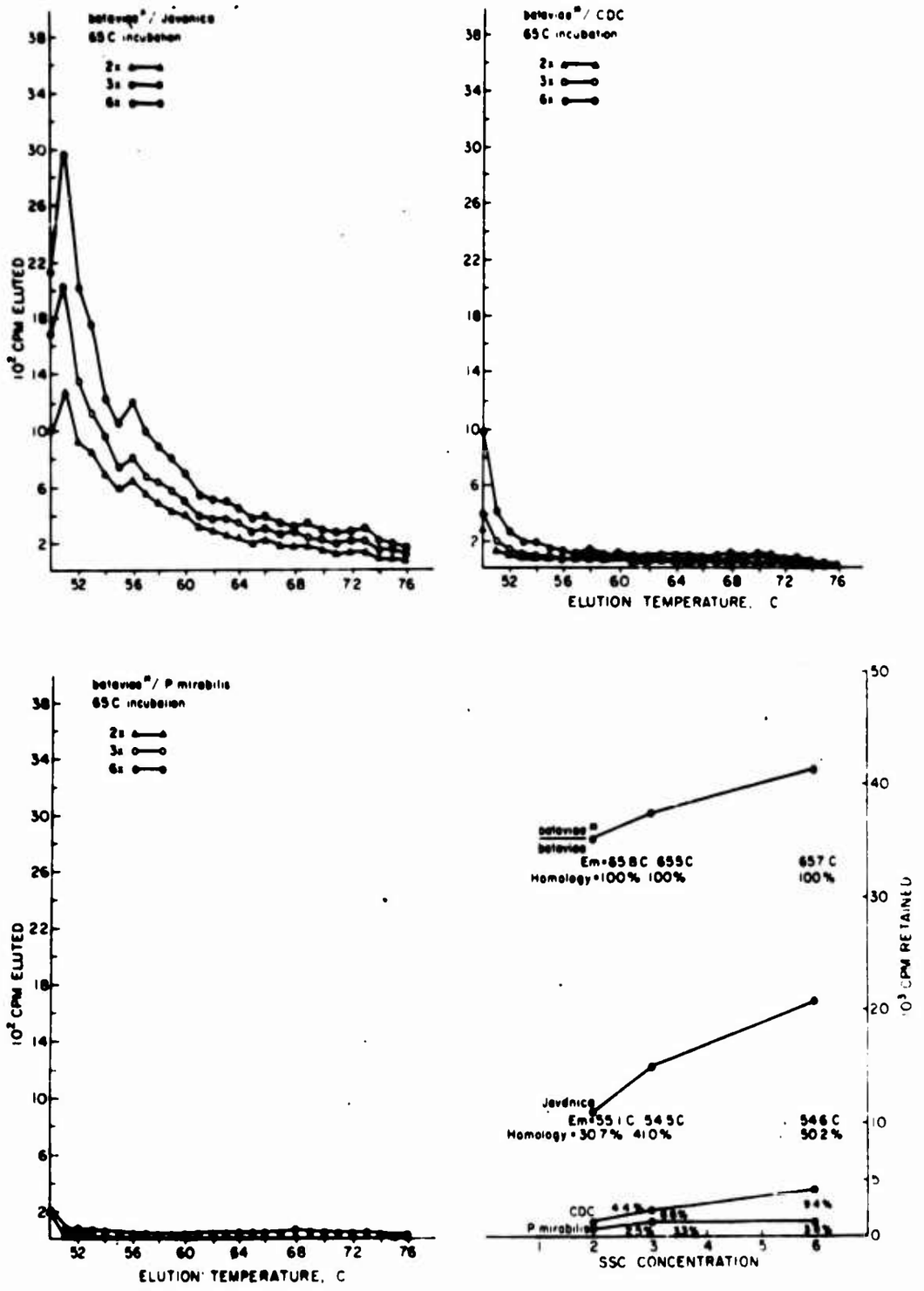
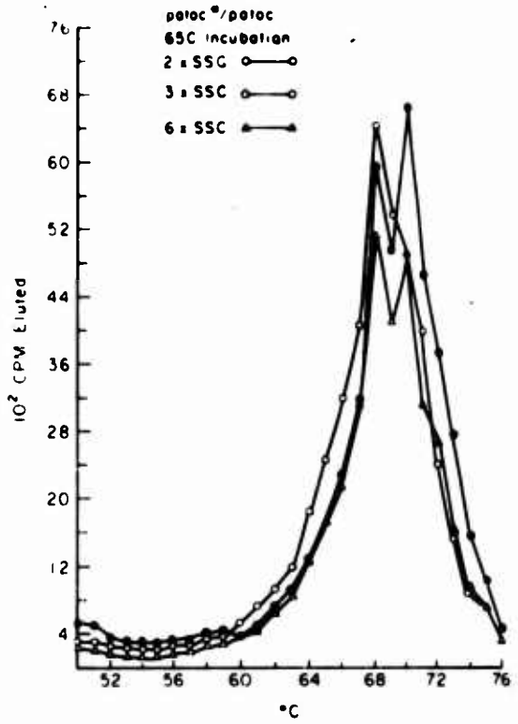
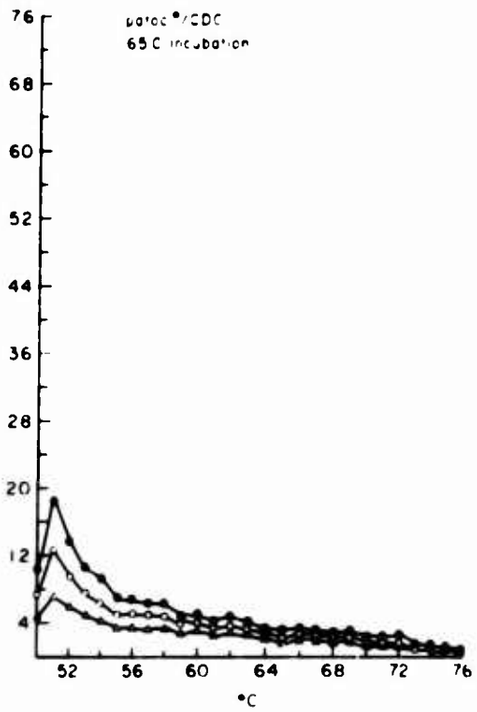

2x
3x
6x
10^2 CPM ELUTED
ELUTION TEMPERATURE, C
Em=65.8C 65.5C
Homology=100% 100%
65.7 C
100%
Em=55.1C 54.5C
Homology=30.7% 41.0%
54.6 C
50.2%
10^3 CPM RETAINED
SSC CONCENTRATION

Figure 3


patoc*/patoc
65C incubation
2 x SSC
3 x SSC
6 x SSC
10² CPM Eluted
°C


patoc*/CDC
65C incubation
°C

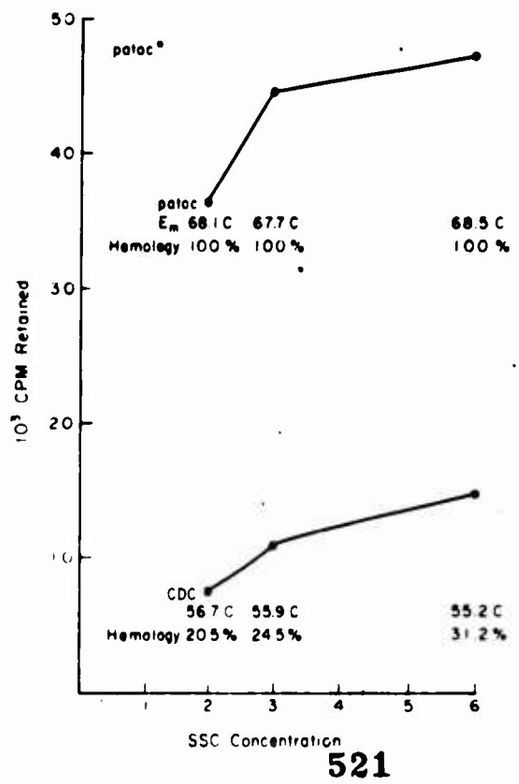
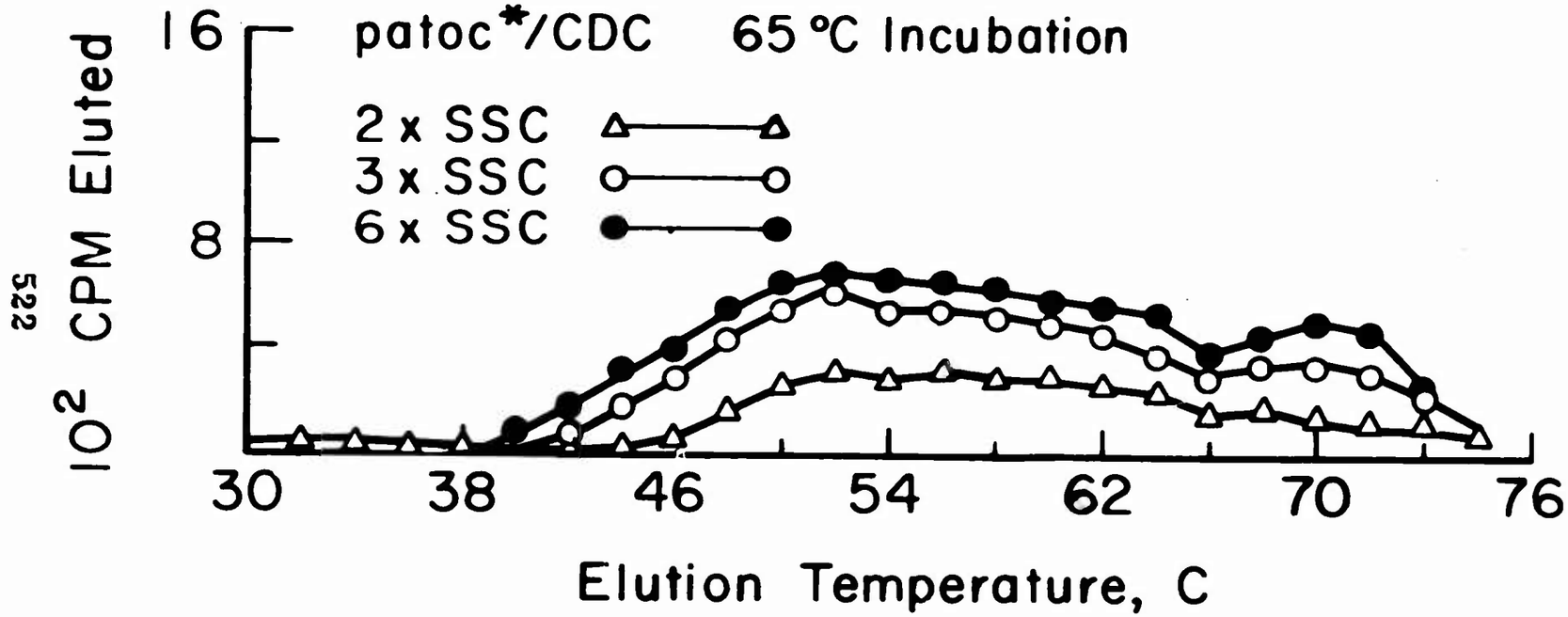

patoc*
patoc
Em 68.1C 67.7C 68.5C
Homology 100% 100% 100%
CDC
56.7C 55.9C 55.2C
Homology 20.5% 24.5% 31.2%
10³ CPM Retained
SSC Concentration

Figure 4


patoc*/CDC 65 °C Incubation
2 x SSC
3 x SSC
6 x SSC
16
8
10^2 CPM Eluted
522
30
38
46
54
62
70
76
Elution Temperature, C

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 170, Militarily important diseases transmissible between animals and man

Literature Cited.

References:

1. Haapala, D. K., Rogul, M., Evans, L. B., and Alexander, A. D.: Deoxyribonucleic acid base composition and homology studies of Leptospira. J. Bacteriol. 98: 421-428, 1969.

Publications:

2. Kaufman, A. F., Alexander, A. D., Allen, A. H., Cronin, R. J., Dillingham, L. A., Douglas, J. D., Moore, T. D., Meliodosis in imported non-human primates. J. Wildlife Dis. 6: 211-219. 1970.

3. Alexander, A. D., Wood, G., Yancey, F., Byrne, R. J., and Yager, R. H. Cross neutralization of leptospiral hemolysins from different serotypes. Infection and immunity 4: 154-159, 1971.

4. Hamson, L. E., Reynolds, H. A., and Evans, L. B. Leptospirosis in Swine caused by serotype grippotyphosa. Am. J. Vet. Research 32: 855-860, 1971.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	3. REPORT CONTROL SYMBOL
DA OA 6448	71 07 01	DD-DR&E(AR)636

4. DATE PREV SUMRY	5. KIND OF SUMMARY	6. SUMMARY SCTY	7. WORK SECURITY	8. REGRADING	8A. GROUP INDTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 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	3A061102B71Q	00	171
b. CONTRIBUTING				
c. [redacted]	CDOG 1412A(2)			

11. TITLE (Procede with Security Classification Code)

(U) Development of Biological Products of Military Importance (09)

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: 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	71	6	170
FISCAL YEAR — SUBSEQ	72	6	

19. RESPONSIBLE DOD ORGANIZATION

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Washington, D. C. 20012

b. ADDRESS:

c. RESPONSIBLE INDIVIDUAL
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TELEPHONE: 202-576-3551

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Division of Communicable Disease and Immunology

b. ADDRESS: Washington, D. C. 20012

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TELEPHONE: 202-576-5208
SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

ASSOCIATE INVESTIGATORS
NAME: Berman, S. L.
NAME: Altieri, P. L. DA

PII Redacted

21. GENERAL USE

Foreign Intelligence Not Considered

22. KEYWORDS (Procede each with Security Classification Code)

(U) Biological Products; (U) Chikungunya Virus; (U) Freeze-Drying; (U) Meningococcus Polysaccharidas; (U) Q Fever; (U) Vaccines

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Procede text 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) 70 07 - 71 06 Investigations have continued on the development of new and improved biological products for military use. - 1. Pilot scale methods for the production of purified high molecular weight group B meningococcal polysaccharide have been developed. However, the product failed to elicit an immunogenic response in man. 2. Primary chick embryo suspension cultures have been employed for the preparation of high titer chikungunya virus, viral antigens, and a highly potent formalin-inactivated chikungunya vaccine. 3. Pilot scale lots of purified Q fever vaccine were produced from freshly harvested infected yolk sac membrane by the genetron-extraction and zonal centrifugation procedure. 4. Preliminary studies on formalin inactivation of bacterial and viral suspensions indicate that the bulk of the formalin added is chemically bound to a constituent of the organism and/or the suspending medium. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Jul 70 - 30 Jun 71.

* Applicable to contractors under guidelines approved

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. FORMS 4844 AND 488

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 171, Development of biological products of military importance

Investigators.

Principal: Joseph P. Lowenthal, ScD
Associate: Sanford Berman, PhD; Patricia L. Altieri, BS; Arthur White, PhD; Doria Dubois, MS; Albert Groffinger

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 Polysaccharides.

During this period studies have been carried out on the development of pilot scale methods for the preparation of purified high molecular weight polysaccharides derived from Neisseria meningitides, group B, for use in the immunization of man against meningococcal meningitis.

a. A lot of group B polysaccharide vaccine was prepared from an eight hour culture of N. meningitidis, strain B-11, by the procedures developed in this laboratory for the preparation of groups A and C polysaccharide vaccines (Annual Report, 1969). The resulting purified B material had the characteristics of the group B polysaccharide previously described (1), and its molecular weight was in the range of the molecular weights of the groups A and C polysaccharides which proved to be immunogenic in man. However, unlike the A and C preparations, the group B product failed to elicit an immunological response in humans.

b. Additional studies were directed towards the preparation of higher molecular weight forms of the group

B specific polysaccharide, on the assumption that the larger molecular species are immunogenic. Close examination of the characteristics of cultures of strain B-11 in the modified Watson & Scherp medium showed that, as the incubation time was extended from 6 to 14 hours, the yield of polysaccharide increased and the pH of the culture dropped from 6.3 to 5.15, as shown in Table I.

TABLE I

Effect of Incubation Time on Final pH of Culture and Yield of Group B Polysaccharide

Incubation Time (Hours)	Final pH of Culture	Yield of Final Product (mgs/liter)
6	6.3	3.4
8	5.5	11.5
10	5.25	18.5
12	5.15	33.5
14	5.15	35.2

Cultures of strain B-11 were grown in 15 liter volumes of modified Watson & Scherp medium in 20 liter carboys on a reciprocal shaker, incubated at 36°C.

The molecular weights of the purified polysaccharides obtained from the cultures harvested at each of the designated time periods were determined by gel filtration on Sephadex G-200. In each instance, the average molecular weight was greater than 100,000, the minimum considered to be acceptable for the groups A and C polysaccharides. However, as the incubation time increased the ratio of the larger molecular weight components to the smaller components decreased. Thus there appeared to be a relationship between the pH of the culture and the amount of larger molecular weight component present in the culture, suggesting that exposure to acid conditions results in the degradation of the polysaccharide molecule. Consequently, studies were carried out on methods for altering the pH cycle during the growth of the B-11 culture.

c. Preliminary studies showed that increasing the phosphate content of the medium two- and four-fold prevents the pH of the culture from dropping below pH 6.0,

and results in the stabilization of the pH at about 6.5 after 14-16 hours incubation. Other experiments demonstrated that a 300% increase in the casamino acids concentration and an 80% decrease in the dextrose concentration of the medium resulted in a rise in the final pH, after 16 hours incubation, to 7.8. When these modified media were used on a pilot-scale level (15 liters of medium per 20 liter carboy), the following results were obtained:

TABLE II

Effect of Modified Media on Final pH of Culture and Yield of Group B Polysaccharide after 16 Hours Incubation

Medium	Final pH of Culture	Yield of Final Product (mgs/liter)
2X Phosphate[a]	6.5	50.5
4X Phosphate[b]	6.6	25.5
3X CA+0.2X Dextrose[c]	7.8	11.6

[a]Phosphate content of medium of Watson & Scherp increased 2 fold.

[b]Phosphate content of medium of Watson & Scherp increased 4 fold.

[c]Casamino acids content of medium of Watson & Scherp increased 3 fold, and dextrose content decreased to 1/5 of original concentration.

As shown in Table II, cultivation of the B-11 strain of N. meningitidis in a medium with an increased phosphate content, or with an increased casamino acids and reduced dextrose content, resulted in a reversal of the usually observed pH cycle (Table I). In addition, marked differences in the yield of purified polysaccharide were obtained, with significantly reduced yields from the more alkaline culture fluids, even though the growth of the organism was enhanced under alkaline conditions.

Molecular weights of the purified polysaccharides obtained from the three cultures were determined by gel filtration on Sephadex G-200. The results indicated that the average molecular weights of the polysaccharides produced in the 2X and 4X phosphate media were equivalent to that of the group C polysaccharide which proved to be

immunogenic for man, and the average molecular weight of the polysaccharide produced in the 3X casamino acids - 0.2X dextrose medium was significantly larger. Accordingly, each of these purified polysaccharide preparations were processed to vaccines suitable for human use. However, they also failed to elicit an immunogenic response.

d. Current studies are directed toward the development of new methods for processing the group B material, and examination of less highly purified polysaccharide preparations. It is postulated that the B polysaccharide may require an additional component, which is removed or destroyed during current processing procedures, in order to manifest immunogenicity.

2. Viral Antigens and Vaccines.

Studies on modifications of usual tissue culture methods for the production of viral antigens and vaccines have continued during this period. Previous investigations, employing Eastern Equine Encephalomyelitis (EEE) virus in chick embryo fibroblast cell cultures as a model system, have demonstrated that greater yields of hemagglutinating (HA) and complement fixing (CF) antigens and significantly more potent vaccines were obtained when the EEE virus was grown in suspension cultures of chick embryo cells rather than in monolayers in stationary or roller bottles (Annual Report, 1970). Consequently, a series of experiments were carried out to determine whether the suspension culture method can be applied to other arthropod-borne viruses of interest to the military for the production of high titered infectious virus and viral antigens, and the preparation of potent vaccines.

a. Suspension cultures of primary chick embryo cells (CEC) were infected with Chikungunya (CHIK) virus, strain 168, at a multiplicity of 10, and the culture fluids were harvested after 18 hours incubation. The results of titrations on the culture fluids for infectious virus, HA and CF activities are given in Table III, along with the results of titrations of fluids from infected monolayer cultures of African green monkey kidney cells (GMKC), the culture system generally used for cultivating CHIK virus (2):

TABLE III

Comparison of Infectivity Titers, HA and CF Titers of CHIK Virus Strain 168 on Chick Embryo Cell Suspension Cultures and Green Monkey Kidney Cell Monolayer Cultures

Cell System	Infectivity Titer*	HA Titer	CF Titer
CEC Suspension	11.0	10,240	128
GMKC Monolayer	7.0	64	8

*Log_{10} suckling-mouse $ICLD_{50}$ per ml.

It is apparent that significantly higher yields of infective virus and HA and CF antigens are obtained in suspension cultures of primary chick embryo cells. Furthermore, the HA and CF antigenic activities were stable for at least 8 months when stored at 4 C.

b. An inactivated CHIK vaccine was prepared by the addition of formalin to the culture fluids of infected chick embryo cell suspension cultures, employing the procedure previously used in this laboratory for the preparation of inactivated EEE vaccine of tissue culture origin (3) The potency of this experimental vaccine was compared with that of a reference CHIK vaccine prepared from virus propagated in GMKC (2). The results are summarized in Table IV.

TABLE IV

Comparison of Formalin-inactivated CHIK Vaccines Prepared in CEC Suspension Cultures and in GMKC Monolayer Cultures

Preparation	Vaccine Dilution	Response to Challenge Surv/Total	ED_{50}* (ml)
CEC Suspension	1/3	12/13	
	1/9	16/17	0.044
	1/27	7/14	
	1/81	0/14	
GMKC Monolayer	Undil.	17/18	
	1/3	14/17	0.197
	1/9	2/16	

*Calculated by the method of Probit Analysis; Finney, D. J., 1952. Statistical methods in biological assay. Hafner Publ. Co., N. Y.

These results show that the vaccine prepared from virus propagated in CEC suspension cultures was approximately 4 times as potent as that prepared from the virus propagated in GMKC.

Studies on the serologic responses elicited by these 2 vaccines are currently in progress.

3. Q Fever.

a. In earlier studies on the use of the continuous flow zonal centrifuge for the preparation of purified Q fever vaccines (Annual Reports, 1969, 1970), vaccines were prepared from a supplemental inactivated rickettsial yolk sac membrane suspension that had been associated with a former project (1963) and stored at 4 C since that time. With this aged material, one part of Genetron was sufficient for the extraction of 10 parts of the crude yolk sac membrane suspension, yielding an extract which was satisfactory for passage through the orifices of the continuous flow zonal centrifuge. When freshly prepared inactivated yolk sac membrane suspensions were processed in this manner, however, difficulty was encountered in passing the extract through the continuous

flow zonal rotor. In order to obtain satisfactory extracts it was found necessary to increase the volume of Genetron used to 2.5 parts per 10 parts of yolk sac membrane suspension. Studies were therefore carried out to determine the effect of this increased Genetron requirement on the yield of purified rickettsial antigen.

b. Five liters of freshly harvested yolk sac membrane suspension, containing inac vated phase 2 rickettsiae, were treated with Genetr. (2.5 parts per 10 parts suspension) and processed through the density gradient zonal centrifuge. Fractions of 50 ml each were collected and the sucrose concentration, density and complement-fixing activity of each fraction was determined. As in previous preparations, the bulk of the antigen was found in fractions 7 through 10, corresponding to densities of 1.228 to 1.299. These fractions were pooled to make up the Genetron zonal vaccine. The product was diluted on the basis of antigenic activity, to permit comparison with a Genetron-zonal vaccine prepared from the aged yolk sac suspension, and a reference (ether-extracted) phase 2 vaccine. The results of chemical assays on these vaccines are recorded in Table V.

TABLE V

Comparative Assays on Vaccines with Equivalent Antigen Content (CF Titer = 8)

Vaccine	Nitrogen (mg/ml)	Protein (mg/ml)	Lipid (mg/ml)
Genetron-Zonal (2.5 parts Gen.)	.011	.063	.055
Genetron-Zonal (1 part Gen.)	.011	.042	.078
Reference (Ether-extracted)	.013	.187	.560

Both Genetron-Zonal vaccines were similar in nitrogen, protein and lipid content, indicating that the larger volume of Genetron used in the extraction of the freshly prepared yolk sac membrane suspension did not adversely affect the product. When compared with the

ether-extracted reference vaccine, the Genetron-Zonal vaccine is significantly lower in protein and lipid content.

c. Additional studies have demonstrated that the method described above is also applicable to the preparation of a purified phase 1 Q fever vaccine from freshly harvested yolk sac membrane suspensions.

4. Formalin Inactivation of Bacterial and Viral Suspensions.

a. The use of formalin for the inactivation of bacterial, rickettsial and viral suspensions during the preparation of vaccines has been a common procedure for many years. The presence of some residual free formalin in the final product of a fluid preparation, although causing a slight stinging sensation when injected into the tissues, is generally considered to be an advantage because of its preservative action. However, in freeze-dried biologicals, the presence of free formalin during the freeze-drying procedure can have a deleterious effect on the antigen(s). Consequently, it has been common procedure in this laboratory, as well as in other biological laboratories, to neutralize the free formalin in the preparation prior to freeze-drying. This is generally accomplished by titrating the free formalin content of the preparation by the NIH method, and adding sufficient sodium bisulfite to just neutralize the formalin present. Since one mole of formaldehyde combines chemically with one mole of sodium bisulfite, the amount of bisulfite required for neutralization varies linearly with the volume and concentration of formalin.

b. Addition of an excess of bisulfite is undesirable because of toxic effects. Consequently, the titration of free formalin must be accurately performed immediately prior to neutralization. However, in the case of some of the biological materials studied in this laboratory, the situation appears to be complicated by the reversible combination of formaldehyde with some constituent of the preparation. The results obtained with formalin-inactivated Vibrio cholerae, Inaba substrain, suspensions are given in Table VI.

TABLE VI

Neutralization of Formalin in V. cholerae (Inaba) Suspensions

% Formalin Added	% Free Formalin (by Titration) Before Neutral.* (a)	After Neutral.** (b)	Total % Formalin (a)+(b)
0.1	0.085	0.025	0.110
0.2	.158	.053	.211
0.3	.204	.082	.286

*Titration performed 48 hours after addition of formalin.
**Titration performed after sufficient sodium bisulfite added to completely neutralize the free formalin content given in column (a).

These results indicate that, when sodium bisulfite is added to neutralize all of the free formalin present, some or all of the bound formalin is released and becomes available as free formalin. Therefore, in this suspension, to completely neutralize the "free" formalin, sufficient bisulfite must be added to neutralize all of the formalin originally added.

c. Additional studies are being carried out with these and other biological suspensions to elucidate this problem.

Project 3A061102B71Q COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 171, Development of biological products of military importance

Literature Cited.

References:

1. Gotschlich, E. C., Liu, T. Y., and Artenstein, M. S.: Human immunity to the Meningococcus. III. Preparation and immunochemical properties of the group A, group B and group C Meningococcal Polysaccharides. J. Exper. Med. 129:1307-1385, 1969.

2. Harrison, V. R., Binn, L. N., and Randall, R.: Comparative immunogenicities of Chikungunya vaccines prepared in Avian and Mammalian tissues. Am. J. Trop. Med. & Hyg. 16:786-791, 1967.

3. Lowenthal, J. P., Berman, S., and Grogan, E. W.: Eastern Equine Encephalomyelitis vaccine prepared in cell cultures. Science 134:3478, 565-566, 1961.

Publications:

1. Berman, S., Altieri, P. L., Groffinger, A., and Lowenthal, J. P.: Freeze-drying various attenuated strains of Pasteurella Pestis. Cryobiol. 7:40-43, 1970.

2. Berman, S., Altieri, P. L., Groffinger, A., and Lowenthal, J. P.: Pilot-scale production of group A and group C Meningococcal Polysaccharide Immunogens. Inf. & Immun. 2:640-643, 1970.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1 AGENCY ACCESSION: DA
2 DATE OF SUMMARY:
REPORT CONTROL SYMBOL: DD-DR&E-RH(A)

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: ☒ YES ☐ NO
9 LEVEL OF SUM: A WORK UNIT

10 NO CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY				
b. CONTRIBUTING				
c.				

11 TITLE (Precede with Security Classification Code)
( ) Ser-[illegible] Malaria Infections ( )

12 SCIENTIFIC AND TECHNOLOGICAL AREAS
3200 Therapeutics [illegible]

13 START DATE	14 ESTIMATED COMPLETION DATE	15 FUNDING AGENCY	16 PERFORMANCE METHOD

17 CONTRACT GRANT
a. DATES/EFFECTIVE: DA — 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: PRECED.		
FISCAL YEAR: CURRENT		

19 RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Inst[illegible] of Research
ADDRESS: Washington, D.C. [illegible]

RESPONSIBLE INDIVIDUAL
NAME: Buescher, COL, E. L.
TELEPHONE: 202-576-3541

20 PERFORMING ORGANIZATION
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NAME: [illegible], E. [illegible]
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SOCIAL SECURITY ACCOUNT NUMBER: [redacted]

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PII Redacted

21 GENERAL USE
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22 KEYWORDS (Precede EACH with Security Classification Code)
(U) Antibody; (U) Antigens; (U) Complement Fixation Tests; (U) Fluorescent Antibody Techniques; (U) Parasitic Diseases; (U) [illegible]diagnosis; (U) [illegible]; (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) During military operations in Asia, troops [illegible] are exposed to a variety of infectious diseases not encountered in U.S. Our research tests [illegible]. These infections either [illegible] pre-existing [illegible] deficiencies to development of [illegible] and/or improvement of existing methods. Procedures critically evaluated for [illegible], value for therapy, and relationship to course of disease. Serological procedures [illegible] required in diseases in which causative agent is difficult to demonstrate.

24. (U) CF, FA, and HA techniques used to determine efficacy of [illegible] procedures and to evaluate specificity and sensitivity of the purified products. Also, new serologic techniques are developed (e.g., FAFA). Technical problems include [illegible] times, limited availability of [illegible] and their separation from host tissues. Improved technology in one case often facilitates research even in unrelated areas.

25. (U) 70 07 - 71 06. Work accomplished to date provides specific, sensitive, serodiagnostic tests for variety of important diseases (parasitic, fungal, bacterial, viral). SAFA test continues to be superior to tuberculin tests for detection of active simian tuberculosis. Efficacy of SAFA test for detection, monitoring and appraising effectiveness of therapy in human TB being investigated. [illegible] for testing dried sera in CF further improved. CF tests for malaria evaluated with sera from servicemen experiencing attacks after return to U.S. Specificity and sensitivity of malaria CF tests comparable to IFA tests, and superior to IFA and HA for experimental. Comparative studies being continued on subjects acquiring natural [illegible]. For technical report see Walter Reed Army Institute of Research Annual Progress Report, Jul 70 - 30 Jun 71.

Available to contractors upon originator's approval

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

Project 3A061102B71Q, COMMUNICABLE DISEASES AND IMMUNOLOGY

Task 00, Communicable Diseases and Immunology

Work Unit 172, Sero-recognition of microbial infections

Investigators.
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Associate: Andre J. Toussaint, M.S.; Lois A. Simonton, B.S.; Barbara J. Flemmings, B.S.; Robert W. Sanders; Albert E. vonDoenhoff, B.S.

Description.

This task is concerned with the mechanisms and patterns of immune responses. In vitro and in vivo methods are used to study host response to antigens. In vitro studies involve the development, improvement, and evaluation of procedures for detection of host antibodies. The studies also entail isolation, purification and identification of antigens by chemical and serological methods. In vivo studies include: (1) investigations on the ability of antigens to stimulate serologically detectable antibodies, (2) cellular level immune response to microbial infection, and (3) production of specific antisera by infection and/or experimental antigens or antigen fractions. Antigens that show a high level of serological sensitivity and specificity are evaluated for immunogenicity.

Progress.

1. Soluble antigen fluorescent antibody (SAFA) tests for serodiagnosis of infectious diseases. Details of the development and progressive technical improvements of the SAFA technic have been presented in previous reports on this Work Unit. The procedure continues to show considerable promise for the serodiagnosis of a variety of infectious diseases. Two technical improvements were introduced during the present reporting period. One was increasing the fixation period for the M. tuberculosis antigens from 10 to 30 minutes, and the other, use of a BSA control for each serum examined for M. tuberculosis antibodies. The latter proved to be particularly important because certain sera, for reasons unknown at this time, showed a high level of nonspecific reactivity with the BSA control disc. Retrospective analysis of these sera revealed that this nonspecific reactivity made the results of the test equivocal. Therefore it was imperative that sera with these properties be identified and the results interpreted accordingly.

a. Simian tuberculosis. The need for a reliable immunodiagnostic test for early detection of simian tuberculosis has long been recognized. Results of initial investigations on experimentally infected monkeys suggested that the SAFA procedure using antigens derived from M. tuberculosis might fulfill this need (see previous report on this Work Unit). It was observed that the SAFA test was superior to the standard

tuberculin test for early detection of infection; not only did the SAFA test show greater specificity, it consistently became reactive earlier (14 - 74 days) in infection than did the tuberculin reaction.

In these studies, the experimentally infected animals were challenged with a large number of organisms (5000 viable tubercle bacilli) to assure infection and permit investigations on the mechanisms of natural transmission. However, the incidence of transmission to the cagemate controls was exceedingly low; cross infection occurred in only 4 of the control animals. This suggested that airborne transmission may be considerably less than had been previously surmised. It was believed that the unexpectedly low rate of transmission in these studies was due primarily to the design and scrupulous cleaning of the cages, which prevented contamination of the food with urine and feces of the infected animals that regularly shed viable tubercle bacilli in their excreta. Nevertheless, these studies did not provide an opportunity to evaluate the diagnostic tests in animals receiving a minimum infective dose, such as that which would be involved in most naturally acquired infections.

This deficiency was remedied in subsequent studies on monkeys challenged with intratracheally administered graded doses, ranging in log increments from 5000 to 5 viable tubercle bacilli. Thus, 4 groups consisting of 6 monkeys each were inoculated respectively with 5000, 500, 50, and 5 viable organisms. Each inoculated monkey had a cagemate control.

These latter studies have just been concluded and were accomplished in collaboration with investigators in the Industrial Health and Safety Directorate, Fort Detrick, and the Division of Veterinary Medicine, WRAIR. At specified intervals following administration of the inoculum, anterior-posterior and lateral radiographs, intradermal tuberculin tests performed both intrapalpebrally and on the abdomen, gastric lavages, and SAFA tests using 3 M. tuberculosis antigens ("A" protein, "C" protein and polysaccharide) were performed on each inoculated and control monkey. Observations were made at weekly intervals during the first 4-week post-exposure period, bi-weekly for the succeeding 5 months, and at monthly intervals thereafter. Complete necropsies were performed on the monkeys that died during the course of the study, and on those that were sacrificed at the conclusion of the investigations one year after initiation of the study. Regardless of whether an animal expired or was sacrificed, efforts were made to demonstrate the presence of tubercle bacilli. Representative samples of tissues were triturated and cultured on appropriate media. Other portions of the tissues were processed for histopathological examination.

It is noteworthy that all of the inoculated monkeys, even those receiving only 5 viable tubercle bacilli, became infected. However, the most striking feature of the study was the extremely high rate of transmission to the cagemate controls; 20 of the 24 controls became infected. Since it was postulated that the low rate of transmission

observed in the earlier study was due in part to the regular scrupulous cleaning of the cages, it was decided to test this hypothesis in the latter study by cleaning the cages at monthly rather than bi-weekly intervals. Other than this, the procedures for the care and handling of the animals were the same as those employed in the previous study. The high rate of transmission that occurred in the latter study shows that good housekeeping practices are an essential factor in controlling outbreaks of tuberculosis in nonhuman primate colonies.

The various diagnostic tests employed in the most recent study were evaluated in terms of sensitivity and specificity. The results are summarized in Table 1. Sensitivity denotes the ability of a test to detect individuals with infection, and can be expressed as the number of infected individuals giving positive reactions in the test, divided by the total number infected, and multiplying by 100. Specificity, on the other hand, is the ability of the test to classify as negative those individuals that are not infected. This can be expressed as the number of noninfected individuals that are negative in the test, divided by the total number of noninfected subjects, and multiplying the quotient by 100. A total of 48 monkeys was used in these studies. Of this group, 41 became infected either by artificial inoculation or by exposure to an inoculated animal. Seven of the controls remained uninfected. In each case, infection was confirmed or excluded by culture and histopathologic methods.

The SAFA test rated very high with respect to sensitivity and specificity. Its sensitivity was 95.1%, detecting 39 of the 41 infected monkeys. Its specificity was 100%; all 7 noninfected animals were non-reactive in the test. The intrapalpebral tuberculin test was less effective from both standpoints. It showed a sensitivity of 75.6%, detecting 31 of the 41 infected animals. Additionally, its specificity was very low; 3 of the 7 noninfected animals converted during the observation period. The tuberculin test performed on the abdomen had the lowest sensitivity of all of the diagnostic procedures employed (59.8%), detecting only 24 of the 41 infected monkeys. Its specificity (85.7%) was somewhat better than that of the palpebral tuberculin test, but still was less than that observed in the other diagnostic tests. X-rays showed a sensitivity of 95.1% and specificity of 100% whereas the gastric lavages were 92.7% sensitive and 100% specific.

It was recognized that some of the animals included in the group just evaluated received an unusually heavy challenge (500-5000 viable organisms), and that the response of such animals could be different from that characteristic of naturally acquired infections. This could influence the overall results. Therefore, a similar evaluation was conducted on the cagemate controls in whom the infections were acquired by exposure to an experimentally infected animal. These results are summarized in Table 2.

Table 1

Evaluation of Results With All Monkeys of Study
(Total = 48; Infected = 41; Noninfected = 7)

SAFA

Sensitivity = 95.1% (pos. 39/41 infected)
Specificity = 100% (neg. 7/7 noninfected)

IDP

Sensitivity = 75.6% (pos. 31/41 infected)
Specificity = 57.1% (neg. 4/7 noninfected)

IDA

Sensitivity = 59.8% (pos. 24/41 infected)
Specificity = 85.7% (neg. 6/7 noninfected)

X-ray

Sensitivity = 95.1% (pos. 39/41 infected)
Specificity = 100% (neg. 7/7 noninfected)

Gastric Lavage

Sensitivity = 92.7% (pos. 38/41 infected)
Specificity = 100% (neg. 7/7 noninfected)

Table 2

Evaluation of Results With Cagemate Controls
(Total = 24; Infected = 18; Noninfected = 6)

SAFA

Sensitivity	=	100%	(pos. 18/18 infected)
Specificity	=	100%	(neg. 6/6 noninfected)

IDP

Sensitivity	=	88.9%	(pos. 16/18 infected)
Specificity	=	50.0%	(neg. 3/6 noninfected)

IDA

Sensitivity	=	77.8%	(pos. 14/18 infected)
Specificity	=	83.3%	(neg. 5/6 noninfected)

X-ray

Sensitivity	=	94.4%	(pos. 17/18 infected)
Specificity	=	100%	(neg. 6/6 noninfected)

Gastric Lavage

Sensitivity	=	88.9%	(pos. 16/18 infected)
Specificity	=	100%	(neg. 6/6 noninfected)

This group was composed of 24 monkeys, 18 became infected and 6 remained noninfected. With these animals with naturally acquired infections, the sensitivity as well as the specificity of the SAFA test was 100%. The intrapalpebral tuberculin test showed a sensitivity of 88.9%, but again its specificity was low, only 50%. As before, the sensitivity of the tuberculin test performed on the abdomen was lower (77.7%) than that exhibited by any of the other diagnostic procedures. However, its specificity (83.3%) again was better than that of the palpebral test. X-rays detected all but 1 of the infected animals, and 16 of the 18 infected monkeys were identified by gastric lavage. Both of the latter procedures showed a specificity of 100%.

Thus in studies to date, the SAFA test has shown considerable promise for the early diagnosis of simian tuberculosis. It is more sensitive and more specific than the conventional intradermal tuberculin tests conducted intrapalpebrally or on the abdomen, and is at least as good as serial radiographs or gastric lavages for detecting animals with active disease. Experience thus far suggests that a reactive SAFA test is highly indicative of current or recent active tuberculosis. Apparently, sub-clinical infections do not elicit production of SAFA-reactive antibodies, at least to detectable levels. Moreover, in contrast to the tuberculin test, conversion of the SAFA test in the absence of active disease has not been observed. It is believed that incorporation of the SAFA test with the procedures used for routine screening and monitoring, and the application of more rigid housekeeping standards for the care of the animals, could appreciably reduce the risks of serious outbreaks of tuberculosis in non-human primate colonies.

The practice of performing SAFA tests for tuberculosis on all non-human primates during their quarantine period at the WRAIR has been continued. A total of 983 non-human primate sera (965 monkeys, 8 chimpanzees, and 10 baboons) were examined during this reporting period. All of the chimpanzee and baboon sera were nonreactive in the SAFA tests. However, 5 of the monkeys reacted with one or more of the antigens employed. Two reacted only with "A" protein antigen, one with the polysaccharide, and two with both antigens. These animals were immediately isolated for further observation. Repeated intrapalpebral tuberculin tests were uniformly negative, but all of the monkeys ultimately expired. One of the animals appeared to have advanced disseminated tuberculosis, but the other monkeys showed no gross evidence of the disease. Unfortunately, detailed necropsies were not performed. Therefore, the failure to observe lesions in the latter monkeys does not necessarily mean that they did not have tuberculosis. To overcome this deficiency in the future, provisions are being made to perform detailed necropsies, including cultural and histopathological examinations, on all animals that are isolated because of reactivity in either the SAFA or tuberculin tests. Use of the SAFA test for routine screening and monitoring for tuberculosis in the non-human primate colony is being continued.

b. Human tuberculosis. In view of the encouraging results and apparent potential of the SAFA test for detection of simian tuberculosis, investigations have been initiated to determine the value of the procedure for the serodiagnosis of human tuberculosis. These studies are being conducted in collaboration with investigators from the Department of Epidemiology, WRAIR, and from the Tuberculosis Service, Fitzsimons General Hospital. The major objectives of these investigations are: 1) To determine the sensitivity and specificity of the SAFA test for the diagnosis of human tuberculosis; 2) To compare the SAFA test results with those obtained by other diagnostic technics (e.g. hypersensitivity, radiology, cultures, etc.); and 3) To evaluate the SAFA test as an indicator of therapeutic response.

Three groups of patients are being studied. These include: Group I - All patients admitted to the Tuberculosis Service of Fitzsimons General Hospital during the 12-month period. Included in this group will be skin test convertors who are managed as outpatients. Patients must be followed for a minimum of 6 consecutive months to be included in the final evaluation. Group II - Patients randomly selected from the non-tuberculosis chest disease service at the Hospital. Group III - All persons skin tested for tuberculosis in the Fitzsimons Immunization Clinic during the study period.

The standard clinical evaluation normally performed on all admissions to the Tuberculosis Service will provide the following information on patients in Group I: TBC smear and culture, X-ray studies, M. tuberculosis and fungal skin testing, protein and immune globulin electrophoresis, and fungal CF studies. In following each patient in this Group, culture results, X-ray findings, extent of disease, level of clinical activity, and chemotherapy for each month of hospitalization will be recorded.

For patients in Group II, the diagnostic evaluations will include the majority of studies performed on the individuals in Group I. Those in whom the diagnosis of tuberculosis is made will be admitted to Group I.

For Group III, histories of prior skin testing, type of skin test currently employed (TTT, Mono-Vac), and the results of the test will be recorded. Similarly, the results of repeat tests in those found to be positive will be recorded. Convertors will be admitted to Group I.

All sera collected for examination in the SAFA tests will be coded and stored at -60°C until tested. Specimens will be collected according to the following schedules:

Group I - An admission specimen (pre-treatment) will be obtained from each patient and at monthly intervals thereafter, for a maximum of 12 months. Efforts will be made to obtain an 18-month specimen from the patients who are SAFA positive at the 12-month period.

Group II - An admission and discharge specimen will be obtained from each patient in this Group.

Group III - A specimen will be obtained at the time the skin test is applied. Additionally, a specimen will be collected from those individuals not skin tested because of a history of prior skin test sensitivity.

At the conclusion of the studies, the data will be analyzed as follows:

1. Analysis will be made to determine the relative sensitivity and specificity of the SAFA tests, tuberculin tests and radiographs for detection of human tuberculosis.

2. Analysis will be made to determine which of the employed diagnostic tests became positive earliest in the course of the disease, and which of the SAFA antigens was superior for early detection and for monitoring therapy.

3. By analyzing the results obtained in Groups II and III, determine the incidence and magnitude of nonspecific SAFA reactions in patients with non-tuberculous pulmonary disease or without any pulmonary disease. Some of these individuals obviously will be tuberculin positive.

4. Determine whether there is a correlation of the SAFA test titers with clinical and radiological response to therapy in Group I patients.

To complement the foregoing investigations, arrangements have been made to obtain a representative number of sera (at least 50) from non-tuberculous patients with sarcoidosis. These sera will be examined in SAFA tests with the M. tuberculosis antigens to provide additional information concerning the specificity of the serologic procedures.

The data obtained in these investigations will be evaluated and discussed in subsequent reports on this Work Unit.

c. Echinococcosis. Initial studies on the suitability of the SAFA procedure for the serodiagnosis of echinococcosis in sheep, dogs and humans were concluded during the present reporting period. These investigations were conducted in collaboration with Dr. J. F. Williams, Centro Panamericano de Zoonosis, Ramos Mejia, Argentina. Preliminary results of these studies were summarized in the previous report on this Work Unit. Hydatid fluid from the cyst of an infected sheep served as antigen for the SAFA, indirect hemagglutination (IHA) and Latex agglutination (LA) tests performed in these preliminary studies. None of the procedures were satisfactory for the serodiagnosis of hyatid disease in sheep because of a high level of nonspecific fluorescence inherent in the SAFA tests, and the large number of nonspecific reactions obtained

with the IHA and LA tests. Moreover, the IHA and LA tests failed to detect antibodies in the majority of experimentally or naturally infected dogs. The SAFA test, however, showed some promise for the serodiagnosis of canine echinococcosis; among a total of 149 infected dogs, 115 (77%) reacted in the SAFA test, and no false positive reactions were observed in any of the parasite-free inbred beagles that were tested. This high level of specificity, however, was not observed in tests on sera from mongrel street dogs residing in pounds located in Buenos Aires or Washington, D.C. Although these latter animals did not harbor any species of Echinococcus, they were infected with a variety of other helminths and 38% reacted in the SAFA test with hydatid fluid antigen. The SAFA test was the most specific of the immunodiagnostic procedures evaluated for the diagnosis of human echinococcosis; no false positive reactions were observed. However, the SAFA test was somewhat deficient in sensitivity; 61% of the infected individuals reacted in the SAFA test whereas 73% reacted in the IHA, 86% in the LA, and 94% in the immunoelectrophoresis test. The IHA and LA tests, however, showed considerable nonspecific reactivity in the noninfected controls.

It was obvious that serodiagnostic tests for echinococcosis required further improvement. This was particularly true with regard to the serodiagnosis of ovine and canine infections. Gore, et al[1] recently reported that antigens extracted from scoleces of E. granulosis were superior to those obtained from hydatid fluids for detection of human hydatidosis. The present studies were continued to determine whether the use of scolex antigen would improve the specificity and sensitivity of the tests for nonhuman echinococcosis, particularly ovine and canine infections. Although we have not received the clinical histories on all of the animals studied, it was apparent that the scolex antigen was superior to the hydatid fluid antigen for testing sheep and dog sera. SAFA tests with the scolex antigen showed a much lower level of nonspecific fluorescence, gave fewer positive reactions, and missed very few experimentally infected animals that were detected with the less specific hydatid antigen.

Shortly after the conclusion of these studies, Dr. Williams resigned from CEPANZO to accept a position at the Michigan State University. This has precluded complete analysis of results of the necropsy and parasitological examinations. However, these data should be available in the near future, and details of the concluding phase of these investigations will be presented in the following report on this Work Unit.

2. Serodiagnosis of American trypanosomiasis (Chagas' disease). In the previous report on this Work Unit, it was noted that the Department of Serology, WRAIR, was participating in a critical evaluation of Trypanosoma cruzi antigens being conducted by a PAHO Study Group on Chagas' Disease Antigens. The principal objective of this Study Group was to select a standard antigen for universal use in the serodiagnosis of Chagas' disease, and for reference in evaluating new antigens and methods. Three of the original 8 candidate antigens showed excessive nonspecific reactivity in tests with non-chagasic sera and were

eliminated from further consideration as [illegible] standardization. It is noteworthy that 2 of the latter, giving [illegible] false positive reactions, were being widely employed for routine [illegible] diagnosis and their use strongly advocated by a number of influential investigators in South America; apparently they were very impressed with the high serum titers obtained with these antigens and had conveniently availed studies on the specificity of the products. It is believed that demonstration of this deficiency is a major contribution of the Study Group.

During the present reporting period the six participating laboratories evaluated the remaining 5 candidate antigens for sensitivity in quantitative tests with sera from documented cases of Chagas' disease. Critical review of these data revealed that 2 of the antigens, one of which was the purified protein antigen submitted by the Department of Serology, WRAIR, were superior to the others. The Group therefore recommended that these two antigens be more extensively evaluated for nonspecific reactivity, specific reactivity, reproducibility, and stability. New lots of each now are being prepared for this purpose. Ultimately, one of the two will be designated the Standard Antigen and recommended for general use in routine serodiagnosis.

3. Development and improvement of serologic methods and reagents. Efforts to further improve serologic methods and reagents were continued during the present reporting period.

a. A new method for critical evaluation of antigen stability. The conventional method for evaluating antigen stability is to periodically test the product with a standard reference serum and observe whether the antibody titer decreases during storage of the antigen. The results obtained by this method, however, often are obfuscate and difficult to interpret because of the titer fluctuations that occur as a result of the varied stability of complement (C') in tests conducted on different days. Therefore, the investigator must make an intuitive guess concerning whether variations observed in the repeated assays are significant.

This problem can be eliminated by employing the new methodology developed for critical evaluation of antigens. These methods are based on the linear relationships between the amounts of immune complexes formed and the amounts of C' fixed, and are presented in detail in the previous report on this Work Unit. It was noted in the earlier report that variations of reactivity due to C' deterioration can be eliminated by expressing the results in terms of the Index of Reactive Capacity (I.R.C.) of the antigen in question. The I.R.C. is the ratio of the antigen titer and the serum titer (i.e. T_A/T_S) and the rationale for its use is given in the previous report. The efficacy of this procedure for evaluating antigen stability is illustrated by the data presented in Table 3, which summarizes the results of periodic assays on a T. cruzi antigen conducted during a 12-month period of storage. As was expected, the numerical values of the antigen and serum titers fluctuated considerably in the assays conducted on different days. However, the

Table 3

Evaluation of Antigen Stability

in Terms of Index of Specific Reactivity (I.R.C.)

Time Months	Serum No.	Antigen Lot No.	Complement Lot No.	Titers		I.R.C. (Titer Ratio, T_A/T_S)
				Antigen (T_A)	Serum (T_S)	
0	G 7856	A	F72	4700	114	41.2
2	G 7856	A	F75	4130	100	41.3
4	G 7856	A	F77	4850	118	41.1
6	G 7856	A	F80	5100	123	41.5
8	G 7856	A	F82	4050	98	41.3
10	G 7856	A	F85	3700	90	41.1
12	G 7856	A	F86	4500	109	41.3

antigen/serum titer ratios, i.e. the I.R.C. values, remained constant, indicating that the specific reactivity of the antigen had not changed during the period of storage. It is noteworthy that this methodology may be employed with any antigen-antibody system that fixes complement.

b. A modified complement fixation procedure for tests on dog sera. It was noted in the previous report on this Work Unit that dog sera frequently became anticomplementary during the heat inactivation (56°C for 30 min) required to destroy the native complement. This problem was overcome by decomplementing the sera by absorption with immune complex (sensitized sheep erythrocyte stromata), thus avoiding the requirement for heat inactivation.

Methods developed for preparing the stromata and for decomplementing the serum were presented in detail in the earlier report. Further improvements of technic were introduced during the present reporting period. The first innovation was designed to improve the efficacy of the decomplementation procedure. It was observed that shaking the serum-sensitized stromata mixture during the early stages of absorption significantly improved the effectiveness of decomplementation. Therefore, the present technic for decomplementation consists of shaking the mixture on a mechanical shaker for 15 minutes at room temperature, and then allowing the tubes to remain at room temperature for an additional 45 minutes without shaking. With this procedure, residual complement levels rarely are sufficient to influence the results of the diagnostic complement fixation tests; to date we have encountered only one absorbed serum that gave greater than 5% hemolysis in the controls for detection of residual C'.

The other technical modification pertained to improvement of the procedures for removing the sensitized stromata from the serum following decomplementation. During the course of continued studies, it was noted that certain fresh sera were anticomplementary following absorption with the sensitized stromata. Since these sera had been carefully collected and processed, it was believed that this probably was indicative of incomplete removal of the sensitized stromata following decomplementation. Subsequent studies revealed that this was the case; it was observed that the anti-complementary activity could be removed by high speed centrifugation of the absorbed serum. The procedure for removing the sensitized stromata therefore was modified as follows: Following the absorption reaction, the serum-sensitized stromata mixture was centrifuged at 3°C for 20 min at 1000 rcf, and the supernatant serum removed from the button of sedimented stromata. The absorbed serum then was recentrifuged at 55,000 rcf for 30 min to remove the minute particles of stromata that remained in the initial supernate. The decomplemented serum then was used without further treatment in the diagnostic complement fixation tests. Recent studies on sera from dogs that were artificially immunized with canine herpes vaccine revealed that the foregoing treatment did not remove the specific complement fixing antibodies.

In contrast to the earlier initial studies, it was observed that heat-inactivation of the decomplemented sera usually did not produce the anticomplementary activity generally encountered with heat-inactivated unabsorbed sera. These unexpected findings may provide a basis for characterizing the component(s) responsible for the anticomplementary activity of the latter. Studies along these lines are in progress, and include investigations on the effects of absorption with unsensitized erythrocyte stromata, absorption with untreated (intact) erythrocytes, and absorption with erythrocytes stabilized by pretreatment with pyruvic or other aldehyde. In addition, the efficacy of the modified complement fixation test for evaluating the quality of canine herpes vaccines is being investigated. Details of the results of these studies will be presented in the following report on this Work Unit.

c. Preservation of Treponema pallidum in the frozen state. A method for preserving T. pallidum in the frozen state was developed and reported in detail in the previous report on this Work Unit. The efficacy of this procedure for prolonged storage of treponemes was further evaluated during the present reporting period. For this evaluation, a glycerolized T. pallidum suspension containing 200 treponemes/hdf with 98% motility, was divided in 1-ml aliquants, shell-frozen, and stored at -70°C according to the prescribed procedures. At monthly intervals a sample was thawed, counted, checked for motility, and the infectivity determined by intratesticular inoculation into a rabbit. The most recent observations were made following storage for 13 months. The sample thawed at this time contained 200 treponemes/hdf (the same as the original suspension) with 92% motility; the latter represented only a 6% decline from that of the original material. Intratesticular inoculation of the suspension into a rabbit produced a typical orchitis. Seven days after inoculation, the rabbit was sacrificed and the treponemes were harvested by the procedures normally used for obtaining antigen for the TPI test. The harvest contained 40 treponemes/hdf and 98% were motile. Moreover, this harvest gave valid results when used as antigen in routine TPI tests. Although the treponeme count in this harvest was approximately one-half that normally obtained from rabbits sacrificed 7 days after receiving a fresh inoculum, succeeding passages produced harvests with treponeme concentrations in the normal range (80-120 organisms/hdf). These studies have shown that T. pallidum preserved by these methods may be stored for more than one year without significant loss of motility or infectivity. Storage in this manner assures the availability of a satisfactory inoculum at all times and provides insurance against loss of the strain due to inability to obtain satisfactory rabbits (often a problem in early spring) or to unavoidable accidents to the infected animals.

d. Evaluation of the micro-hemagglutination test (MHA-TP) for syphilis. In 1966, Tomizawa and Kasamatsu[2] reported development of a new hemagglutination test for the serodiagnosis of syphilis. These investigators employed a sonicate of virulent T. pallidum (Nichols strain) adsorbed on formalinized sheep cells as the antigen in their tests, and as in the FTA-ABS test, treated the serum with a sonicate of the

avirulent Reiter treponeme to block any Treponema group antibodies that might be present. These investigators also observed that the sensitized sheep cells could be lyophilized, and this led to the development of a test "kit" that was produced commercially and widely used in Japan. It was generally believed that the hemagglutination test gave results comparable to those obtained with the FTA-ABS test.

More recently, investigators at the VDRL, CDC, adapted the procedure to the Microtiter system and reported that it could be performed manually or by automation.(3,4) Since the reagents for the manual and automated microhemagglutination tests now are available through an American distributor, it was deemed important to obtain first hand information concerning the advantages and limitations of the procedure. This was accomplished by performing parallel MHA-TP, TPI, FTA-ABS and CMF tests on a group of 70 sera submitted to this laboratory for treponemal antibody tests, and comparing the results. The findings are summarized in Table 4.

Although the microhemagglutination (MHA-TP) test obviously was superior to the cardiolipin microflocculation (CMF) test, the procedure showed some deficiencies in specificity and sensitivity. Six of the non-syphilitic sera in the group reacted in the MHA-TP test. Admittedly, the magnitude of these reactions was not great (1+ or 2+). Nevertheless, these tests would have been considered positive according to the criteria prescribed for interpreting the results. On the other hand, 3 of the primary syphilitic sera failed to react in the MHA-TP test. In tests on sera in other categories, the hemagglutination test compared favorably with the TPI and FTA-ABS tests. The MHA-TP test gave strong reactions with the 12 sera from individuals with late syphilis and reacted with only 1 of the 6 sera from biologic false positive reactors.

On the basis of these findings, it is apparent that the MHA-TP test cannot replace the TPI or FTA-ABS test for definitive diagnosis of syphilis. However, it may warrant some consideration as a possible substitute for the CMF test in certain situations, but even this application may be limited because of the considerably greater cost of reagents for the MHA-TP test.

e. Preservation of complement in the liquid state. Commercially prepared lyophilized guinea pig complement (C') generally is marketed in 3-7 ml volumes, and frequently this exceeds the amount required for CF tests conducted on a given day. Since the rehydrated C' is quite labile unless stored at -60°C, the portion not used on the day of rehydration usually must be discarded. This results in a considerable waste that is of particular concern when lots of the reagent that are certified to be free from various viral and rickettsial antibodies are involved. The latter are difficult to obtain and are considerably more expensive than the C' that is used in general diagnostic serology.

Table 4

TPI, FTA-ABS, MHA-TP, and CMF tests* on
70 sera submitted for treponemal antibody tests

	Number of reactors and nonreactors in indicated test			
	TPI	FTA-ABS	MHA-TP	CMF
Reactive	33	38	42	33
Nonreactive	27	32	28	37
NSI**	10			

*Tests identified as:
TPI = T. pallidum Immobilization.
FTA-ABS = Fluorescent Treponemal Antibody-Absorbed.
MHA-TP = Microhemagglutination - T. pallidum.
CMF = Cardiolipin Microflocculation.

**NSI = Nonspecific Immobilization (invalidating results).

Studies were initiated to determine whether this problem could be overcome by use of Richardson's solution[5] as a C' preservative. This solution is composed of boric acid, sodium borate and sodium chloride dissolved in a saturated sodium chloride solution; sodium azide is added as a bacteristatic agent. Some adjustment of the original reported concentrations of the components was necessary to bring the pH of the preserved serum mixture to the proper range (pH 6.0-6.5). Also, it was determined that the two solutions described by Richardson could be prepared as a single reagent and an appropriate amount added to the serum. The formula of the modified Richardson's solution and procedures for its use in the preservation of C' are given in detail in Table 5.

To evaluate the stability of C' preserved in the modified Richardson's solution, a pool of fresh guinea pig serum was combined with the solution according to the prescribed procedures, dispensed in 1-ml aliquants, and stored at 3°C. At approximately weekly intervals a sample was titrated for hemolytic activity. In addition, the stability of the C' under conditions obtaining in diagnostic CF tests was determined by preparing standardized 5-unit C' from the sample and checking the controls immediately and after overnight storage at 3°C. The results of this evaluation are summarized in Table 6.

Comparison of the titers of the non-preserved C' and the preserved C' titrated on day 0 revealed that the addition of the Richardson's solution resulted in a slight loss of hemolytic activity. An additional minor reduction of titer was noted in the preserved C' after storage for 1 week. However, no further loss of hemolytic activity was noted during the succeeding seven weeks of storage. Although the initial loss of activity observed with the preserved C' obviously is not desirable, it is noteworthy that the reduction of titer is not of sufficient magnitude to preclude use of the product in diagnostic CF tests; in a number of subsequent evaluations of different lots of fresh and lyophilized guinea pig sera (C'), titers of the preserved products after storage for two months always exceeded 500 C'H50, the minimum level considered acceptable for the product used for preparing the standardized C' employed in diagnostic CF tests.

The stability of the preserved C' under conditions obtaining in the diagnostic CF tests also was investigated. At each observation period, a solution containing 5 C'H50/0.3 ml was prepared by the standard procedures. Two sets of 1-unit controls were prepared from the standardized C'; the hemolytic activity of one set was checked immediately, and that of the other set after overnight incubation at 3°C. The results of these studies also are summarized in Table 6. In no instance did the per cent hemolysis of the overnight control differ by more than 10% from that of the initial control. These results are comparable to those regularly obtained with non-preserved C'. Thus it is apparent that the Richardson's solution does not adversely affect the stability of C' used in diagnostic CF tests.

Table 5

Preservation of Complement in Modified Richardson's Solution

Modified Richardson's Solution

Borax ($Na_2B_4O_7 \cdot 10H_2O$)	0.04 M	1.53 gm
Boric acid (H_3BO_3)	0.08 M	0.49 gm
Sorbitol ($C_6H_{14}O_6$)	0.29 M	5.28 gm
Sodium azide (NaN_3)	0.06 M	0.39 gm

Dissolve the above components in saturated NaCl solution and q.s. to 100 ml with the saturated salt solution.

Note: It is imperative that only Reagent Grade quality chemicals be used for preparing the solution. U.S.P. Grade is unsatisfactory.

Preservation of Complement

Combine 8 parts of serum (complement) with 2 parts of Richardson's solution. Mix and store at 3-5°C in a stoppered tube. Complement preserved and stored in this manner will maintain its hemolytic activity for a number of months.

Use of Preserved C' in CF Tests

For use in CF tests, dilute 1.0 volume of the preserved C' with 7.0 volumes of distilled water. This will restore the isotonicity of the solution and will represent a 1:10 dilution of the original serum. It should be noted that the diluted C' exhibits the same lability shown by unpreserved serum and must be handled accordingly.

Table 6

Stability of complement preserved in modified Richardson's solution and stored at 3°C.

C' lot 62670 Days of storage	Titer (C'H50/ml)	1-unit controls* (per cent hemolysis)	
		Initial	Overnight (3°C)
Non-preserved	1250	49	40
0	988	48	38
7	823	45	40
14	869	52	45
22	837	48	40
28	811	50	43
36	886	53	44
42	832	46	38
50	843	51	44
60	820	48	39

*Prepared from 5-unit C' standardized for use in diagnostic CF tests.

Studies were conducted to determine whether the Richardson's solution in any way influenced the specific reactivity of diagnostic complement fixation tests. Standardized 5-unit C' was prepared from non-preserved C' and fro C' that had been preserved with the Richardson's solution. Each was used in parallel tests on a group of sera from patients with Chagas' disease. Without exception, the titers obtained with the two complements were identical. In view of the foregoing studies, it is apparent that Richardson's solution is an effective preservative for C'. It permits storage of the reagent for as long as two months in the liquid state, and can be safely used to avoid undue waste of this critical reagent.

f. Evaluation of the complement fixation test for malaria. Methods for effective separation of malaria parasites from host erythrocytes and for isolation of specific complement fixing antigens from the parasite harvests were developed in another work unit assigned to the Department of Serology, WRAIR. Details of these procedures have been published.(6,7) During the present reporting period, the complement fixation (CF), indirect fluorescent antibody (IFA) and indirect hemagglutination (IHA) tests were compared in examinations of sera from 62 soldiers that developed clinical malaria after return to CONUS. With the exception of 1 individual with falciparum malaria, all of these patients had parasitologically proven vivax malaria. These studies were conducted in collaboration with investigators from the Parasitology Section, Center for Disease Control.

In each case, sera were collected when clinical symptoms appeared and then at regular intervals for 1 year. Antigens from P. knowlesi (for detection of vivax antibodies) and from P. falciparum were used in the CF tests. P. falciparum and P. vivax served as antigens in the IFA tests. The IHA procedure utilized a crude extract of P. knowlesi for sensitization of the red cells. The sera were grouped in 4 categories based on the time after appearance of clinical symptoms that the specimens were drawn. These groups were composed of sera collected 0-13 days, 15-58 days, 7 months, and 1 year after onset of disease. The results of these studies are summarized in Table 7.

It is noteworthy that the CF and IFA tests were equally effective for early detection of antibodies. With the group of sera collected 0-13 days after the onset of symptoms, 87% reacted in the CF test and 89% were positive in the IFA test. In tests on the sera in the 15-58 day category, the IFA was slightly more sensitive, detecting 98% of the cases whereas the CF reacted with 93%. Results obtained with the sera collected 7 months after the onset of disease suggested that following cure the CF test may revert to negativity before the IFA test; 64% of this group were seronegative in the CF test whereas 47% remained positive in the IFA test. However, examination of the results obtained with sera collected 1 year after radical cure revealed that this was not the case. The frequency of reactions was essentially the same in both tests, 24% with the CF and 26% with the IFA.

Table 7

Results of CF, IFA, and IHA tests on sera
from returnees developing malaria within CONUS

Time after onset	Complement Fixation Tests			
	Negative		Positive	
	No.	%	No.	%
0 - 13 days	7	13	46	87
15 - 58 days	4	7	53	93
7 months	39	64	22	36
1 year	28	76	9	24

Time after onset	Indirect Fluorescent Antibody Tests			
	Negative		Positive	
	No.	%	No.	%
0 - 13 days	6	11	47	89
15 - 58 days	1	2	57	98
7 months	33	53	29	47
1 year	28	74	10	26

Time after onset	Indirect Hemagglutination Tests			
	Negative		Positive	
	No.	%	No.	%
0 - 13 days	10	19	43	81
15 - 58 days	5	9	53	91
7 months	11	18	51	82
1 year	9	26	25	74

Review of the results obtained with the IHA tests revealed certain inherent deficiencies of this procedure. The IHA was less sensitive than the CF or IFA tests for early detection of infection; the IHA test was positive on only 81% of the sera collected 0-13 days after onset of clinical symptoms. Moreover, reactivity following radical cure persisted much longer in the IHA test than in the CF or IFA tests. With the sera collected 1 year after treatment, 74% remained positive in the IHA test. These comparative studies are being continued in tests on a large number of sera from a group of Californians that acquired vivax malaria by sharing needles.

The recent acquisition of a strain of P. vivax that produces 5-10% parasitemias in intact Aotus monkeys for the first time has provided P. vivax parasites in quantities sufficient for preparing complement fixing antigen. This antigen is being included in the evaluation now in progress. Since the majority of these subjects have not traveled or resided in a malaria endemic area, and thus have not been exposed to P. falciparum, these studies provide an unusual opportunity to compare the efficacy of the knowlesi and vivax CF antigens for detecting vivax antibodies, and for studying the cross reactions between falciparum antigens and vivax antibodies in the CF and IFA procedures. Results of these investigations will be reported in detail in the next report on this Work Unit.

g. Evaluation of complement fixation and fluorescent antibody tests for anti-nuclear antibodies. For years, complement fixation (CF) and fluorescent antibody tests (FAT) have been used to detect anti-nuclear antibodies that are produced in many collagen-vascular diseases, especially systemic lupus erythematosus (SLE). However, reports of critical evaluations and comparisons of the two procedures are essentially nonexistent. In view of this lack of definitive information, selection of one procedure over the other usually has been based solely on the personal preferences of the individual responsible for performing the tests.

During the present reporting period, a collaborative study was initiated with investigators in the Clinical Laboratory, WRGH, to evaluate the relative specificity and sensitivity of the two procedures and to correlate the findings with the clinical data on each patient. The 174 sera evaluated in these studies were selected from those submitted to the Clinical Laboratory for FAT examination for anti-nuclear antibodies. Portions of each were forwarded to the Department of Serology, WRAIR, for CF tests. The FAT performed in the Clinical Laboratory was an indirect fluorescent antibody procedure employing calf thymus nuclei as antigen. The CF tests performed by the Department of Serology utilized a nucleo-protein extract of calf thymus as antigen. Both tests were performed independently without knowledge of the results obtained in the other laboratory.

A total of 162 patients were evaluated in these studies. Thirty-nine reacted in the FAT whereas only 16 gave reactions in the CF test. Among the 10 of the group that reacted in both tests, 9 had a clinical diagnosis of SLE. The exception was a patient with chronic active hepatitis and was believed to have had a variety of "autoantibodies" at high titer. It is noteworthy that all patients with active SLE reacted in both tests. However, these individuals uniformly became seronegative in the CF test following cortico steroid therapy. This latter phenomenon was not observed with the FAT.

Although the data from these studies leave many questions still unanswered, certain generalizations can be made. It appears that the CF test had considerable pathognomic value for appraising the clinical state in SLE and acute liver disease, but is of little or no value for the diagnosis of other collagen-vascular diseases or rheumatic disorders. The FAT, on the other hand, appears to react in a much broader spectrum of collagen-vascular diseases and often is positive in cases of rheumatoid arthritis and scleroderma.

The group of patients that reacted in the FAT but presented no other evidence of collagen-vascular disease is of special interest. It is suggested that long-term longitudinal studies on this group would be worthwhile to determine whether this unexplained reactivity in the FAT is indicative of occult collagen-vascular or rheumatic disease that ultimately will exascerbate at some future time. Unfortunately, other priorities do not permit a study of this nature at the present time.

Summary and Conclusions.

1. The soluble antigen fluorescent antibody (SAFA) procedure continues to show excellent potential for the serodiagnosis of a variety of infectious diseases. Recent innovations of methodology have further improved the sensitivity and specificity of the procedure.

a. Studies on the use of the SAFA test for the serodiagnosis of tuberculosis have been continued. In investigations on monkeys infected with graded doses of M. tuberculosis and those acquiring the disease by exposure to an inoculated animal, the SAFA test was superior to tuberculin tests conducted intrapalpebrally or on the abdomen. The SAFA test detected 39 of the 41 infected animals whereas the intrapalpebral tuberculin test detected 31 of the infected monkeys and the tuberculin test performed on the abdomen was positive in only 24 of the group. In contrast to the palpebral tuberculin test that showed conversion in 3 of the 7 uninfected controls, no false positive reactions were obtained with the SAFA test. Use of the SAFA test for screening and monitoring tuberculosis in the animals of the nonhuman primate colony at WRAIR is being continued.

Comprehensive investigations on the potential of the SAFA test for the serodiagnosis of human tuberculosis have been initiated. The principal objectives of these studies are to determine the specificity

and sensitivity of the SAFA test for the diagnosis of human tuberculosis, to compare the SAFA test results with those obtained by other diagnostic technics, and to evaluate the SAFA test as an indicator of therapeutic response.

b. Studies on the suitability of the SAFA test for the serodiagnosis of canine, ovine and human echinococcosis were continued. Use of a fraction from E. granulosis scoleces rather than hydatid fluid as antigen markedly improved the specificity and sensitivity of the tests, particularly when dog and sheep sera were examined.

2. Critical evaluation of antigens for the serodiagnosis of American trypanosomiasis (Chagas' disease) was continued. Two of the original 8 antigens remain candidates for consideration as the standard reference antigen. These 2 antigens are being more extensively evaluated for nonspecific reactivity, specific reactivity, reproducibility and stability.

3. Efforts to further improve serologic methods and reagents were continued during the present reporting period.

a. The new quantitatively standardized complement fixation procedure was shown to provide an excellent method for critically evaluating antigen stability. With this procedure, the specific reactivity can be expressed independently from the influence of complement deterioration, thus eliminating the confusion caused by titer fluctuations normally encountered in tests conducted on different days. This is accomplished by expressing the specific reactivity as the ratio of the antigen and serum titers.

b. The modified complement fixation procedure for tests on dog sera was further improved. Decomplementation of the serum by absorption with immune complex (sensitized erythrocyte stromata) continued to be the method of choice for avoiding the requirement for heat inactivation. It was observed that shaking the serum-sensitized stromata mixture improved the efficacy of absorption. Also, high speed centrifugation was necessary to completely remove the stromata following absorption. The absorbed sera were not anticomplementary, and tests on sera from dogs artificially immunized with canine herpes vaccine revealed that this treatment did not remove the specific complement fixing antibodies.

c. Studies on the preservation of T. pallidum in the frozen state were continued. Glycerolized suspensions of the organisms showed essentially no loss of motility or infectivity after storage at -70°C for 13 months. Storage in this manner assures the constant availability of a satisfactory inoculum for preparing antigen for the TPI and FTA-ABS tests for syphilis.

d. A new microhemagglutination test for syphilis was evaluated. Parallel microhemagglutination, cardiolipin slide flocculation, TPI and FTA-ABS tests were performed on 70 sera submitted for treponemal antibody

tests, and the results were compared. Although the microhemagglutination test was superior to the standard cardiolipin test, the procedure showed some deficiencies in specificity and sensitivity. Six of the nonsyphilitic sera reacted in the microhemagglutination test, and 3 of the primary syphilitic sera failed to react. On the other hand, the test gave strong reactions with the 12 sera from individuals with late syphilis and reacted with only 1 of the 6 sera from biologic false positive reactors. It was concluded that the microhemagglutination test in certain situations might serve as a substitute for the cardiolipin test, but could not replace the TPI or FTA-ABS test for definitive diagnosis of syphilis.

e. A procedure for the preservation and storage of complement in the liquid state was developed and evaluated. Guinea pig serum could be stored at 3°C for 2 months with only a modest loss of hemolytic activity. The preserved complement was shown to be satisfactory for use in diagnostic CF tests and its stability under conditions obtaining in the diagnostic tests was comparable to that of untreated complement. The procedure can be used to avoid undue waste of a critical reagent.

f. The complement fixation (CF) test for malaria was evaluated in tests on sera from a group of servicemen who experienced clinical malaria after return to CONUS. The results were compared with those obtained in indirect fluorescent antibody (IFA) and indirect hemagglutination (IHA) tests. The CF and IFA tests were equally effective in detecting antibodies in the sera collected 0-13 days after the onset of symptoms. The IFA, however, gave a slightly higher number of reactions with the group of sera collected 3 weeks after onset. The CF test showed more reversions to negative than did the IFA test on sera collected 7 months after treatment. However, the number of reactors in the CF and IFA tests was essentially the same with the sera collected 1 year after radical cure. The IHA test was less effective than the CF or IFA tests for early detection of antibodies, but once developed, antibodies reactive in the IHA test persisted for a much longer period than those reactive in the CF and IFA test. The studies are being continued on sera from a group of sera from individuals who acquired vivax malaria by sharing needles.

g. The complement fixation and fluorescent antibody tests for anti-nuclear antibodies were evaluated on sera from patients with a provisional diagnosis of collagen-vascular disease or rheumatoid disorder. The complement fixation test proved to have considerable pathognomic value for appraising the clinical state in SLE and acute liver disease, but was of little or no value for the diagnosis of other collagen-vascular diseases or rheumatic disorders. On the other hand, the fluorescent antibody test appeared to react in a much broader spectrum of collagen-vascular diseases and often was positive in cases of rheumatoid arthritis and scleroderma.

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Literature Cited

1. References.

(1). Gore, R. W., Sadun, E. H., and Hoff, R. Echinococcus granulosis and E. multilocularis: Soluble antigen fluorescent antibody test. Exptl. Parasitol., 28: 272-279, 1970.

(2). Tomizawa, T., and Kasamatsu, S. Hemagglutination tests for diagnosis of syphilis. A preliminary report. Jap. J. Med. Sci. Biol., 19 : 305-308, 1966.

(3). Cox, P. M., Logan, L. C., and Norins, L. C. Automated, quantitative microhemagglutination assay for Treponema pallidum antibodies. Appl. Microbiol., 18 : 485-489, 1969.

(4). Logan, L. C., and Cox, P. M. Evaluation of a quantitative microhemagglutination assay for antibodies to Treponema pallidum. Am. J. Clin. Path., 53 : 163-166, 1970.

(5). Richardson, G. M. Preservation of liquid complement serum. Lancet, 2 : 696-697, 1941.

(6). D'Antonio, L. E., vonDoenhoff, A. E., and Fife, E. H. A new method for isolation and fractionation of complement fixing antigens from Plasmodium knowlesi. Proc. Soc. Exper. Biol. & Med., 123 : 30-34, 1966.

(7). D'Antonio, L. E., vonDoenhoff, A. E., and Fife, E. H. Serological evaluation of the specificity and sensitivity of purified malaria antigens prepared by a new method. Mil. Med., 131 (Suppl., Sept.) : 1152-1156, 1966.

2. Publications.

1. Colwell, E. J., Legters, L. J., and Fife, E. H. Splenomegaly and malaria in the Central Highlands of South Vietnam. Am. J. Trop. Med. & Hyg., 19 : 741-746, 1970.

2. Fife, E. H., Kruse, R. H., Toussaint, A. H., and Staab, E. V. Serodiagnosis of simian tuberculosis by soluble antigen fluorescent antibody (SAFA) tests. Lab. Animal Care, 20 : 969-978, 1970.

3. Colwell, E. J., Ortaldo, H. R., Schoenbechler, M. J., Barbaro, J. F., and Fife, E. H. Trichinella spiralis and Schistosoma mansoni: Specificity of in vitro, leukocyte-mediated histamine release from rabbit platlets. Exptl. Parasitol., 29 : 263-270, 1971.

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