AD

US ARMY MEDICAL RESEARCH INSTITUTE
OF INFECTIOUS DISEASES

ANNUAL
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FY: 1980

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UNITED STATES ARMY MEDICAL RESEARCH INSTITUTE
OF INFECTIOUS DISEASES
FREDERICK, MARYLAND 21701

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Biological warfare	Rickettsial diseases	Pathology	Host
Viral diseases	Laboratory animals	Defense	
Prophylaxis	Parasite	Metabolism	
Identification	Biochemistry	Malaria	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A report of progress on the research program of the U. S. Army Medical Research Institute of Infectious Diseases on Medical Defense Against Biological Agents (U) for Fiscal Year 1980 is presented.

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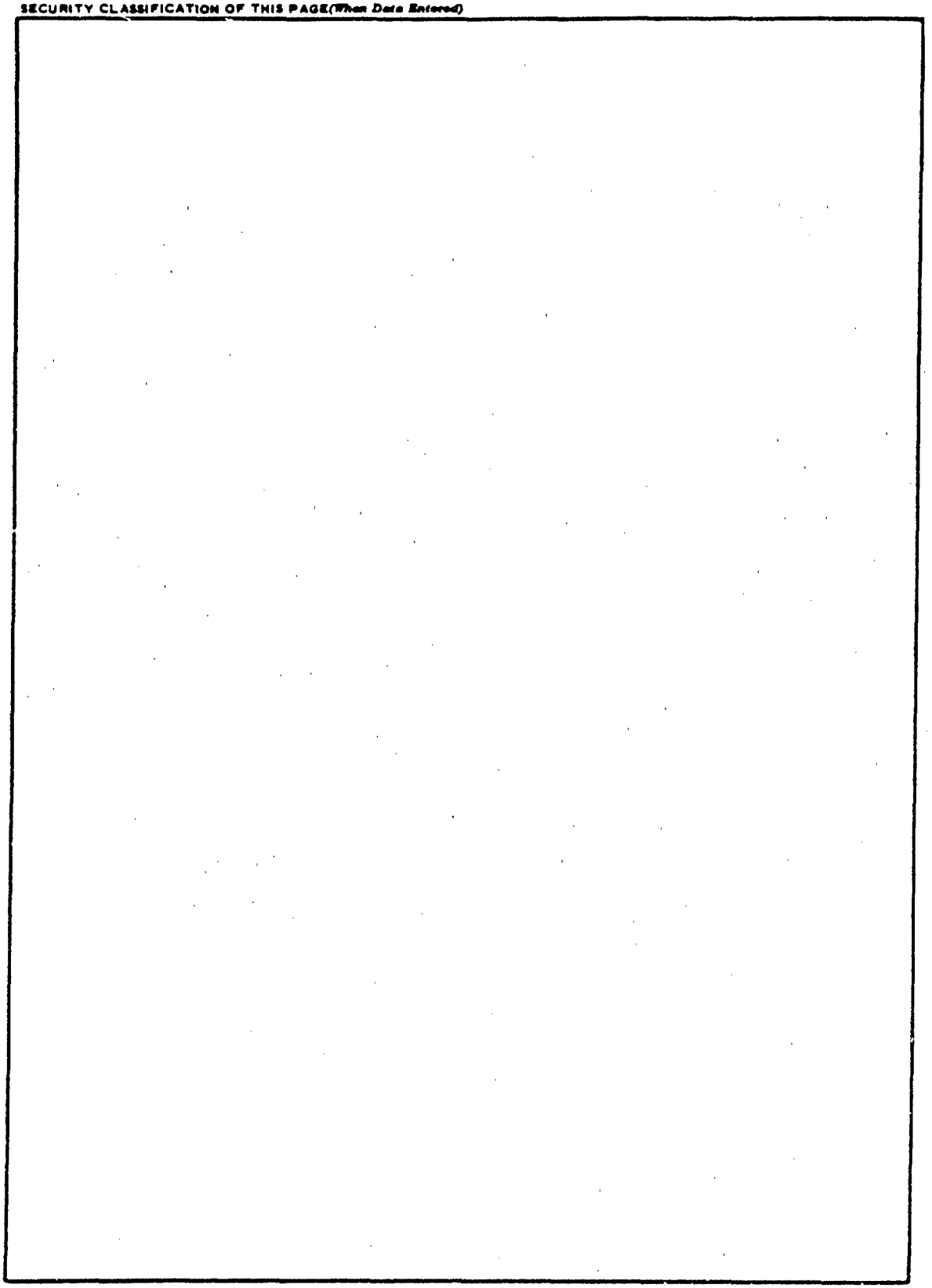
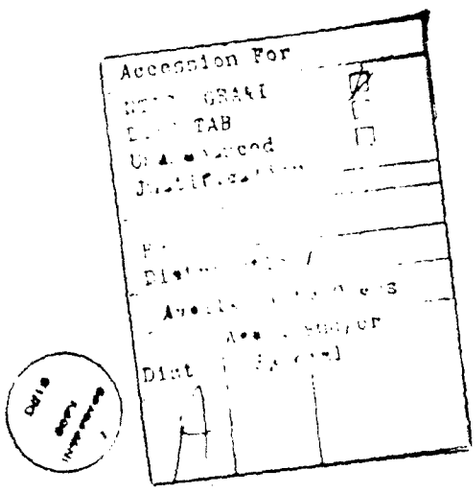


EDITOR'S NOTE

This FY 1980 Annual Progress report is a general review of research activities of the U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, conducted on Medical Defense Against Biological Warfare Agents (U) under Projects 3M162776A841 and 3M161102BS03 and In-House Laboratory Independent Research, Project 3A161101A91C.

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 Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.



FOREWORD

I. USAMRIID's MISSION

The formal mission tasking USAMRIID reads as follows:

Perform studies on the pathogenesis, diagnosis, prophylaxis, treatment, and epidemiology of naturally occurring infectious diseases of military importance with emphasis on problems associated with the medical defense against biological agents and on those microorganisms which require special containment facilities.

By DOD directive and further Army guidance, USAMRIID performs its Biological Agent Medical Defense research in support of the needs of the three services. This mission, and all work done at USAMRIID, is in keeping with the spirit and letter of both President Nixon's 1969 and 1970 Executive Orders renouncing the use of biological and toxin weapons, and the U.N. Convention (Against) . . . Bacteriological (Biological) and Toxin Weapons . . . of 1972.

II. DISSEMINATION OF INFORMATION:

All work conducted at USAMRIID is unclassified. Results are published in peer-reviewed scientific literature, when accepted, as well as in annual reports. Results of value to organizations outside the U.S. Department of Defense are shared willingly, often in the hope that such sharing will result in additional information on the validity of scientific results or the efficacy of new products, such as vaccines or other biologicals or drugs. Numerous intra-U.S. and international collaborations exist and are encouraged to expand. USAMRIID prints a cumulative bibliography of published articles, which may be obtained by a request to the Editor, USAMRIID, Fort Detrick, Maryland 21701.

III. THE STRATEGY OF THE PROGRAM:

A. The program rests on the judgment that both natural infectious diseases and potential biological warfare threats exist which could seriously interfere with the functions of U.S. forces. The first requirement for constructing the USAMRIID program is to arrive at an assessment as to which microbial and toxin agents are the highest priority threats. Those agents for which existing medical defenses are adequate are set aside. Those agents being addressed by other agencies within the U.S. or elsewhere are likewise set aside. From the refined list the available resources are applied in priority derived from considerations of the severity of their threat and the scientific feasibility of developing improved medical defenses against the agent.

B. The agents being addressed during the period of this report were:

Bacterial

B. anthracis
F. tularensis
L. pneumoniae
S. pneumoniae
S. typhimurium
P. pseudomallei

Viral

Lassa fever virus
Ebola fever virus
Korean hemorrhagic fever virus
Rift Valley fever virus
Bolivian hemorrhagic fever virus (Machupo)
Argentinian hemorrhagic fever virus (Junin)
Dengue fever virus
Congo/Crimean hemorrhagic fever virus
Sandfly fever virus
Eastern encephalitis virus
Western encephalitis virus
Venezuelan equine encephalitis virus
Japanese B fever virus
Chikungunya virus
Tacaribe virus
Pichinde virus
Yellow fever virus
Influenza virus
Saint Louis encephalitis virus

Rickettsial

C. burnetii

Parasitic

P. falciparum

Toxins

Pseudomonas A
Diptheria
Botulinum A-G
Anthrax toxins
Staphylococcal enterotoxin

IV. Goals

For each of the agents being addressed, the goals were:

A. Pathogenesis: Sufficient knowledge of the biology of the agent and the responses of the infected or intoxicated host (man, as well as available animal models) was gathered to provide a basis for progress in the applied goals which are listed below. Useful cell cultures, organ cultures, and a variety of laboratory animal models must be developed and exploited for the insight they can provide on the pathogenic processes in man, since information from human cases for many of the diseases of concern is limited.

B. Improved Diagnosis: Since the choice of medical interventions for either the prevention or the treatment of infectious/toxic disease can only be optimized when the precise infecting/intoxicating agent is known, the ability to make a rapid and specific identification of the causative agent is an important component of a system for medical defense. Ideally, there should be the capability to confirm the identity of agents isolated from the environment, to detect antigen in appropriate clinical samples taken early in the course of disease, and also to detect antibodies from later clinical cases or convalescents. The technology used should be suitable for use throughout the military medical system, including field facilities operating with austere resources. Not only must the agents of major concern be identifiable, but those more common agents which must be considered in a full differential diagnosis must also be identifiable.

C. Prevention: Prevention of infectious disease by immunization is the most effective, convenient, and economical means to reduce the impact from disease on military forces. This goal, then, commands nearly half of USAMRIID's resources. Vaccine development is expected to continue as a major USAMRIID theme, since technological advances often allow the improvement of vaccines which were once state-of-the-art accomplishments. The application of modern biology to vaccine development is presenting opportunities and challenges not foreseen a few years earlier. Passive immunization, active immunization using killed or living attenuated whole agent, or immunization with sub-unit antigens achieved by older or newer methods are options which must be comparatively evaluated for each agent, to arrive at the optimum immunizing method for military forces in various scenarios.

D. Treatment: The unexpected natural disease outbreak can preempt the opportunity to use prevention and leave treatment as the major medical means to limit damage to the individual and to maintain military force effectiveness. For many of the diseases of concern to USAMRIID, specific treatments which will reverse pathology have not yet been developed. Therefore, treatment strategy must consist of optimal supportive care to give the host defenses sufficient time to respond and overwhelm the disease insult. For these reasons research on improving treatments at USAMRIID has emphasis on developing new specific treatments and on maximizing the effectiveness of supportive care of the infected patient.

V. SUMMARY OF TRENDS IN FY 80

A. Since many programs at USAMRIID are multidisciplinary and carried out by multiple investigators in loose and shifting consortia over several years, the material covered by this annual report may give only a fragmentary insight of the overall program and its progress. In the following sections trends and accomplishments are highlighted.

B. General Progress Highlights during FY 80: FY 1980 was a most productive year. Many of the program changes implemented during the two preceeding years reached maturity and generated much new data. A new synthetic medium was developed for consistently growing high titers of Legionella pneumophilia and with no loss in virulence. A rat model for studying Rift Valley fever virus was refined and the basis for genetic susceptibility of the disease in their host was established. Ribavirin, immune plasma or poly ICLC (an inducer of natural interferon) were shown to be

useful in the treatment of the hepatic form of RVF; however, none of these treatments were effective if the infection became encephalitic. In studies concerned with manipulating the metabolic and endocrine systems of the body to the advantage of the host during infection, it was shown that marked loss of body protein could be prevented by parenteral administration of appropriate combinations of amino acids, carbohydrates and lipids. Entomology studies revealed the mechanisms by which Keystone and Saint Louis encephalitis viruses survive the winter. Aerobiological research demonstrated that both Machupo and Lassa fever viruses possess aerosol stability and are infectious by the aerosol route. This information confirms the need for this Institute to develop appropriate vaccines to protect. U. S. Military populations. In addition, key aerosol assessment studies were initiated for Legionella pneumophilia to determine its risk to our military.

Ties were strengthened with the Chemical Systems Laboratory, Aberdeen Proving Ground, Maryland in the testing and evaluation of the "Field Biological Alarm System". Contractual arrangements were made to convert a portion of the 2,000 liters of high-titer, human anti-botulinum plasma, reported last year, into hyperimmune immunoglobulin. In volunteer studies, performance decrements during a mild, self limiting viral disease (Sandfly fever) were measured. This type of information is of vital importance if Biological Warfare (BW) is employed against U. S. Forces. Vaccine development studies were particularly productive. In an exciting breakthrough, the attenuated strain of Junin virus was found to protect monkeys and laboratory rodents against both the Argentine and Bolivian forms of hemorrhagic fever. A candidate vaccine virus seed was selected for Chikungunya, a disease of explosive potential as either a BW agent or natural disease threat to U.S. military forces in Africa and Southeast Asia. Studies with selected pure strains of the Dengue-1 virus were continued and additional tests were developed in an effort to identify "virulence" markers.

Finally, the strains of RVF virus recently isolated in Egypt were compared with the older parent strains, and were shown to have comparable antigenic markers. This indicates that the newly produced USAMRIID vaccines should protect equally well against all strains of RVF virus.

C. Research areas given added emphasis during FY 80: FY 1980 can be best characterized as a year of maturation, a holding period for new starts, which the massive redirection of programs and realignment of priorities initiated in FY 1978 and FY 1979 began to produce. Priority I research continued to emphasize: (a) the hemorrhagic viruses including Argentine, Korean and Bolivian hemorrhagic fevers, Rift Valley fever, Lassa and Ebola virus infections; (b) the bacterial diseases caused by Bacillus anthracis and Legionella pneumophilia; and (c) the botulinum toxins A to G. The two most extensive and expensive studies were concerned with Legionnaire's disease and Rift Valley fever. The need to expand anthrax studies was demonstrated; however, expansion of this effort must await the free-up of current program commitments.

Priority II studies include research on Chikungunya, Dengue-1, TBE and VEE viruses, as well as the Staphylococcal enterotoxins and a rickettsia, Coxiella burnetii.

Priority III studies included research on tularemia and Pseudomonas pseudomallei.

D. Research Areas completed or for which efforts are diminishing in FY 80: One last effor is being made to develop an effective vaccine for Pseudomonas pseudomallei. Thus far, a variety of experimental vaccines, killed whole organism, live attenuated organism, or extracts from the bacterium have failed to produce any measure of protection. These studies will be discontinued in FY 81, unless some progress is made during the current year.

Studies comparing Phase I versus Phase II Q fever vaccines had to be curtailed because of the continuing inadequacy of the current assay system. The current assay, fever response in guinea pigs followed by testing for complement fixing antibody, is too crude and too erratic to detect meaningful differences between these two vaccines or to measure differences among lots within the same vaccine product. A more sensitive and consistent assay must be developed before meaningful progress can be made on Q fever vaccine studies.

E. List of significant accomplishments for FY 80:

1. USAMRIID's ILIR program for FY 80 ranked second out of 421 projects and 38 laboratories by a senior panel of advisors, Office of the Assistant Secretary of the Army.

2. A three day workshop entitled, "Receptor-Mediated Binding and Internalization of Toxins and Hormones" was held at USAMRIID on 24-26 March 1980. The workshop attracted leading authorities in these important areas of medicine including the Belgium Nobel Laureate, Dr. Christian de Duve. USAMRIID derived significant benefit from the workshop because: (a) the validity of its research on toxins and hormones was confirmed; (b) new experimental approaches were discussed; (c) new lines of scientific communication and collaboration were established.

3. More advances were made in finding out how Pseudomonas exotoxin A and the closely related diptheria exotoxin produced their specific toxicity. In vitro studies in cultured cells employed radioactively labeled exotoxin molecules to demonstrate that specific cellular receptors exist for each exotoxin.

4. A new synthetic medium has been devised for culturing high levels of Legionella pneumophilia with no loss in virulence. This represents a significant advance because, now, sufficient quantities of bacteria can be produced to permit solubilized components to be characterized and the cell wall, cell membrane and internal components to be purified in order to find the most antigenic component of the bacterium to prepare a safe and effective vaccine.

5. Rift Valley fever virus was studied in animals and the rat was identified as a key for investigating the genetic aspects of host susceptibility. Two types of the disease were demonstrated: acute death of liver cells caused by unchecked virus growth; and late brain inflammation due to virus growth plus an, as yet, undefined mechanism of disease progression.

Ribavirin, antibody obtained from recovering animals or poly ICLC (an inducer of native interferon) were shown to be useful in the treatment of the liver form of the disease; however, none were effective against infection that had reached the brain.

6. Lymphocyte traffic studies have demonstrated that several adjuvants accelerate the movement of lymphocytes into regional lymph nodes. CP-20-961 seems to be the most effective, has the least toxicity, and the best chance to be used as an adjuvant for man.

7. Six strains of St. Louis encephalitis virus have been isolated from hibernating adult Culex pipiens mosquitoes. How these mosquitoes became infected has not been fully determined, but early results suggest that an infective bloodmeal eaten before hibernation begins, rather than passage of virus by the female to her eggs (transovarial transmission), is the cause.

8. Aerosol research studies were initiated to determine the aerosol infectivity and stability properties of the new bacteria that causes Legionnaires' disease (Legionella pneumophila). A respiratory disease model was developed in guinea pigs with response criteria consisting of fever, weight loss and development of antibodies. The lethal dose of inhaled organisms was insignificantly lower than that of injected organisms. This difference may explain why the organism can be disseminated by water cooled air conditioning systems. It also showed that monkeys can also be used to study the progression of Legionnaires' disease.

9. A solid support assay medium for the chemical binding of antibodies against VEE virus was developed for use in a chemiluminescent immunoassay system The system was xtremely fast for detecting the virus, within 10 hours; morecver, the system was extremely sensitive, requiring only 100 to 1,000 infectious units of virus.

10. The anthrax program is currently being expanded by redirection of programmed resources with the development of improved techniques for producing, purifying and characterizing anthrax toxins. Particular emphasis is being devoted to the preparation of larger quantities of protective antigen (both in-house and contract with Michigan Department of Public Health). More protective antigen is needed in order to intensify current studies which are concerned with its purification and chemical characterization.

11. Methods were devised to quantitate delayed hypersensitivity reactions and to detect the magnitude of "suppressor" or "helper" functions of different transfused lymphocyte populations in mice inoculated with the live, attenuated tularemia vaccine. Protection against highly virulent tularemia organisms appeared to require both T- and B-lymphocyte activity.

12. The preparations and testing of Chikungunya 181 master seed, production seed and vaccine are now underway in conformance to GLP and will require extensive animal and human testing.

13. While ribavirin was previously shown to have the ability to protect test animals if given before signs of illness appeared, a major new finding showed that it was effective even though treatment was not begun until after the onset of clinical illness due to Machupo virus in monkeys and guinea pigs, RVF infection in mice and hamsters, and to a limited degree, yellow fever infection in monkeys.

14. The Institute acquired several fixed and transportable P-4 containment plastic human isolators (Vicker's) for the hospital care and safe transport of patients suffering from highly contagious, often lethal infectious diseases. In cooperation with the US Air Force, these units were tested with normal volunteers under long-flight conditions to simulate the evacuation of a contagious patient from Panama to USAMRIID. The test was most successful and established this unique mode of medical evacuation as an achievable reality for future patients.

15. The phase-III testing of a large number of experimental vaccines was continued in the laboratory workers of USAMRIID and other collaborating institutions. These vaccines were administered primarily for the safety of "at-risk" laboratory workers and included live attenuated TC-83 VEE vaccine, inactivated EEE and WEE vaccines, inactivated Phase II Q fever vaccine, attenuated live tularemia (LVS) vaccine, anthrax vaccine, inactivated RVF vaccine, inactivated Chikungunya vaccine and polyvalent botulinum toxoid.

16. A new radioimmune assay was developed which is more simple, rapid and sensitive than conventional plaque neutralization tests for identifying viruses.

17. Isotachophoresis technology was developed and applied to rapidly identify subclasses of IgG, one of the major human components of antibody defense. Since efficient, as well as inefficient, patterns of immune response may follow vaccination, the new isotachophoresis procedure can be used to screen immunized individuals for the appropriateness of their immune response, so that corrective action may be implemented when possible.

18. An enzyme-linked immunosorbent assay (ELISA) was modified and successfully applied to arbovirus identification. The test demonstrated sufficient serospecificity to identify subtypes of Venezuelan equine encephalomyelitis virus.

VI. EXTRAMURAL RESEARCH:

While this report deals principally with USAMRIID's in-house effort, total program progress is the result of the combination of the in-house effort augmented and supplemented by efforts by contractors from academia and industry. Individual contractor's research is synopsized in reports which are filed with the Defense Technical Information Center (DTIC). A list of contracts in place during FY 80 is included as Appendix C. Readers who want specific contract report should make request to DTIC.

VII. QUESTIONS:

Questions or comments about this report are welcomed and may be addressed to:

Commander
USAMRIID
Fort Detrick, Frederick, MD 21701

AUTHOR INDEX

ANNUAL PROGRESS REPORT - FY 1980

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PROJECT NO. 3M162776A841: MEDICAL DEFENSE AGAINST BIOLOGICAL AGENTS (U)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6410	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. GRADE SECURITY	7. REGRADING	8a. GROUP SUM'RY	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10a. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	001
b. [illegible]				
c. [illegible]	STOG 80-7.2:3			

11. TITLE: (U) Evaluation of experimental vaccines in man for BW defense

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 10	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE

	FY	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
PRECEDING FISCAL YEAR	80	1.0	279
CURRENT FISCAL YEAR	81	1.0	112

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Barquist, R. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Medical Division
ADDRESS: USAMRIID
Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic participating)
NAME: Ostroy, P. R.
TELEPHONE: 301 663-7281
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Provide KEY with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Vaccines; (U) Prophylaxis; (U) Therapy; (U) Infectious diseases; (U) Human volunteers

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

23 (U) Evaluate experimental vaccines developed by USAMRIID, various contractors, organizations or other governmental agencies. Assess effect of antimicrobials, various drug regimens and immune plasma in treatment of militarily important infectious diseases. This work unit is an essential element in a comprehensive program for medical defense against BW agents and other infections of unique military importance by allowing testing in man of newly developed experimental vaccines, new drugs for chemoprophylaxis or therapy and immune plasma or globulins.

24 (U) Test vaccines, experimental drugs and newly developed hyperimmune plasma and/or globulins are given to human volunteers after both full safety testing in animal models and approval under strict protocol conditions which have undergone evaluation by scientific review and medical ethics review.

25 (U) 79 10 - 80 09 - Human clinical vaccine trials were conducted with inactivated Rocky Mountain Spotted Fever and Rift Valley Fever vaccines and a live attenuated Dengue-2 vaccine. Evaluation of a new antimalarial drug was conducted in human volunteers with both falciparum and vivax malaria. Comprehensive studies were conducted on a new botulinum toxoid as well as the collection and evaluation in man of Botulinum Immune Plasma of human origin. One person was admitted to the special isolation suite for possible Lassa fever exposure and received hyperimmune plasma and ribavirin. No clinical Lassa fever developed. The special Vickers isolation equipment was tested further with several exercises both within and outside of USAMRIID.

* (If available in connection upon authorization's approval)

BODY OF REPORT

Project No. 3M162770A871; (3M162776A841) Prevention of Military Disease Hazards (U)

Task No. 3M162770A871 BD: Evaluation of Experimental Prophylactic and Therapeutic Regimen in Man

Work Unit No. 871 BD 147, (841 00 001) Evaluation of Experimental Vaccines, Prophylaxis and Therapy in Man for BW Defense Against Infectious Diseases of Special Military Importance

Background:

This work unit is a comprehensive research effort incorporating all areas of human volunteer testing and evaluation, as well as utilization of experimental vaccines, antimicrobial drugs, hyperimmune plasma, and special medical isolation procedures in man. This work unit incorporates studies of prophylaxis and therapy against both potential biological warfare threats, as well as infectious diseases of special military importance. The Medical Research Volunteer Subjects (MRVS) Program has enabled USAMRIID to actively conduct clinical studies involving human volunteers.

Progress:

Vaccines

Rocky Mountain Spotted Fever. Protocol FY 80-1, an addendum to FY 78-4, "To Assess Booster Dose Efficacy and Safety of the Rocky Mountain Spotted Fever Vaccine, Undiluted (IND 862)" was performed. Seven (7) volunteers who had previously been volunteers on FY 77-2 (The Clinical Evaluation of the Two Dose Schedule of the Inactivated RMSF Vaccine, 1.3 dilution) and/or the FY 78-4 (The Clinical Evaluation of the Two Dose Schedule of the Inactivated RMSF Vaccine, Undiluted), were administered one additional 0.5 ml dose of the undiluted vaccine. This third "booster" dose was given between 16 and 24 months after their first 2 doses. All 6 complained of local pain; 3 complained of pain lasting 2 days while 2 complained of pain lasting 3 days. In one case the pain was described as moderately severe and that person also noted a 6 x 6 cm area of induration which was similar to that noted after receiving the botulism vaccine which also contained formalin. Two individuals noted erythema and one person noted transient temperature elevation to 99.6°F orally. A large boost of the IFA titer was noted while the microagglutination (MA) titer remained constant. Lymphocyte transformation results were invalid due to technical difficulties.

The interlot comparisons of potency and stability performed by Merrill-National Laboratory for the RMSF Lots 1 through 3 were received. Analysis showed that Lot 1 of the Merrill-National (MN) vaccine is only about one-half as efficacious as Lot 1 of the USAMRIID-prepared vaccine, Lot 2 of the MN vaccine being only about 2/3 as efficacious as the USAMRIID-prepared vaccine Lot 1. Lot 3 of the MN vaccine may be similar to USAMRIID's. There is a quantative difference of rickettsial organisms per lot. Merrill-National Lot 1 has only 3.1×10^7 rickettsia/ml,

whereas MN Lots 2 and 3, and USAMRIID's Lot 1 all have between 1.1 and 1.3 x 10^8 ml. Based on this information it was determined to use only MN Lots 2 and 3 for safety testing in immune volunteers.

Project FY 80-4, "To Assess the Efficacy and Safety of Merrill-National Rocky Mountain Spotted Fever Vaccine, Lots 2 and 3, an addendum to FY 76-1, The Proposal for the Clinical Evaluation of a Two-Dose Schedule of Inactivated RMSF Vaccine, Undiluted (IND 862)" was tested in 4 USAMRIID employees, both civilian and military. The volunteers were selected on the basis of previously elevated MA or IFA titers to RMSF. After baseline physical examinations and laboratory work were satisfactorily completed, each person was inoculated SC with 0.5 ml of the 1:10 dilution of either lot. There were no local or systemic reactions. MA titers failed to show any change at either 7 or 28 days after the inoculation. Only one of 4 persons showed at least a 4-fold titer rise by the IFA method. In summary, MN Lots 2 and 3 appear safe to use in immune subjects, although efficacy and immunogenicity cannot adequately be determined.

USAMRIID was host to a conference called by Robert Edelman, M.D., Chief, Clinical Studies Branch, The Microbiology and Infectious Disease Program, National Institute of Allergy and Infectious Disease on 9 November 1979 in order to discuss a draft of the protocol for the RMSF vaccine efficacy trial at the University of Maryland. Representatives from the Center for Vaccine Development of the Division of Infectious Disease at the University of Maryland; The Bureau of Biologics; The National Institutes of Health (NIH) and USAMRIID were present. Relevant human and animal studies with RMSF vaccine were reviewed. The data obtained from FY 80-1, assessing the booster dose efficacy and safety was forwarded to Dr. Edelman and was used to help in gaining approval for the NIH challenge study. Upon completion of the testing, the safety and immunogenicity of MN Lots 2 and 3 vaccine will be provided to NIH and the University of Maryland for the challenge study.

Q Fever Vaccine. No progress was made in evaluating the Formalin-Inactivated, Dried Henzerling Strain, Phase I Q Fever Vaccine, NDBR 105 (IND 610), due to a prolonged illness and subsequent resignation of the principal investigator.

Dengue Virus Vaccines. The following studies on Immunization with Live Attenuated Dengue Virus Vaccine were done in collaboration with the Department of Virus Diseases, WRAIR.

Protocol FY 80-2, Study No. 3: Response to Varied Doses of DEN-2 (PR-159/S-1) Given Subcutaneously to Adult Volunteers with Prior Yellow Fever Immunization. Sixteen adult volunteers all of whom had previous yellow fever immunization were divided into 4 groups. Three groups of 5 volunteers each received SC 0.5 ml of the candidate DEN-2 vaccine at one of the following dilutions: 10^{-1} containing 4.3 x 10^3 PFU, 10^{-2} containing 5.5 x 10^2 PFU or 10^{-3} containing 7.0 x 10^1 PFU. One other volunteer received a placebo. The recipient of placebo and one of the recipients of the 10^{-3} dilution of vaccine were found to have low levels of DEN-2 neutralizing antibody against the vaccine parent strain, PR-159.

Biweekly serum samples were obtained; viremia was demonstrated in 2 subjects on days 10 and 14, respectively. Each had received the 10^{-1} dilution of vaccine and were unique in the study group in that they had previously received only one yellow fever immunization within 4 months of the DEN-2 vaccine trial. One of these was the only volunteer that experienced a fever $\geq$38 C that could be attributed to the DEN-2 immunization.

Five of the 14 seronegative recipients seroconverted, including those who were viremic. The 50% immunizing dose for yellow fever immune subjects calculated on the total experience from studies 1 and 3 is approximately $10^{3.3}$ PFU. Antibody titers on day 30 were consistent with those seen after a primary infection; however, considerably greater cross-reactivity was found for other flaviviruses, than is seen following a primary DEN-2 infection in a flavivirus nonimmune individual. The antibody responses appeared to be sustained. All 5 of the subjects who seroconverted maintained both HI and neutralizing antibody titers 6 months following immunization. In 4 of them neutralizing antibody titers were greater than 1:100.

None of the volunteers reported local reactions at the injection site. A variety of febrile illnesses were reported by 3 of 16 volunteers during the 21 days following vaccination. Only one illness could be attributed to DEN-2 immunization. Two of 5 of the seroconverters had leukopenia at some time during the 21 days. The results suggest that DEN-2 (PR-159/S-1) vaccine is safe and contained $10^{3.3}$ PFU median immunizing doses calculated on the basis of 19 yellow fever immune recipients.

Protocol FY 79-1, Study No. 4: Response of Previously Dengue-2 Immunized Adult Volunteers to a Booster Dose of DEN-2 (PR-159/S-1) Vaccine. Twelve adult volunteers who had been inoculated with the DEN-2 (PR-159/S-1) vaccine 4.5 to 18 months previously, were divided into 2 groups and reinoculated SC with undiluted ($1.1\text{-}5.8 \times 10^5$ PFU/0.5 ml) vaccine. Volunteers were followed as outpatients for 21 days and were required to fill out symptom checklists, to take oral temperatures daily and to obtain routine laboratory tests each week.

None of the volunteers developed fever (oral temperature >100 F) but 7 of 12 noticed soreness at the inoculation site between days 0 and 2. One volunteer also noted some redness of the inoculation site from days 0-2. Five volunteers experienced brief headaches. One volunteer developed unexplained abdominal pain with pyuria; another was treated for bronchitis during week 2.

Only 2 volunteers developed a 4-fold rise in serum neutralizing antibody following revaccination; both persons had seroconverted after the first dose of vaccine. There is no evidence that revaccination is either harmful or would increase the level of protection of recipients against wild-type dengue-2 infections.

Protocol FY 80-3, Study No. 5: Responses to Administration of DEN-2 (PR-159/S-1) Vaccine by an Intradermal Route to Adult Volunteers. Eight (8) adult volunteers with no previous reported exposure to flavivirus received 0.5 ml of either undiluted DEN-2 vaccine (PR-159/S-1) containing 1.2×10^5 PFU (6) or placebo (2). The vaccine was administered using a jet injector gun (Scientific Equipment Co., NY) equipped with an intradermal nozzle. There were no long-term local reactions following vaccine administration by this route.

Three individuals developed antibody following immunization; one recipient had only HI antibody, which was detected at 30 days but had disappeared by 60 days. Another recipient had an HI titer by 30 days, developed neutralizing antibody by 60 days but lost both antibodies by 6 months. The third person developed high antibody titers by both tests by 30 days which persisted throughout the follow-up period. This subject had a high titered, broadly cross-reactive antibody response, which suggested a previous experience with flavivirus. The low conversion rate (3/6) and the lack of sustained antibody response in individuals without previous experience with flavivirus indicates that intradermal inoculation has no advantages over the SC route of vaccine administration.

Rift Valley fever (RVF)(Protocol FY 78-1. During this period, experience continued to be gathered with the vaccine NDBR-103. It was demonstrated that the administration of two 0.5-ml doses of Lot 6, Run 1, did not result in attainment of reliable level of immunity in human subjects. No difference was detected among the various lots of the vaccine when given as three 1-ml doses. However, the number of persons tested, 2 and 3/lot, in the interlot comparison did not permit high confidence in the significance of these comparisons.

During this reporting period, 22 persons at USAMRIID received the NDBR-103 vaccine as part of their inoculation schedule for work-related exposure risks. Of these, all but 5 developed adequate PRN titers of $\geq$1:40. Each of the subjects received doses of 1 ml of Lot 6, Run 1, given on days 0, 10, and 28. Of the 5 who failed to develop adequate titers, 3 developed titers of 1:10 and 2 developed levels of 1:20 when measured on or about day 42. One of these, WHE, subsequently had a laboratory exposure to RVF virus. At the time of the accident his titer was 1:40; after the accident the titer rose to 1:640. During this time several blood samples were drawn, none of which revealed any viremia. There was no clinical illness associated with this laboratory exposure. Of those vaccinated at USAMRIID, none reported any reaction to the vaccine.

Eighty-three persons were vaccinated at the U.S. Department of Agriculture, National Animal Disease Laboratory (NADL), Ames, Iowa with Lot 6, Run 1 of NDBR-103; of these, 4 developed titers <1:40. Thus, from a total of 105 recipients of 1 ml of Lot 6, Run 1, 9 failed to develop adequate antibody titers. Pre- and post-immunization sera were collected on RVF vaccine immunized United States, Canadian and Swedish military personnel serving with United Nations Forces in the Sinai Peninsula. Results are not yet complete from these studies. No significant adverse reactions have been reported from these trials.

The first human test of the vaccine TSI-GSD-200 (Protocol FY 79-5) involved testing of Lot 1, Run 1, in doses of 1.0, 0.3, and 0.1 ml given SC on days 0, 10, and 28 to 4 and 5 volunteers. The results of this experiment through day 182 are included in Table I. Table II tabulates maximum titers for each recipient on or before day 91 in relation to the dose. Previous experiments in hamsters have indicated that this response is linear. When plotted and analyzed by least-squares, the regression coefficient was consistent with that observed in animals under similar test circumstances, giving some measure to the variability in the human response to a given dose of the vaccine. Based on these data, it was elected to test all subsequent lots at a dose of 0.3 ml so that any significant difference among the lots could be detected and so that if need for operational employment arose, the most efficient dose of the vaccine could be utilized rather than uniformly recommending a possibly wasteful, 1-ml dose.

Partial results of vaccination of humans with 0.3 ml of TSI-GSD-200 lots 1-8 on days 0, 10, and 28 are now available and are presented in Table III. However, some sera were not collected due to unavailability of subjects. Lots 3 and 7 appear to be significantly less efficacious than Lots 1, 2, 4-6, and 8.

As tabulated in Table IV the mouse potency test ($r=0.33$) did not correlate with the human potency results ($r=0.65$). More useful laboratory animal models are under study at this time.

TABLE I. PRN_{80} RESPONSE OF HUMANS FOLLOWING INJECTION OF 1.0, 0.3, OR 0.1 ml OF TSI-GSD-200 LOT 1 ON DAYS 0, 10 AND 28

SUBJECT	DOSE (ml)	RECIPROCAL PRN_{80} BY DAYS				
		14	35	42	91	182
Cross	1.0	160	320	320	80	20
Delong	1.0	20	40	80	>40	40
Lovett	1.0	40	1280	1280	160	80
McGookin	1.0	NA	NA	640	160	160
Blagg	0.3	80	320	160	80	40
Blount	0.3	20	20	160	40	40
Useldinger	0.3	<20	40	40	5	<5
Virga	0.3	5	80	40	40	<10
Beauchamp	0.1	80	160	160	40	40
Doty	0.1	<5	<5	<40	<5	<5
Joffe	0.1	20	80	160	>10	40
LeBlanc	0.1	<5	<5	<40	>20	20
Voelmeck	0.1	<5	<5	<40	<5	NA

TABLE II. HIGHEST TITER MEASURED ON OR BEFORE DAY 91 IN RESPONSE TO TSI-GSD-200 VACCINE GIVEN ON DAYS 0, 10 AND 28

DOSE	HIGHEST PRN_{80}	GEOMETRIC MEAN TITER	% NON-RESPONDERS
1.0 ml	320 80 1280 640	380	0
0.3 ml	320 160 40 80	112	0
0.1 ml	160 <5 160 20 <5	14	40

TABLE III. RESULTS OF IMMUNIZATION WITH RIFT VALLEY FEVER VACCINE, DRIED, INACTIVATED TSI-GSD-200 FROM VARIOUS LOTS AS MEASURED BY PRN_{80} IN VERO CELL CULTURE. DOSAGE WAS 0.3 ml INJECTED SC ON DAYS 0, 10 AND 28

LOT - SUBJECT	RECIPROCAL PRN_{80} TITER BY DAYS								
	0	7	14	21	28	35	42	91	182
Lot 1 - Day 0 was 29 Mar 79									
Useldinger	<5	<5	<20	<20	<10	<20 (40)[a]	40	5	<5
Blagg	5	20	80	160	320	320	160	80	40
Blount	5	5	20	40	-	20	160	40	40
Virga	5	5	5	-	80	80	40	40	<10
Lot 2 - Day 0 was 13 Apr 79									
Flores	<5	<5	20	40	40	160	320	80	
Savage	<5	<5	160	160	80	160	160	40	
Barriner	<5	-	10	40	40	160	320	20	
Young	<5	<5	<5	<20	<5	<20	20	<20	
Boucher	<5	<5	-	40	80	320	320	40	
Lot 3 - Day 0 was 13 Apr 79									
Wakefield	<5	10	10	40	40	160	20	20	
Lohr	<5	5	20	40	80	40	80	20	
Boudman	<5	5	5	20	40	80	80	80	
Gross	<5	5	5	20	-	80	40	<40	
Lot 4 - Day 0 was 28 Aug 79									
Garrett	<5	5	320	-	≥320	1280	1280		
Marrero	<5	<5	40	-	-				
Rivera	<5	5	80	-	320	1280	1280		

Lot 5 - Day 0 was 22 May 79									
Gwiazda	5	10	40	640	-	-		1280	160
Marsh	5	5	20	640	320	640		640	40
Silvis	5	10	20	320	320	1280		1280	-
Sprouse (2 doses only)	5	20	80	320	320	320		-	-
Lot 6 - Day 0 was 29 Oct 79									
Bunner	-	5	-	-	-	320		-	-
McCall	5	10	-	-	-	640		-	-
Short	5	5	-	-	-	640		640	-
Smith	5	5	-	-	-	320		640	-
Lot 7 - Day 0 was 29 Oct 79									
Carson	5	-	-	-	-	20		40	
Hatch	5	-	-	-	-	20		10	
Hardy	-	-	-	-	-	80		-	
Mason	5	5	-	-	-	160		-	
Lot 8 - Day 0 was 29 Oct 79									
Murrell	5	5	-	-	-	320		320	
Ladouceur	-	5	-	-	-	160		320	
Elder	5	-	-	-	-	-		640	
Suggs	-	5	-	-	-	80		-	

[a] Repeat test.

Malaria. During the last year phase II studies of the experimental antimalarial drug halofantrin (WR 171,669) were continued (Protocol FY 80-7). Results of the studies are summarized in Table V. To date, halofantrin has resulted in cure of malaria at all dose levels tested. From studie now in progress it appears that this drug will be effective in a single dose. No significant toxicity has been encountered in any of the volunteers.

Botulinum toxoids. The production and evaluation of new and improved botulinum toxoids presents many problems. Toxoids are available, in limited quantity, for inducing immunity to only 5 (A-E) of the 7 (A-G) immunologically distinct types of botulinum toxin. The equine antitoxins currently available for the treatment of botulism are responsible for adverse reaction in approximately 21% of recipients.

The immunogenicity and reactogenicity of two lots (MDPH A-2, MDPH B-1) of a newly bottled pentavalent botulinum toxoid in 52 volunteers were evaluated and compared to the currently used Parke-Davis-produced investigational pentavalent botulinum toxoid (Project No. FY 79-4). There was no significant difference between the incidence of local reactions in volunteers immunized with either of the two new MDPH lots nor were there any significant differences between local reactions occurring in volunteers immunized with either of the MDPH lots of toxoid and the local reaction in those volunteers immunized with the Parke-Davis toxoid. No systemic reactions occurred in any immunized volunteers.

There was no statistically significant difference demonstrated between the immunogenicity, as measured by serum neutralizing activity, of the two MDPH toxoid lots for Types A, B, or E toxin. There were no significant differences between

TABLE IV. HUMAN AND MOUSE POTENCY DATA FOR LOTS 1-8 OF TSI-GSD-200

LOT	RUN	RECIPROCAL LOG TITER DAY 42 IN MAN	REPLICATIONS MOUSE ED_{50} (ml)[a]	
			1	2
1	1	2.05	0.0055	0.0045
2	2	2.21	0.0050	0.0035
3	1	1.98	0.0050	0.0050
4	2	3.11	0.0110	0.0045
5	1	2.89	0.0110	0.0050
6	2	2.74	0.0030	0.0090
7	1	1.68	0.0080	0.0040
8	2	2.43	0.0090	0.0120

[a] Mouse ED_{50} measured by Salk Institute using method of Reed & Munch.

TABLE V. PHASE II STUDIES OF WR 171,669. FOLLOW-UP AT 60 DAYS

PATIENT NUMBER	DRUG DOSE	TOXICITY	PARASITE COUNT/mm^3 AT TREATMENT	PARASITE COUNT/mm^3 HIGHEST	PARASITE CLEARANCE TIME (h)	DEFERVESCENCE (h)
P. vivax (Chesson)						
1	250 mg Q6H x 12	None	850	1040	64	50
2		Mild nausea, diarrhea	700	1010	69	62
P. falciparum (Smith)						
1	250 mg Q6H x 12	None	220	222	28	90
2		"	1200	1200	70	108
3		"	1620	1620	71	81
4	250 mg Q6H x 8	Mild diarrhea	230	230	27	54
5		None	190	600	40	98
6		"	150	430	63	104
7	250 mg Q6H x 4	"	270	280	63	104
8		"	230	420	69	104
9		"	60	330	62	98
10	500 mg Q12H x 2	"	30	350	56	102
11		"	250	260	44	94
P. falciparum (Buchanan)						
1	250 mg Q6H x 8	Abdominal cramps, single loose stool	750	980	46	104

the immune response elicited against Type A and Type E toxins in volunteers immunized with either of the MDPH toxoids and those immunized with the Parke-Davis toxoid. However, the immune response elicited in volunteers by both MDPH lots, A-2 and B-1, to Type B toxin was significantly ($P<0.01$) greater than the response elicited in volunteers immunized with the Parke-Davis toxoid.

To date, over 80 volunteers have been immunized, under the auspices of Project No. FY 79-3, with pentavalent botulinum toxoid for the purpose of evaluating the immunologic response to the booster administration of botulinum toxoid, adsorbed, pentavalent (ABCDE) (IND 161), and to qualify the volunteers for participation in a plasmapheresis program which has yielded over 1200 liters of Botulism Immune Plasma (Human) IND #1332.

Half-life values (Project FY 79-7) for the neutralizing activity of five separate units of Botulism Immune Plasma (Human) when infused into 5 volunteers ranged from 11-17 days to 42-49 days with a group average of 21-27 days. In 4 of 5 volunteers the actual period of "protection", expressed as the number of days the recipients' titer to Type A toxin remained above 0.25 IU/ml of serum, equaled or exceeded a predicted period of "protection."

In an effort to evaluate the immunologic response of a booster, administration of monovalent (Type B) botulinum toxoid singularly and in conjunction with the booster administration of a pentavalent (ABCDE) botulinum toxoid, 62 volunteers were immunized under Protocols FY 80-5 and FY 80-6 with 0.5 ml of the newly bottled MDPH botulinum adsorbed monovalent type B toxoid. The immunologic responses induced in the volunteers are currently under evaluation.

All of the volunteer projects involving botulinum toxoids or botulism immune plasma were performed in collaboration with Work Unit No. 871 BA 123, Development of Effective Countermeasures Against Poisoning with Microbial Toxins of Military Importance.

Hospitalized Exposures

Lassa fever (LAS). There was one admission to the clinical isolation facility during this year resulting from an accidental exposure of a subject to LAS virus. The subject was treated in the usual manner with immune plasma, and at her own request, received ribavirin. There were no complications from the administration of ribavirin; however, an allergic reaction due to immune plasma occurred. The urticarial reaction responded promptly to antihistamines. The patient was discharged after 21 days of isolation in good health. No signs or symptoms of LAS developed.

Korean hemorrhagic fever (KHF). A senior civilian microbiologist was treated with immune plasma for a possible exposure. No disease occurred. There were multiple admissions of healthy volunteers to the clinical isolation unit as part of simulated exposure exercises.

Evaluation of the Vickers Aircraft Transit Isolator.

Several healthy volunteers were admitted to the Vickers Isolation Units as part of simulated exposure exercises.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6419	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GROUP MBR'S	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	003
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE: (U) Mechanisms of immunoprophylaxis against aerosol-disseminated respiratory diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 08	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS ($ in thousands)
PRECEDING FISCAL YEAR 80	1.0	176
CURRENT FISCAL YEAR 81	0	0

19a. RESPONSIBLE DOD ORGANIZATION
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ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS: (U) Military medicine; (U) BW defense; (U) Respiratory pathogens; (U) Airborne infections; (U) Franciscella tularensis; (U) Animal models

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS:

23 (U) Characterize host immunity induced against respiratory infections by administration of microbial antigen by aerosol, intranasal and parenteral routes to appropriate animal models. Determination of optimal methods of immunoprophylaxis is an essential element in military medicine as a major determinant of resistance against potential BW respiratory pathogens.

24 (U) Immunogenic determinants for resistance against aerosol-disseminated respiratory disease are being studied in the Fischer 344 inbred rat/tularensis model system. The rats vaccinated by different routes (aerosol and parenteral) are then treated with an immunosuppressant drug before and after vaccination to evaluate the contribution of the various effector arms of immunity to the host's defense against respiratory tularemia.

25 (U) 79 10 - 80 09 - Immunosuppressant cyclophosphamide (CY) pretreatment of Fischer-344 rats vaccinated with either the live vaccine strain (LVS) or an inactivated vaccine of Franciscella tularensis resulted in a significant suppression of humoral agglutinins correlative to suppression of serum IgM levels. Macrophage inhibition factor (MIF) activity for CY-vaccinated and non-CY-vaccinated rats was indistinguishable from nonvaccinated control rat responses, indicating that MIF was not of adequate sensitivity for use as a correlate of cellular immunity in these rats. Skin test reactivity as an indicator of delayed type hypersensitivity in vaccinated rats also was unaffected by CY treatment.

In other studies, 98 - 100% of rats passively transferred with whole immune serum from rats vaccinated 3 times with LVS survived an aerosol or intraperitoneal challenge with virulent F. tularesis. Of control rats receiving normal rat serus, 92 - 100% died. Passive transfer of various fractions of immune serum protected 34 - 100% of the rats to subsequent challenge. Terminated for management efficiency. Continued in W.U. 871 BB 149. (DAOG3813)

DD FORM 1498 (1 MAR 88) 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 88 (FOR ARMY USE) ARE OBSOLETE

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BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BB: Prevention of Bacterial and Rickettsial Diseases of Potential BW Importance

Work Unit No. 871 BB 125: Mechanisms of Immunoprophylaxis Against Aerosol-
(841 00 003) Disseminated Respiratory Diseases

Background:

Microbial pathogens capable of causing significant incapacitation or death when inhaled as small particle aerosols (SPA) have long been considered potential BW threats (1). Ideally, protection against such airborne diseases would be provided by immunoprophylaxis. In attempts to induce immunity against aerosol disseminated patnogens, however, one must consider the relative importance and interactions among humoral, local and cell-mediated immunity. All the component arms of immunity and their interrelationships may well be dependent on the method used to stimulate host protective immunity. The live vaccine strain (LVS) of Francisella tularensis is being used to vaccinate inbred Fischer-344 rats (F344) to investigate basic mechanisms of host immunity against lethal respiratory tularemia. The effectiveness of aerosol vaccination and various parenteral routes to induce infection related antibody and cell-mediated immune responses important for protection against aerosol challenge with virulent F. tularensis is being compared and characterized. In addition, efforts have been directed towards studying the effects on host immunogenesis and protective immunity of (a) an immunosuppressant, cyclophosphamide (CY), which suppresses the induction of humoral antibody and may potentiate the cellular immune response and (b) passive transfer of various fractions of tularemia immune serum in the F344 rat.

Progress:

Previously, we reported that treatment of F344 rats with CY administered IP at a dose of 50 mg/kg body weight 3 days prior to vaccination and on days 7 and 18 after vaccination with either LVS or the inactivated Foshay type vaccine (FO) resulted in total or almost total suppression of humoral agglutinins (USAMRIID Annual Report, 1979). Despite the lack of significant levels of serum agglutinins, practically all of the CY-treated LVS- and FO-vaccinated rats survived aerosol and IP challenge with virulent F. tularensis. In fact, the survival rate of the CY-treated vaccinated rats was not different from that of vaccinated control rats that had high levels of serum antibodies. All nonvaccinated control and CY-treated rats died within 14 days after challenge.

These data indicated that the serum antibody produced by a single LVS vaccination may not be a critical component of the immune system in providing full protection against lethal respiratory tularemia. Since arlier studies showed that humoral antibodies induced by LVS vaccination were predominantly IgM, experiments were performed to determine the effect of CY treatment on immunoglobulin levels of LVS- and FO-vaccinated rats. Efforts also were directed towards determining whether CY treatment of vaccinated rats would result in a

significant increase in the macrophage migration inhibition factor (MIF) or skin test response for delayed type hypersensitivity (DTH) as indicators of cellular immunity. The results of serum Ig and serum agglutinin assays for vaccinated rats pretreated once with CY are shown in Table I. Ig content of sera were determined by the Mancini radial immunodiffusion procedures employing specific rat anti-Ig serum fractions procured from Miles Labs, Inc. Serum agglutinins were measured by microagglutination (MA) on days 7, 14, and 21 after vaccination. The values shown for IgM represent the mean diameter of precipitin rings obtained for duplicate serum samples and are representative of the relative concentration of IgM in the serum samples. Since CY treatment neither depressed nor increased IgM or IgA levels over baseline, these values were excluded from Table I.

TABLE I. ANTIBODY RESPONSE OF VACCINATED RATS WITH AND WITHOUT CY PRETREATMENT

TREATMENT (n=8)	IgM BY DAYS (mm diam.)			GEOM. MA TITER BY DAYS (reciprocal)		
	7	14	21	7	14	21
CY-LVS	4.69	5.08	4.83*	16	80	254
LVS	5.08*	5.73*	4.95*	271	642	270
CY-FO	4.68	4.78	4.60	56	70	34
FO	5.16*	4.74	4.54	113	88	44
CY control	4.60	4.71	4.68	0	0	0
Room control	4.53	4.71	4.64	0	0	0

*P < 0.01, ANOV, vs. values in same column.

Pretreatment of the LVS- and FO-vaccinated rats with CY did not cause any rise of decrease of IgM when compared to the baseline values measured for either of the nonvaccinated groups of rats. In contrast, IgM levels were significantly higher at all assay periods for the LVS-vaccinated rats not treated with CY. The elevated levels of IgM for the LVS rats for 21 days corroborated our previously reported findings. For FO-vaccinated rats not treated with CY, a significant increase of IgM occurred only on day 7 and thereafter declined to baseline values. This rather weak IgM response is attributed to the poor immunogenicity of the inactivated FO vaccine and the probably rapid elimination of the FO vaccine from the host. The relative poor antigenicity of the FO vaccine also can be recognized by comparing the agglutinin titers measured for the FO and LVS rats. Even with CY treatment, vaccination with FO always resulted in much lower serum titers than those obtain in rats vaccinated with LVS. A similar pattern was observed with FO and LVS rats were treated with CY. Since humoral tularemia antibodies are predominatly IgM (2), one would expect reasonable correlation with MA titers. The data in Table I substantiate this correlation. Lack of increase in IgM levels generally coincided with low MA titers.

Data also were obtained on possible use of skin test responses for DTH and degree of MIF activity as indicators for cellular immunity in CY-treated and non-CY-treated vaccinated rats. The rats were pretreated with CY (50 gm/kg) 3 days before vaccination with either LVS given IP at a dose of $1 \times 10^{4.5}$ cells

of the FO vaccine. Skin tests were performed by ID injection of 0.1 ml of FO vaccine. Skin tests were performed by ID injection of 0.1 ml of FO vaccine containing 1.0 μg of bacterial nitrogen (~ 1 x 10^8 dead cells). Skin reactions were assessed by measuring skin-fold thickness with skin calipers at the site of injection 4, 24, 48, and 96 h and 1, 2, and 3 weeks postvaccination. The data shown in Table II are based on the 24-h reading at which time maximum skin reactivity was observed. Each data point is a mean derived from assays of 5 rats.

TABLE II. MIF AND SKIN TEST RESPONSE OF VACCINATED RATS WITH AND WITHOUT CY PRETREATMENT

	RESPONSE BY WEEKS					
	No. with MIF/Total			Skin Test Positivity[a]		
TREATMENT	1	2	3	1	2	3
CY-LVS	1/5	0/5	0/5	3+	2+	+
LVS	1/5	1/5	1/5	3+	2+	+
CY-FO	1/5	0/4	0/5	+	-	-
FO	0/5	1/5	0/5	+	-	-
CY controls	0/5	0/4	0/2	-	-	-
Room controls	0/5	0/4	1/5	-	-	-

[a]Based on 1+ to 4+ 8-24 h.

Blood samples drawn prior to skin tests were assayed for MIF activity using the indirect MIF assay procedure. Only the LVS rats showed definitive skin reactions indicative of DTH; DTH persisted for > 2 weeks. Skin test reactivity of these LVS rats was not affected in any manner by CY pretreatment. Only minimal skin test response (1+) was measured for FO rats. This is not unexpected for animals administered an inactivated vaccine. Treatment of these rats with CY, however, did not augment the skin test response as has been reported for FO vaccinated guinea pigs (3).

Perhaps the 50 mg CY dose given in our experiments was not sufficiently high to cause any intensification of DTH. Published reports record doses administered to mice, guinea pigs and outbred rats to be from 200-300 mg/kg body weight. CY dosages at this level, however, are lethal for F344 rats.

MIF activity also was not augmented by CY treatment in vaccinated rats. In fact, results for vaccinated rats, with and without CY, were no distinguishable from the results obtained for nonvaccinated control rats. Only an occasional rat showed minimal to moderate MIF activity as had been observed in previous experiments. These results indicated that MIF is not a sensitive enough correlate for cellular immunity in F344 rats.

To infestigate further the relative role and interaction of humoral and cellular immunity in respiratory tularemia, research was initiated on the effects of passively transferred F. tularensis antiserum in F344 rats. These studies also would provide baseline data for anticipated passive transfer experiments

wherein F344 rats would serve as recipients for serum or spleen cells or lymphocytes from vaccinated donor rats in which humoral or cellular immunity would be modified by appropriate immunosuppressant agents, e.g., cyclophosphamide, antithymocyte serum. In addition, these studies could provide additional information on the still controversial problem of the degree of resistance conferred against tularemia by passively administered antibody in recipient animals.

Immune serum for passive transfer was obtained from F344 rats vaccinated IP with LVS at 1, 2, and 3 weeks at dose levels of 10^4, 10^5, and 10^7 cells, respectively. Two other groups of rats were injected on a similar schedule for control purposes. One control group received normal rat serum, the other group was injected with sterile, modified casein partial hydrolysate (MCPH), the solution used as the growth and suspension medium for LVS. One week after the 3rd injection, all rats were bled out, serum separated, pooled for each group, heat-treated at 50°C for 30 min, and assayed for LVS agglutinin titers. For the passive transfer studies, recipient F33 rats were injected IP with 0.7 ml of either the LVS immune serum (anti-LVS), normal rat antiserum (NRS) or MDPH antiserum at 16-18 h preceding aerosol challenge. This time-lapse provided for the serum to be distributed through the body of the recipient host. All recipient rats then were challenged by exposure to aerosols of virulent F. tularensis SCHU-4 or by the more stressful IP route. The survival response data accumulated for 3 experiments are shown in Table III.

TABLE III. SURVIVAL RESPONSE OF PASSIVELY IMMUNIZED RATS TO CHALLENGE WITH F. TULARENSIS, SCHU-4

PRECHALLENGE SERUM (titer)	ROUTE CHALLENGE (log_{10} dose)	NO. SURVIVED/TOTAL	%	MEAN TIME OF DEATH
Anti-LVS (1:1548)	Aerosol (5.4)	28/29	97	6
	IP (4.7)	13/13	100	-
Normal Rat Serum (0)	Aerosol (6.1)	1/11	9	4.2
	IP (4.5)	1/9	11	4.7
MCPH (0)	Aerosol (6.1)	0/9	0	5.3
	IP (4.5)			

All but one of the 42 rats which received the LVS immune serum survived both types of challenge. None of the animals appeared ill during the 30-day observation period. Of the control rats that received the normal rat serum, only 10% survived and all of the rats passively transferred with MCPH died by day 6.

Of additional interest were the assay data (Table IV) obtained from the LVS-antiserum rats at selected days post challenge. Control rat data are not

shown as concentrations of challenge organisms in tissues were at typically high levels and early deaths occurred. It is important to note that specific tularemia serum antibody was detected by MA in the antiserum of recipient rats for a 3-day period after passive transfer.

TABLE IV. RESPONSE OF LVS PASSIVELY IMMUNIZED RATS TO CHALLENGE WITH F. TULARENSIS, SCHU-4

ASSAY DAY	MEAN MA TITER (reciprocal)	NO. POSITIVE (log_{10} organisms) Lung	Spleen
Aerosol challenge with $10^{5.5}$ (n=4)			
1	28	4 (4.6)	0
2	30	4 (5.6)	0
3	10	4 (6.2)	2 (4.7)
7	40	4 (5.0)	3 (3.6)
10	125	4 (4.5)	3 (3.9)
IP challenge with $10^{4.7}$ (n=2)			
1	10	0	0
2	20	0	0
3	10	2 (4.7)	0
7	20	2 (3.7)	1 (3.5)
10	80	2 (4.9)	1 (4.9)

No tularemia antibodies were detected in rats given either normal rat serum of MCPH serum. The presence of preformed antibodies, although at a low level may well have prevented early splenic infection. In fact, the temporal pattern of spleen and lung infection observed in the passive transfer rats closely resembled the tissue infection patterns usually observed to actively immunized animals following either aerosol or IP challenge with virulent F. tularensis.

The reason for the survival of the passively immunized rats observed over the 3 experiments is currently undefined. Kostiala et al. (4) have reported that passively transferred immune serum is devoid or protective immunity. Their cierion of protection, however, was based only on the magnitude of decrease in the level of growth in the liver and spleen of the LVS organisms used to challenge the passively immunized rats. Survival after challenge with virulent F. tularensis was not studied by them. Thorpe and Marcus reported that the passive transfer of immune tularemiz serum mitigated the normal course of infection but not the mortality in guinea pigs, rabbits and mice (5). However, their method of vaccination consisted of one SC injection of an attenuated strain of F. tularensis, followed 3 weeks later by SC injection with 10^3 to 10^4 cells of a virulent strain;

animals were bled out 3 weeks later. It is possible that in our experiments, the regimen of 3 consecutive weekly vaccinations with increasing doses of LVS may have stimulated the production of soluble lymphokines, or as suggested in a personal communication with A. O. Anderson, formerly of Pathology Division, a soluble low MW T-cell receptor which, being present in the transferred serum, conferred protection. The 3-week vaccination schedule also may have stimulated production of IgG antibodies and combined with IgM, which is produced within the first week of vaccination, resulted in a significant cumulative resistance to tularemia. We conducted studies, therefore, to attempt to determine the critical factors responsible for the protection afforded the passively transferred rats in our experiments.

A preliminary study was initiated in collaboration with MAJ D. Reichard, Physical Sciences Division, on the capacity of various fractions of F. tularensis antiserum injected IP in F344 rats to protect against lethal tularemia. Serum fractions consisted of dialyzed immune serum (Code I) to remove low MW soluble T-cell receptors and other soluble lymphokines, a $(NH_4)_2SO_4$ precipitate containing total Ig components (Code II), a supernatant fraction from the precipitated phase (Code III), a purified IgG (Code IV, and a purified IgM fraction (Code VI), and 2 nonspecific precipitated factors (Code V and Code VII). In addition, whole LVS immune serum was treated with 2-mercaptoethanol (2-ME) to eliminate IgM but retain potential soluble protective factors (Code IX). For control purposes, whole normal rat serum (Code VIII) and whole LVS immune serum (Code X) were included. Rats were passively immunized by IP injection with 0.7 ml of either whole tularemia antiserum or normal rat serum or with the various fractions derived from the immune serum. All recipient rats were challenged IP with 0.5 ml of 2×10^5 cells of virulent F. tularensis, SCHU-4, 16-18 h after passive transfer. Just before IP challenge, blood was obtained from 2 rats of each group via orbital sinus bleeding; serum samples were assayed for the presence of LVS serum agglutinins. All animals were observed twice daily for morbidity or mortality for a 21-day period. The response data obtained for these rats are shown in Table V.

TABLE V. EFFECT OF PASSIVELY TRANSFERRED SERUM FRACTIONS ON SURVIVAL OF F. TULARENSIS-CHALLENGED RATS (10^5 SCHU-4) (n=6/group)

Code	TRANSFER SERUM	MA Titer	RECIPIENT MA Titer	SURVIVAL No.	SURVIVAL %	MEAN DAY OF DEATH
I	Immune	320	20	6	100	-
II	NH_4SO_4 ppt	640	40	6	100	-
III	NH_4SO_4 super.	0	0	3	50	6.97
Fraction						
IV	0.01 M IgG	40	0	3	50	4.94
V	0.08 M nonspec. ppt	20	0	2	34	5.66
VI	0.16 M IgM	80	0	3	50	6.31
VII	1.0 M nonspec. ppt	20	0	2	34	4.91
Controls						
VIII	Normal	0	0	0	0	6.41
IX	2-ME-treated	80	20	5	83	6.03
X	LVS immune	1024	20	6	100	-

All rats passively immunized with whole LVS immune serum (Code X) survived the challenge while all control rats given normal rat serum died within 10 days. Of interest was that the dialyzed immune serum and the precipitate also conferred total protection, indicating that if a soluble lymphokine (t-cell receptor) was the essential protective factor, it's MW was high enough to be retained by the dialyzing membrane. It was learned later that the dialyzing membrane used retained all protein with a MW > 12,000; it had been planned to removed all soluble protein with a MW of $\leq$ 20,000. Also of importance with the full protection offered by the reconstituted precipitate fraction containing all immunoglobulins. This suggests that, at least in the rat, although IgM *per se* may not be a critical determinant of resistance to tularemia, the combined total Ig portion of serum by the purified IgG and purified IgM as single entities was only 50% of the protection obtained with the total Ig fraction. This observation also is compatible with the premise that IgG is subsequently induced by the 3X vaccination procedure and in combination with the earlier produced IgM, which is still readily detectable at 28 days, results in a cumulative protection above that conferred by the individual IgG and IgM fractions. Although the nonspecific precipitate fractions showed some protection (34% survival), this can be explained by assuming probably contamination of these fractions with IgG and IgM as indicated by the MA titers of 1:20. Results obtained with immune serum treated with 2-ME is of interest as this appears to be additional evidence of the possible protective effect of IgG antibody and/or possible lymphokines, if one assumes that the IgM antibody indeed was neutralized

by the 2-ME treatment. The 1:80 agglutinin titer obtained for this fraction, therefore, would be accounted for by IgG. On the basis, it again appears that IgM, per se, is not an essential immune defense element against lethal tularemia.

It is emphasized that the data on serum fractions possibly transferred in F344 rats were derived from one experiment in which small number of animals were used. However, the data point up some interesting and pertinent approaches to delineate the factors present in tularemia immune serum that may be responsible for the protection obtained in passively immunized rats. Confirmatory experiments are in progress to accumulate animal responses. Experiments also will be conducted to study the protective effect of serum fractions obtained from tularemia vaccinated rats treated with immunosuppressant agents, as well as investigating further the stimulation of IgG and IgM antibody by varying vaccination regimens and their subsequent role in host immune defense mechanisms.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

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79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

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

23 (U) Models for lethal arenavirus infections in primates and rodents are developed for use in testing the protective efficacies of antiviral drugs and immune serum therapy regimens. Experimental vaccines are developed and tested in animal models. Attenuated virus strains are characterized using biological and biochemical markers of virulence. These investigations will contribute to development of more effective vaccines to protect military personnel.

24 (U) Direct and indirect effects of virus replication in animal tissues and cell cultures are assessed. Humoral and cellular immune responses are measured and correlated with alter disease patterns.

25 (U) 79 10 - 80 09 - Rhesus, cynomolgus, and African green monkeys were lethally infected with a virulent strain of Lassa (LAS) virus. Capuchin and squirrel monkeys were inapparently infected. Inbred, strain 13 guinea pigs, were also lethally infected. The disease course in all lethally infected animal models was similar to the human disease, as measured by virus replication, histopathology, immunofluorescence, enzyme fluctuations, and hematologic parameters. Treatment of cynomolgus monkeys with a combination of ribavirin plus immune serum was effective even when delayed until day 10, while serum or ribavirin alone were ineffective after day 6. Higher ribavirin doses were toxic. A neutralization antibody assay was developed, which depends on a heat-labile factor in immune serum. A live attenuated LAS strain from Rhodesia holds promise as a vaccine candidate. Publications: J. Infect. Dis 141:580-589, 1980; Abstr. Annu. Meeting ASM, T134, p. 257, 1980. In Manual of Clinical Microbiology, 3d ed., pp. 884-890, 1980; In Manual of Clinical Immunology, 2d ed., pp. 667-671, 1980.

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BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards
(3M172776A841):

Task No. 3M162770A871BC: Prevention of Viral Diseases of Potential BW Importance

Work Unit No. 871 BC 134: Determinants for Virulence and Attenuation of Arbo- and Arenavirus Vaccine Candidates
(841 00 009):

Background:

Lassa virus is an arenavirus which has been associated with severe, often fatal infections of man. Outbreaks of Lassa (LAS) fever have been reported in Nigeria and Sierra Leone, and serologic evidence suggests the presence of Lassa virus in Guinea and Senegal (1-3).

Management of clinical cases has been largely symptomatic and supportive. Specific treatment of this virus disease, which may be associated with mortality rates as high as 40% in hospitalized patients, has until recently not been attempted. Immunotherapy has been tried in a small number of patients (4), with equivocal success and essentially no supporting virologic data to evaluate critically treatment efficacy. Animal models are needed to understand the pathogenesis of LAS virus, and to test promising antiviral drugs and immunotherapy regimens. An effective vaccine would also reduce the risk to laboratory workers and hospital personnel exposed to this life-threatening virus. This work unit is focused on the development of useful animal models, candidate vaccines and assessment of antiviral drugs and immunotherapy regimens to prevent and control LAS fever in man.

Progress:

Table I extends preliminary data reported last year which suggest that rhesus, cynomolgus, and African green monkeys sustain severe, usually lethal infections following LAS virus inoculations, while squirrel and capuchin monkeys sustain only mild infections. Rhesus monkeys inoculated with 12 PFU SC (1.1 $\log_{10}$ PFU) were uniformly killed (6/6) while only 6/10 died at the higher dose. We observed a similar phenomenon among outbred guinea pigs; 35/40 inoculated with 1.3 $\log_{10}$ PFU died, in contrast to 17/40 inoculated with 6.1 $\log_{10}$ PFU. These findings suggest the possibility of an interference phenomenon operational in the intact susceptible animal; this possibility should be investigated in more detail. It also suggests that the rhesus (inoculated with low virus doses) is as useful as the cynomolgus monkey for protective efficacy studies. To determine if other primate species were more susceptible to low vs. high doses of LAS virus, 1.1 $\log_{10}$ PFU doses were inoculated into African green and capuchin monkeys. African greens were uniformly killed by the low virus dose, while capuchins were still uniformly resistant, although all 4 capuchins seroconverted by indirect fluorescent antibody (IFA) test 16-21 days, indicating inapparent infection. Monkeys of all species, including those that died, seroconverted by IFAT, even though thet were usually still viremic, suggesting the

possible role of virus/antibody complexes in the pathogenesis of this disease.

In untreated, surviving rhesus monkeys, seroconversion was titrated by IFAT several weeks before neutralization Table II. Optimization of the neutralization test is described in a following section of this report. However, it is clear that neutralization and IFAT antibodies represent 2 independent responses to LAS infection, both in rhesus monkeys and in cynomolgus monkeys treated wtih ribavirin (Table II).

TABLE II DEVELOPMENT OF NEUTRALIZING AND IFAT ANTIBODIES IN SERA OF SURVIVING RHESUS AND CYNOMOLGUS MONKEYS

	DAYS AFTER INOCULATION	ANTIBODY TITER PRN_{80}	LNI[a]	IFA
Rhesus	20		[b]	2560
	24		[b]	2560
	28		0.4	2560
	43		2.1	2560
	61		2.6	3120
	180		4.1	2620
Cynomolgus[c]			[b]	
	30	<10		5956
	45	<10	2.4	8413
	66	16	2.9	7080
	95	26	2.8	7080
	187	35	4.1	3550
	305	73	>5.3	1170

[a]Geometric mean log neutralization index (1:10 dilution serum) based on 4 monkeys inoculated with 6.1 $\log_{10}$ PFU.

[b]Monkeys still viremic - LNI determination was impossible.

[c]Cynomolgus monkeys treated with ribavirin (30 mg/kg/day) initially on day 4 following virus inoculation.

Further descriptions of the unmanipulated primate models include total and differential leukocyte counts, hemoglobin, SGOT and CPK determinations. In general, fluctuations were mild in capuchin and squirrel monkeys, and pronounced in the lethally-infected primate species, except for hemoglobin which did not vary significantly in any monkeys examined. SGOT and CPK elevations were significantly elevated within 7 days of infection and peaked by day 10, corresponding to the hepatocellular necrosis apparent in HE sections of livers obtained from dead monkeys. Monkeys of all 5 primate species sporadically shed virus from the nasopharynx and in the urine, during the first 4 weeks of infection, suggesting that horizontal transmission might occur between infected and

TABLE III. VIRUS CONCENTRATIONS IN TISSUES OF MONKEYS DYING AFTER LASSA VIRUS INFECTION.

TISSUE	VIRUS CONCENTRATION (LOG_{10} PFU/ml or g)			
	Rhesus (n = 6)	African Green (n = 5)	Cynomolgus (n = 12)	Cynomolgus (3) (ribavirin treated)
Serum	5.6 + 0.6	4.8 ± 0.5	5.5 ± 0.2	2.6 ± 0.2
Liver	7.6 + 0.5	6.0 ± 0.9	7.0 ± 0.5	UD[a]
Spleen	6.7 + 0.3	6.0 ± 0.4	6.3 ± 0.3	5.4 ± 0.2
Kidney	6.5 + 0.3	6.3 ± 0.5	6.8 ± 0.2	UD
Adrenal	6.9 + 0.5	6.3 ± 0.7	7.3 ± 0.3	4.7 ± 0.6
Lung	6.9 + 0.4	6.0 ± 0.7	6.9 ± 0.2	6.2 ± 0.3
Pancreas	6.8 + 0.5	6.1 ± 0.8	6.6 ± 0.2	UD
Heart	NT[b]	5.2 ± 0.5	5.9 ± 0.2	3.3 ± 0.1
Salivary gland	NT	4.8 ± 0.6	5.6 ± 0.2	UD
Lymph node	6.2 + 0.5	5.8 ± 0.3	6.4 ± 0.2	4.0 ± 0.6
Bone marrow	NT	4.8 ± 0.5	6.4 ± 0.2	UD
Brain stem	5.5 + 0.4	4.8 ± 0.6	5.7 ± 0.2	4.2 ± 0.4
Cerebrum	5.0 ± 0.4	4.7 ± 0.5	5.2 ± 0.2	UD
Spinal cord	5.4 ± 0.3	4.7 ± 0.7	5.8 ± 0.3	NT

[a] Undetectable

[b] Not tested.

and susceptible primates in close contact with each other.

The distribution of virus in tissues of dead monkeys was determined by infectivity titrations (Table III); results will eventually be correlated with the distribution of viral antigens detected by immunofluorescence and the severity of histopathologic lesions. In cynomolgus, rhesus, and African green monkeys, significant viral replication was detected in all visceral tissues tested. In contrast, brain stem, cerebrum, and spinal cord (the CNS) contained minute quantities of infectious virus, probably from contained blood. By FA, no virus was detectable in the CNS, with the possible exception of vascular endothelium. Electron microscopy of CNS tissues in thin section is required to resolve the important question of whether LAS virus infects vascular endothelium within the CNS.

The distribution of virus within tissues of cynomolgus monkeys which died in spite of ribavirin treatment (initiated too late on day 7), was somewhat different from untreated monkeys (Table III). Virus was totally excluded from liver, being undetectable by both infectivity titration and direct immunofluorescence. Likewise, virus was apparently excluded from kidney, pancreas, salivary gland, and bone marrow, and titers were reduced in most visceral tissues; however, virus was recovered from brain stem at concentrations exceeding contained blood; it is not possible to determine whether the degree of viral replication observed was of pathologic significance. In contrast, replication in the lung was only slightly reduced. Histologically, often severe interstitial pneumonia was observed, and by immunofluorescence, massive aggregates of LAS antigens were observed within thickened alveolar septae. These results suggest that ribavirin administered parenterally was ineffective against pneumonic LAS, however, it is possible that ribavirin administered by aerosol would be effective in delivering active drug to the lungs where it is critically needed, a testable hypothesis using either monkeys or guinea pigs. (See Work Unit A870 BB 042).

Development of a Neutralization Test for LAS Antibodies. A sensitive and reproducible assay system is required to measure neutralizing antibody in immune plasma for immunotherapy and survey sera, and to evaluate vaccine efficacy. Efforts to develop an assay were stalled until we realized that the neutralizing antibody response develops relatively late in convalescence, and that sera containing high titered antibody measured by IFAT may contain little or no neutralizing antibody.

Using late convalescent, hyperimmune monkey serum, and a late convalescent human serum, we examined several variables in the neutralization test for the purpose of optimizing the procedure, using Vero cells to determine plaque reduction endpoints. We performed serum-dilution and virus-dilution tests using fresh and heated immune serum supplemented with either fresh or heated monkey or guinea pig serum as a complement source (Table IV). Using the virus-dilution procedure and a 1:10 dilution of immune serum, fresh (unheated) serum yielded higher LNI values than heated immune serum; the addition of fresh normal serum restored LNI titers partially. Similar results were obtained for human immune serum. The effectiveness of fresh guinea pig serum equalled or exceeded that of fresh monkey serum. In the serum dilution test, it appeared that fresh normal serum more completely reversed the effect of heating. However, PRN_{80} titers were relatively low even under optimal conditions. It appears that only low dilutions of immune serum effectively neutralize LAS virus.

TABLE IV. NEUTRALIZATION OF LAS VIRUS BY LATE-CONVALESCENT ANTISERA TESTED UNDER VARIOUS CONDITIONS.

Immune Serum		NORMAL SERUM (10%)					NEUT. Ab TITER	
		Monkey		Guinea Pig		Fetal Calf		
Fresh	Δ[a]	Fresh	Δ	Fresh	Δ	Δ	PRN^b_{80}	LNI[b]
Monkey								
+		+					160	>4.2
+			+				40	2.7
+				+			160	>4.2
+					+		40	2.6
+						+	40	>4.2
	+	+					160	1.9
	+		+				20	1.0
	+			+			320	1.7
	+				+		40	1.0
	+					+	20	1.4
Human								
+		+					20	1.7
+			+				10	1.5
+				+			20	2.5
+					+		10	2.5
+						+	10	2.2
	+	+					10	0.8
	+		+				<10	1.2
	+			+			20	1.7
	+				+		<10	1.4
	+					+	<10	1.3

[a] Heated-treated.

[b] Highest serum dilution producing less than 20% of control number of plaques.

[c] Log neutralizing index, using serum diluted 1:10 in HBSS containing 10% normal serum (heated or fresh).

The virus dilution neutralization test thus appears to be more sensitive and reproducible than the serum dilution test. Unfortunately, this test requires a relatively large volume of serum, which presents a special problem for serologic surveys. A reasonable compromise appears to be a "modified VDN" in which a single tube containing serum is challenged with 1000 PFU. If the mixture results in plaques too numerous to count, the LNI is <1. Countable plaques yield LNI between 1 and 3. No plaques imply an LNI >3. Using this modified procedure, we are now examining human sera obtained sequentially following confirmed LAS infection, as well as convalescent monkey sera and survey sera from Sierra Leone.

higher dose of ribavirin (90 mg/kg/day). We assumed, erroneously, that this was a tolerable dose: all 6 monkeys died, but viremias were low. Very little virus was recoverable from the viscera, and no viral antigens were detected in frozen sections stained by FA. Histopathologic examination revealed few viral-induced lesions, but widespread toxicity, especially depletion of erythroblastoid stem cells, suggesting a drug-induced death. Subsequent studies conducted under work unit A871-BE-144 demonstrated the potential of ribavirin to induce these lesions when administered at high dosage levels. Thus, at this point it appears that the most effective treatment of advanced LAS in monkeys is the combination treatment using ribavirin (30 mg/kg/day) + immune serum.

Guinea pig models to supplement primate models for lethal LAS infection. Two models for lethal arenavirus disease in guinea pigs have been developed. One, using adapted Pichinde (PIC) virus, was described in detail last year. In an extension of these studies, we tested the protective efficacy of immune plasma, Table VI. One sample obtained early in convalescence (day 35) had an IFAT titer of 10,240, but negligible neutralizing antibody (LNI = 0.3). The other plasma, obtained day 145, had an IFAT titer of 5,120 but an LNI = 4.4. The "early" plasma failed to protect guinea pigs, viremias of animals treated with undiluted "late" plasma were fully protected; their viremias were substantially reduced. However, "late" plasma was totally ineffective when diluted 1:10, Table VI.

TABLE VI PROTECTION OF STRAIN 13 GUINEA PIGS INFECTED WITH ADAPTED PIC VIRUS BY IP INOCULATION OF IMMUNE PLASMA (1 ml undiluted, days 0,3,6).

GROUP	DEAD/TOTAL (mean day of death)	VIREMIA - LOG_{10} PFU/ML, ± BY DAY 5	8	12	15	19
Untreated controls	8/8 (17.6)	1.8 ± 0.11	3.38 ± 0.35	4.4 ± 0.44	5.0 ±0.54	5.7 ±0.80
"Early" immune plasma (LNI,0.3)	5/5 (20.6)	1.5 ± 0.12	2.36 ± 0.36	4.4 ± 0.26	4.0 ± 0.17	5.7 ±0.26
"Late" immune plasma (LNI,4.4)	0/5	1.7 ± 0.22	1.80 ± 0.29	2.3 ± 0.75	<0.7	<0.7
"Late" immune plasma (1:10)	5/5 (17.6)	1.9 ± 0.12	2.34 ± 0.30	4.4 ± 0.19	6.0 ± 0.24	all dead

Serum and ribavirin therapy initiated late after LAS infection of cynomolgus monkeys. Previous studies suggested that ribavirin or serum therapy was effective when initiated early, but not late after infection. Studies presented in Table V extend these data to include monkey groups treated initially on day 7 or 10 Ribavirin alone in the usual (maximal tolerable dose of 30 mg/kg/day)protected only 4/8 monkeys treated initially day 7. Hyperimmune serum therapy was even less effective, since 5 of 6 monkeys treated initially on day 7 eventually died.

TABLE V EFFECT OF RIBAVIRIN AND/OR IMMUNE SERUM TREATMENT INITIATED 7 OR 10 DAYS AFTER LAS VIRUS INFECTION OF CYNOMOLGUS MONKEYS

DAY AFTER INFECTION	LOG_{10} PFU/ml SERUM ± SE			
	30 mg/kg/day	90 mg/kg/day	Serum Only day 7,10,13	Untreated Control
Ribavirin, day 7				
3-4	2.4 ± 0.31	2.1 ± 0.38	3.2 ± 0.93	
6-7	4.2 ± 0.14	3.1 ± 0.58	4.4 ± 0.13	
9-10	4.1 ± 0.19	2.9 ± 0.53	4.7 ± 0.17	
13-14	4.0 ± 0.23	2.9 ± 0.50	4.9 ± 0.42	
16-17	3.4 ± 0.31	3.6 ± 0.61	2.2	
19-20	3.2 ± 0.50	3.3 ± 0.67	2.4	
23-25	2.3 ± 0.95	3.6 ± 0.28	<0.7	
27-28	<0.7	4.4	<0.7	
DEAD/TOTAL	4/8	6/6	5/6	
Ribavirin & serum, day 10				
3-4	3.1 ± 0.32			2.5 ± 0.51
6-7	4.2 ± 0.24			4.0 ± 0.30
9-10	5.7 ± 0.07			5.5 ± 0.12
13-14	4.3 ± 0.16			5.5 ± 0.17
16-17	3.3 ± 0.14			4.1 ± 0.21
19-20	0.8 ± 0.21			3.0 ± 0.36
23-25	1.2 ± 0.37			All dead
27-28	<0.7			
Dead/Total	0/6			12/12

However, as reported last year, the combination of ribavirin + immune serum initiated day 7 protected all monkeys. We have extended the treatment delay until day 10. All 6 monkeys were protected, although the mechanism by which the antiserum + drug acted additively or synergistically has not yet been determined. It is possible that the effect was simply additive, and that combinations of 2 marginally effective regimens resulted in adequate treatment. To probe this question more deeply, we attempted treatment of monkeys with a

TABLE VII INFECTIVITY AND LETHALITY OF LAS VIRUS FOR GUINEA PIGS INOCULATED SC.

PFU/0.2 ML INOCULATED SC	STRAIN 13 NO./TOTAL			OUTBRED NO./TOTAL		
	DEATHS/TOTAL	SEROCONVERTED	RESISTED CHALLENGE	DEATHS/ TOTAL	SEROCONVERTED	RESISTED CHALLENGE
240,000	5/5	-	-	2/10	8/8	8/8
2,400	5/5	-	-	4/10	6/6	6/6
24	5/5	-	-	6/19	13/13	13/13
2	5/5	-	-	3/10	7/7	7/7
0.2	2/5	0/3	0/3	3/10	2/7	4/7
0.02	1/5	0.4	0/4	NT		

LAS virus also kills strain 13 guinea pigs, Table VII. Similar to the observation for adapted PIC, the infectious dose of Lassa approximates the lethal dose of 1 PFU, since no strain 13 guinea pig that survived in the titration seroconverted. In contrast, outbred guinea pigs were relatively resistant. They were readily infected, since all guinea pigs inoculated with 2 PFU or more seroconverted, but lethality appeared to be dose-independent, since a small fraction of outbred guinea pigs died at each dosage level tested. The uniform susceptibility of strain 13 guinea pigs to Lassa infection makes them preferable to outbred animals for detailed pathogenesis and protective efficacy studies.

Viremia titers based on 8 guinea pigs were remarkably uniform (Table VIII). All guinea pigs died 14-20 days after infection, with viremias approximately 4 Log_{10} PFU/ml. SGOT were elevated by day 7 and continued to increase during the course of the infection. A serologic response, measured by IFAT was detectable by day 7, and continued to increase until death. Again, the possibility of circulating virus/antibody complexes was suggested.

TABLE VIII VIREMIA, SGOT AND IFAT RESPONSES IN SERA OF STRAIN 13 GUINEA PIGS FOLLOWING LAS INOCULATION

DAYS AFTER INOCULATION	VIREMIA Log_{10} PFU/ml $\pm$ SE	SGOT IU/L $\pm$ SE	IFAT TITER
0	<0.7	26 $\pm$ 3	<10
4	2.2 $\pm$ 0.26	25 $\pm$ 7	<10
7	3.8 $\pm$ 0.14	96 $\pm$ 29	8
10	4.4 $\pm$ 0.08	NT	51
13	3.6 $\pm$ 0.20	196 $\pm$ 51	304
17	4.0 $\pm$ 0.33	256 $\pm$ 58	580

A sequential sacrifice study of LAS infected guinea pigs revealed the source of blood-borne virus to be most visceral tissues. Again, CNS tissues (brain) appeared to be largely excluded (Table IX). Immunofluorescent examination of these tissues provided similar information. Surprisingly, however, the extent of histologic distribution seen in H&E sections was minimal; the immediate cause of death is unexplained. However, in most respects, the LAS guinea pig model approximates the primate models and should prove to be useful for sequential sacrifice studies to probe the modes of action of antiviral drugs and immunotherapy regimens, and to test efficacies of candidate vaccines.

TABLE IX LASSA VIRUS CONCENTRATIONS IN TISSUES OF INBRED (STRAIN 13) GUINEA PIGS

TISSUE	LOG_{10} PFU/ml OR g ± SE BY DAY AFTER INOCULATION				
	4	7	10	13	17
Plasma	2.2 ± 0.26	3.8 ± 0.14	4.4 ± 0.08	3.6 ± 0.20	4.0 ± 0.33
Liver	3.7 ± 0.18	4.6 ± 0.21	5.1 ± 0.06	5.6 ± 0.30	5.6 ± 0.21
Spleen	5.7 ± 0.70	7.8 ± 0.11	7.3 ± 0.95	6.3 ± 0.50	6.8 ± 0.06
Pancreas	6.2 ± 0.20	5.9 ± 0.32	6.3 ± 0.20	6.3 ± 0.10	6.5 ± 0.16
Kidney	1.7 ± 0.66	4.5 ± 0.31	5.9 ± 0.06	6.1 ± 0.12	5.8 ± 0.09
Adrenal	4.1 ± 0.46	4.3 ± 0.16	5.3 ± 0.13	5.6 ± 0.20	5.6 ± 0.16
Lung	4.4 ± 0.85	6.1 ± 0.28	5.9 ± 0.16	6.9 ± 0.26	6.9 ± 0.27
Heart	2.5 ± 0.73	4.4 ± 0.23	5.7 ± 0.10	5.3 ± 0.10	3.3 ± 1.22
Lymph node	6.9 ± 0.33	7.7 ± 0.17	7.0 ± 0.13	6.5 ± 0.10	6.0 ± 0.15
Salivary gland	4.1 ± 0.15	6.9 ± 0.15	7.7 ± 0.10	7.4 ± 0.17	6.7 ± 0.23
Brain	<1.7	1.7 ± 0.73	2.4 ± 0.71	2.9 + 0.85	3.6 ± 0.38

Development of Candidate vaccines. We are proceeding to develop both live attenuated and inactivated candidate vaccines. Inactivation kinetics for LAS virus using formaldehyde, cobalt irradiation, and psoralen derivatives were presented in preliminary form last year; the data have been confirmed repeatedly; we are confident that we can totally inactivate the infectivity. Unfortunately, no inactivated LAS vaccine produced to date has elicited a serologic response or protected strain 13 guinea pigs. The problem may be one of insufficient antigenic mass. We will attempt to concentrate LAS virus by ultracentrifugation and/or ultrafiltration to determine if the concept of an inactivated arenavirus vaccine is valid.

A candidate live attenuated LAS virus strain isolated from a Rhodesian mastomys presently holds more promise. In preliminary studies, this strain has inapparently infected and immunized 20 strain 13 guinea pigs and 4 rhesus monkeys. Viremias were not detectable; IFAT responses were observed 14-21 days after Rhodesian LAS inoculation. No perturbations in SGOT, hemoglobin, WBC, or differential leukocyte counts were observed. The 4 rhesus monkeys resisted SC challenge with 1.2 Log_{10} PFU virulent LAS; challenged monkeys failed to develop viremia, and blood parameters did not change. Similarly 10 guinea pigs challenged SC with virulent LAS all survived inapparent infection. The remaining 10 guinea pigs were challenged by aerosol and similarly resisted challenge, although viremias and blood parameters were not measured.

The safety and efficacy of this or similar naturally attenuated Lassa virus strains will be the focus of our vaccine development efforts for the coming year.

Presentations:

1. Jahrling, P. B. Lassa virus: development of animal models. Presented, Am. Soc. Trop. Med. Hyg Meg. 7-10 Nov 79, Tucson, AZ (invited presentation).

2. Jahrling, P. B., and R. A. Hesse Pathogenesis and treatment of Lassa and Pichinde virus infections in guinea pigs. Presented, Annu. Mtg. ASM, Miami, FL, 1-16 May 80 (abstracts, T134, p. 257).

Publications:

1. Jahrling, P. B., R. A. Hesse, G. A. Eddy, K. M. Johnson, R. T. Callis, and E. L. Stephen, 1980. Lassa virus infection in rehesus monkeys: pathogenesis and treatment with ribavirin. J. Infect Dis. 141:580-589.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

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23 (U) Define regulatory mechanisms of lymphocyte recirculation and cellular interaction in tissues during the in vivo immune response; use this information to develop new and more potent adjuvants for use with military vaccines; materials developed in this manner should significantly hasten the interval between vaccination and protection

24 (U) Use morphological and radiolabeled indicators of lymphocyte kinetics to study influence of various factors affecting lymphocyte traffic into lymph nodes during induction of immune responses; assess effector immunity using serological and cellular systems.

25 (U) 79 10 - 80 09 - Lymphocytes exhibit characteristic motile behavior in regard to their morphology, shape, and migration speed. The capping phenomenon is a unique property, where the evidence supports that motile function is involved. New methods developed in this laboratory have made the questions of lymphocyte chemotaxis answerable. Peripherally, lymphocyte recirculation is responsible for immune surveillance, whereas locally, nodal reassortment of T and B cells is important. Recirculation is affected by the recirculatory cell's maturity. HEV proliferation, chemotaxis, and macrophage modification of antigen may function in selective recruitment of specifically sensitized lymphocytes; short-lived recirculating lymphocytes may also be recruited. The similarity of the structural elements of chronic inflammatory lesions to those of nonencapsulated lymphatic tissues led to the finding that large-scale lymphoid cell traffic occurs in these lesions en route to regional nodes. The nature of the antigen and possibly its eventual neutralization at a site of chronic inflammation determines whether discomfort, disease or recovery ensues. Terminated due to transfer of the principal investigator. Publications: J. Reticuloendothel. Soc. 26 (Suppl.):667-680, 1979; Fed. Proc. 39:698, 1980; In fundamentals of Clinical Hematology, pp. 155-197, 1980; two chapters of book, in press, 1980.

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BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M162776A841)

Task No. 3M161102BS10-AQ: Enhancement of Host Defense Against Agents of Potential BW Importance

Work Unit No. S10-AQ-173: Cellular Responses in Lymphatic Tissue Following
(841 0C 010) Immunization

Background:

Gowans and his collaborators provided the first convincing evidence that lymphocytes constantly travel from the blood stream into lymphatic tissues and return to the circulation via major efferent lymphatics. The phenomenon of recirculation is now regarded as representative of normal lymphocyte behavior in most mammals, including man.

Lymphocytes can be divided into two classes, T- and B-cells, with distinctly different functions. The T-cell contribution to humoral immunity was revealed by observations that both thymus and bone marrow cells were needed to restore humoral antibody responses in irradiated mice. The demonstration by karyotype analysis that Jerne plaque-forming cells from such mice were of bone marrow and not thymic origin promoted the concept that T-cells played important "helper cell" functions in humoral immunity. It is currently believed that T-cell help is required for most humoral antibody responses, but some repeating-polymer antigens, such as pneumococcal polysaccharide may directly stimulate B-cells. T-cell help is also believed to be important in the triggering and differentiation of cytotoxic T-cells, nonspecific suppressor cells, and antigen-specific suppressor cells.

Since soluble factors released by immunoregulatory cells can mediate amplification or suppression of immunity, it is not clear whether helper or suppressor T-cells need to contact the respective effector cell directly in order to produce their effects. In the case of B-cell responses, antigens are bound to the surfaces of specifically reactive cells via immunoglobulin molecules which are inserted in the membrane; such antigen-binding activity has been difficult to demonstrate for T-cells. Apparently the T-cell antigen receptor is not an immunoglobulin and is either secreted into the surrounding environment or is weakly held by the lymphocyte membrane. Since T-cells depend heavily on Ia-antigen-bearing macrophages for antigen-reactivity, it is possible that macrophages serve antigen-binding role for T-cells by nonspecifically absorbing onto their surfaces the released T-cell receptor-antigen complexes. Nonphagocytic dendritic and Langerhans cells apparently serve this antigen-binding presentation role as well.

Indications of functional heterogeneity among the classes of lymphocytes and evidence that various kinds of cellular interactions were highly restricted contributed to, rather than weakened, the status of lymphocyte recirculation as an immunological phenomenon. Considering the tremendous diversity of antigens in the environment and the "one-cell one-antigenic determinant" tenet of the clonal selection theory of immunity, it is difficult to see how the appropriate cells

would be in the same place at the same time as antigen without a dynamic process-like lymphocyte recirculation. This constant flux and sorting of immunocompetent lymphocytes guarantees that a significant portion of the total uncommitted lymphocyte population will traffic past antigen/macrophage depots in lymphatic tissues.

Progress:

Intrinsic Migratory Behavior of "Recirculating" Lymphocytes. It has been known for nearly 50 years, that lymphocytes are motile cells which exhibit cycles of spontaneous movement interrupted by resting stages of variable length. However, migration is executed by lymphocytes only with great difficulty, because of a relative deficiency of the cytoplasmic machinery for locomotion. The high nuclear:cytoplasmic ratio is probably responsible for the characteristic morphology of locomoting lymphocytes. Resting lymphocytes isolated from the thoracic duct lymph or peripheral blood are spherical cells with uniformly distributed microvilli on their surfaces. Between 15.6 and 30% of these cells spontaneously move during 10 min of observation of coverslip preparations warmed to 37°C. As migration begins, the lymphocyte probes its surroundings by extending and retracting villous projections and lamellipodia. Adhesion of a lamellipodium to the substratum causes the cell to elongate into the characteristic "hand-mirror" shape with anterior nucleus and posterior uropod. The lymphocyte continuously extends lamellipodia anteriorly and appears to drag its uropod like a "ball and chain" as it contracts and relaxes through cycles of translocation. Waves of cytoskeletal contraction constrict the nucleus and squeeze it into the anterior end of the cell as each contraction propagates from front to rear. The constriction zone, which contains a circumferentially thickened subplasmalemmal microfilament network, remains stationary with respect to the substratum as the rest of the cell moves forward. The cell surface overlying the constriction zone is devoid of microvilli when examined by scanning electron microscopy. Both T and B lymphocytes exhibit spontaneous motility, although rates of locomotion vary. Small T lymphocytes from thoracic duct lymph travel at a mean velocity of 11.9 μm/min while B-cells move more slowly (5.3 μm/min), if at all. B-cells appear to require some stimulation before they move. Exposure of B-cells to antiimmunoglobulin induces capping, which is followed by translocation. B-cells also begin to move after they have been "fondled" by a migrating T-cell. Blast cells of either class move faster than small lymphocytes, their average speed being 24 μm/min. While the migration characteristics of lymphocytes do not appear to be as great as those of neutrophils and monocytes, which move at rates between 30 and 70 μm/min, what movement lymphocytes can manage is obviously sufficient to transport these immunocompetent cells across the vascular barrier into the reticular meshworks and lymphatic channels of lymph nodes, Peyer's patches and spleen. Global redistribution of ligands bound to surface receptors ("capping") is another manifestation of lymphocyte motility. Most capping phenomena appear to involve participation of the cytoskeleton, protein synthesis and cellular respiration, since drugs which affect these activities inhibit capping. However, in rare instances, capping may also result from passive diffusion and aggregation of cross-linked receptors within the fluid membrane. The capping of membrane-associated immunoglobulin molecules by multivalent antiimmunoglobulin antibodies apparently is an inductive signal, which precipitates B-cell locomotion, because it frequently is followed by translational movement. Regardless of whether the B-cell caps or locomotes first, the anti-Ig cap is usually found at the end of the uropod of migrating

cells. This is also true of the red cells stuck to locomoting human T-cells which had been incubated with sheep erythrocytes. These phenomena suggest that oriented movement of surface receptors occurs from front to rear during locomotion. This is not necessarily true during all cases of capping, since certain ligands form caps which are not localized over the centriole or Golgi zone of the cell. Since the centriole is always found in the uropod end of locomoting and capping lymphocytes, it is likely that this structure is responsible for the cytoplasmic and cytoskeletal polarization necessary for these motile functions. A primary cilium generated by the centriole appears to serve this orienting function in other types of eukaryotic cells. The organization of the lymphocyte cytoskeleton is still uncertain, but an umbrella-like network of microtubules may be seen radiating from the centriole around the nucleus toward the lamellipodia of lymphocytes which have become polarized prior to locomoting. A network of 5-nm filaments forms a continuous mat beneath the plasmalemma and is arranged within the core of microvilli as longitudinally oriented fibers. Ten-nanometer filaments appear to extend anteriorly beside the microtubules and can be seen forming loops in the lamellipodia of motile cells. The complex interrelationships of these fibers during various biological activities remain to be determined.

The directed migration of leukocytes along a chemical gradient is generally accepted as playing an important in vivo role in regulating cellular traffic and promoting the accumulation of inflammatory cells at sites of tissue injury. Historically, lymphocytes were not regarded as being capable of chemotaxis, because of their erratic behavior in crude coverslip chambers. Recent evidence of others indicates that mitogen-stimulated T lymphoblasts and some lines of transformed lymphocytes display chemotaxis toward casein hydrolysates, denatured albumin, endotoxin-activated serum, submitogenic doses of plant and microbial lectins. In addition, supernatants isolated from mixed lymphocyte cultures, mitogen-stimulated lymphocytes and activated macrophages have been said to evoke chemotaxis by spleen and lymph node cells. Many of these studies were conducted in systems which cannot distinguish between chemotaxis (directed migration of cells which are oriented by a gradient) and chemikinesis (increased random migration which is greatest on the side facing the gradient), and each of these factors are equally chemoattractive to other leukocytes. Therefore, it is possible that true chemotaxis cannot be demonstrated using single-parameter assays. Preliminary observations using a "three-chamber" agarose system suggest that thoracic duct lymphocytes (TDL) are inefficient in their ability to orient in a gradient of endotoxin-activated serum presented to them by diffusion through agarose. However, TDL populations show significant net orientation and directional migration toward such a gradient when compared to random migration controls. Closer examination of the cellular interactions among these slightly heterogeneous populations indicates that T lymphocytes appear to be about as attracted to nearby B lymphocytes and small macrophages as they are to the artificial gradient. This local interference in gradient-sensing by cellular chemotaxins is probably responsible for the highly erratic migration patterns of lymphocytes moving into chemotactic gradients. Since directed lymphocytes frequently reverse or change orientations by 90° increments, while making net movement toward the gradient source, the chemotropism index for chemotaxing lymphocytes is 0.45-0.50, as expected of a moving neutrophil. However, lymphocytes moving in the absence of a gradient, reverse direction so often that they fail to accumulate any net forward movement and produce chemotropism indices between 0.09 and 0.12. Further studies are needed to determine whether chemotaxis plays any role in regulating the characteristic traffic patterns displayed by lymphocytes in vivo. Specialized

microvascular structures at lymphatic tissue/blood interfaces may have developed during evolution to compensate for the relative inability of lymphocytes to display efficient "goal-directed" forward movement. Diffusion of small quantities of factors produced by macrophages, antigens, immunoblasts and concentrations of other lymphocytes, into vascular lumens, may be all that is needed to provoke the emigration of a circulating lymphocyte. Once inside, other factors and interactions with other cells apparently determine its ultimate tissue distribution.

Circulation of Lymphocytes During an Immune Response. Successful initiation of a specific immune response requires that the lymphocytes must engage and bind the appropriate antigen with its surface receptor. Studies in nonimmune animals have shown that only a tiny minority of the lymphocytes present within a single lymph node are capable of reacting with a given antigenic determinant. If these cells were static or their movements were randomly sorted throughout the body, the likelihood of chance collisions between reactive lymphocytes and the appropriate antigen displayed on an accessory cell would be very remote. However, this is clearly not the case in vivo, where immunocompetent T- and B-cells continually recirculate between blood and lymphatic tissues. Long-lived lymphocytes flowing in the blood show a unique "homing instinct" for lymph node high endothelial venules (HEV). These cells cross HEV and emigrate into the reticular meshwork, where they crawl along reticular cell surfaces, collide with macrophages and other lymphocytes and either stay to initiate proliferation or leave.

Lymphocytes recirculate through other lymph nodes and mucosal lymphatic tissues via HEV, providing a constant form of surveillance by immunocompetent lymphocytes that move through antigen-binding meshworks. This phenomenon probably enables a small depot of antigen to recruit a large number of antigen-specific cells from the body's lymphocyte pool.

When an individual lymphocyte encounters an appropriate antigenic stimulus within the node, it binds the antigen, interacts with other cell types and is trapped in the node, where it gives rise to a clonal burst of proliferation and differentiation of lymphocytes which mediate immune responses. Many of the immature lymphocyte progeny leave the stimulated node 48-100 h after antigen exposure and disseminate to distant nodes, the spleen and other tissues, where they can mature into specific effector cells. The B-cells activated in this response move into the medullary cords of regional and distant nodes, where they mature into antibody-secreting plasma cells.

Although T and B lymphocytes emigrate from the same segments of HEV, they are sorted by unknown mechanisms within the nodal parenchyma. The T lymphocytes establish residence in the deep cortex for relatively short time intervals before moving out into the efferent sinuses. B-cells emigrate into the superficial cortex and probably remain there for longer periods before exiting via sinusoidal pathways. This migration pattern appears to permit the T- and B-cells to interact with antigen-binding macrophages and engage in cellular collaboration before they redistribute into their respective zones within the nodal cortex.

Other factors also influence lymphocyte traffic patterns in the body. The ability of T-cell subpopulations to recirculate appears to depend upon their state of maturation. B-lymphocytes display similar variations in their emigration patterns. The immature B-cells appearing shortly after antigenic challenge may leave the node, but frequently lodge in the spleen and do not recirculate

through the thoracic duct. However, "memory B-cells" arising at the late stages of an immune response recirculate in a typical manner between blood and lymph. The B-cell precursors of IgA secretion also possess distinct and quite different migration pathways. This circuit includes generation of precursor progeny in Peyer's patch follicles, which complete various maturational steps in the mesenteric node and spleen before finally lodging in the lamina propria of the gut as IgA-secreting plasma cells.

In nonstimulated animals, lymphocyte recirculation is characterized by a balanced flux of cellular traffic across afferent and efferent terminals of lymphatic tissues. This kinetic equilibrium is rapidly distorted in the regional nodes draining sites of inflammation, infection or antigenic challenge. This is accompanied by a rapid increase in lymphocyte accumulation with the lymph node cortex, which is not associated with cellular replication. Such nodal enlargement probably reflects the combined result of increased lymphocyte traffic into the node and decreased egress, or markedly increased entry with normal rates of exiting. These early changes are believed to be produced, in part, by changes in blood flow and by release of secretory factors and lysosomal enzymes from activated macrophages, which alter lymphocyte surface adhesiveness and transit times within the node. As this early sequestration of recirculating lymphocytes within the stimulated node subsides, blast cell transformation and mitotic activity appear in the T- and B-cell zones of the cortex, reflecting the antigen-dependent cellular proliferation.

The specificities of the lymphocytes entering lymphatic tissues in the immediate 6- to 48-h period following stimulation are largely unrelated to the antigen which initiated the response. In fact, similarly nonspecific lymphocytic accumulations can be induced by adjuvants which themselves are poorly immunogenic. However, as the sequestered lymphocytes are released into the efferent lymph, they are depleted of cells specifically reactive to the priming antigen. The retention of antigen-specific lymphocytes in regional lymph nodes has been termed "specific recruitment," and was documented using double-label techniques. Specific memory cells generated *in vivo* in the presence of [^{14}C]thymidine were mixed with equal numbers of [^{3}H]thymidine-labeled control memory cells and subsequently transfused to immunized syngeneic recipients. The relative accumulation of specific vs. nonspecific cells was measured in regional and contralateral nodes by scintillation spectroscopy of extracted DNA. These kinds of studies clearly demonstrated increased traffic of specifically reactive cells in antigen-draining nodes which withstood reciprocal specificity controls. The numbers of labeled immunospecific cells available to lodge selectively were small enough to tax the resolving power of the assays, but studies of efferent lymph indicated that specific cell traffic remained elevated throughout the immune response. Specifically reactive lymphocytes enter the efferent lymph in maximal numbers between 72 and 100 h. Peak accumulations of antigen-reactive lymphocytes correlated chronologically with early formation of reactive centers in the cortex and proliferation of new segments of HEV, suggesting that these vascular structures may have some function in selective recruitment, but lymphocyte chemotactic factors or modified antigens secreted by macrophages may also favor the emigration of specific cells to HEV.

The increased migration of lymphocytes into antigen-stimulated lymphatic tissues is not restricted to long-lived recirculating lymphocytes. Lymphocytes in the cortex of the thymus undergo enhanced proliferation within the first 3 days

following peripheral antigen inoculation. The systemic stimulus which causes thymocyte proliferation is presently unknown, but these newly formed cells leave the thymus and accumulate in the marginal zone of the spleen, Peyer's patches, and some lymph nodes. These cells comprised predominantly of $Ly^{(1+,2+,3+)}$ cells may be precursors of antigen-specific suppressor cells which require T-cell help in order to complete their differentiation. Adult thymectomy, which removes the short-lived, cortical thymocytes but not the long-lived recirculating cells, prevents the formation of Freund's adjuvant-induced T-suppressor cell populations, further supporting the suggestion that the cortical thymocytes which lodge in the spleen are precursors of suppressor cells. As early as 6 days after immunization, the numbers of antigen-specific suppressor cells begin to increase until the immune response is terminated.

These changes in lymphocyte traffic combined with local proliferation of immunoreactive cells are responsible for the biphasic 2- to 6-fold enlargement of regional lymph nodes draining sites of antigen inoculation or infection. In the absence of additional free antigen, the suppressive effects of anti-idiotypic antibodies and/or suppressor cells eventually result in gradual reduction in traffic and proliferation. It may take a month or more for the regional lymph node to return to its normal size and physiologic activity. Following secondary exposure to antigen, the kinetics of lymphocyte traffic and immunocyte proliferation in the regional and distant lymph nodes are greatly accelerated, possibly because of the dissemination of foci of immunological memory to nearly all the lymphatic tissues of the body.

Peripheral Sites of Chronic Inflammation. Antigens presented in a form not easily removed by cells of the mononuclear phagocyte system, e.g., emulsified in a nondegradable oily base, result in the formation of chronic inflammatory foci. These lesions possess follicular and diffuse lymphocytic infiltrates in addition to collections of foam, epithelioid and giant cells and other monocyte components of granulomata. Similar collections of diffuse lymphoid tissue may be seen: in lesions resulting from infections by facultative intracellular organisms of viral, bacterial and parasitic origin; in tissues exposed chronically to microbes present in the fluids which bathe them, such as the bladder or renal pelves of patients with chronic bacilluria due to pyelonephritis; in organs which are undergoing chronic rejection following transplantation; and in organs attacked by autoallergic phenomena, such as Hashimoto's thyroiditis and allergic orchitis and encephalomyelitis.

All these chronic inflammatory lesions contain structural elements which are similar to those of nonencapsulated lymphatic tissue. The inflammatory infiltrate which succeeds neutrophils in these sites is composed predominantly of monocytes, macrophages and lymphoid cells. While monocytes and lymphocytes usually arrive at the site simultaneously, large-scale lymphoid cell emigration occurs only after additional microenvironmental structures are generated. Chronic inflammation typically develops in connective tissue sites which are well supplied by lymphatic channels. The inductive stimulus, which is possibly provided by a number of factors released by monocytes and lymphoblastic cells, results in proliferation of fibroblasts and lymphatic and blood vascular endothelium. Specialized vascular segments develop by mitotic division, which morphologically, histochemically and functionally resemble HEV of lymph nodes. Such new vessels lined by plump, esterase-positive endothelial cells arise in chronic inflammatory foci prior to the appearance of dense aggregates of small lymphocytes. Circulating lymphocytes home to these vessels, emigrate from the

blood and accumulate temporarily in the lesion before passing into afferent lymphatics and regional lymph nodes. Lymphoblastic cells also enter the lesion at such sites and differentiate into plasma cells or participate in the formation of germinal follicles. The antibody specificities of these cells are frequently unrelated to the antigens present at the site.

This large-scale lymphoid cell traffic through sites of chronic inflammation has been documented in studies where the outputs of cannulated afferent lymphatics draining adjuvant-induced granulomata were measured or where the distributions of transfused [^{3}H]uridine-labeled lymphocytes were traced in tissues by autoradiography. In the former studies, the afferent lymph output in sheep inoculated with Freund's adjuvant reached 3.1 x 10^7 lymphocytes/h, which is nearly equal to the output of a normal lymph node, and is at least 10 X that of normal afferent lymph. Increased lymphocyte traffic via the granuloma site persisted through the 70 days of the study and probably would have continued as long as the lesion was present. The lymphocytes present in the afferent lymph were identified as predominantly B-cells. This augmentation of B-cell traffic to the regional lymph node via the granuloma site correlated with the pronounced increase in antibody production and with the altered ratios of T to B lymphocytes found in these nodes. The enriched B-cell populations present in the afferent lymph may have been recruited from the circulation, but their formation in germinal follicles is also possible.

Macrophages in the lesion, which are unable to completely remove the antigen-containing material, become activated and secrete mediators which affect the local microenvironment and that of the regional lymph nodes by stimulating lymphocyte division, chemotaxis of monocytes and lymphocytes, vascular permeability, blood flow and cell proliferation. These peripheral sites of chronic inflammation resolve when all the antigen is neutralized and eliminated by phagocytes; they may, therefore, serve a beneficial purpose by establishing new reticular microenvironments which serve the same antigen- and cell-sorting functions as peripheral lymphatic tissues. However, these foci also cause discomfort and disease when the antigen is (a) a normal constituent of the host, (b) not degradable or neutralizable, or (c) capable of changing its antigenic determinants in response to immunological pressure. In addition, hydrolytic enzymes which may be released in the course of phagocytosis, may damage other neighboring structures, such as the articular surfaces of joints in rheumatoid arthritis and the arthritis associated with autoallergic diseases.

Plasmacytomagenesis. Studies of the pathogenesis of peritoneal plasmacytomas in Balb/c mice developed as a result of interest in possible deleterious effects of nondegradable oil vehicles for adjuvants. While at the international symposium on immunological adjuvants, Dr. Michael Potter of NCI and I decided that we would investigate the pathogenesis of the lesions which produced plasmacytomas productive of monoclonal antibody in mice. These lesions are produced by a single IP inoculation of 0.5 ml of mineral oil and routinely develop in 50-60 days. In pilot studies we determined that these lesions were present as early as 18 days postinoculation and had progressed to neoplastic plasmacytomas by 126 days.

Morphologically, peritoneal plasmacytomas developed multifocally as polypoid masses of connective tissue, lipid laden macrophages and plasma cells which protruded from the mesentery and were concentrated along neurovascular and lymphatic bundles which supply Peyer's patches. In a time-course study it was determined that the development of these lesions was signaled by sprouting of specialized

vascular structures from mesenteric vessels on or about days 3 or 4 postinoculation. By day 8, clusters of fully formed vascular polyps were seen, which increased in number at later time intervals.

The fact that these lesions developed multifocally and discretely, but eventually produced monoclonal antibody, is perplexing. One of the questions to be answered is whether these discrete lesions are in fact polyclonal but yield to some dominant controlling influence or are for some reason coordinated in their development by lymphocyte recirculation patterns and antigen exposure by Peyer's patches. Also, it seems that macrophages are the only other leukocyte associated with these plasmacytes. Do the macrophages produce an excess of B-cell stimulating factor which ultimately results in neoplastic differentiation of what would normally result in end stage plasma cells?

Developing Lymphatic Tissues. The peripheral lymphatic tissues of the rat complete their structural development and are populated by lymphocytes during the first few days of extrauterine life. Since certain microenvironments in these peripheral tissues apparently depend upon the cellular or hormonal contributions of the thymus, it is possible to impede the development of these regions in newborn rats and mice by removing the thymus immediately after birth. However, other structures in lymphatic tissues develop normally in the absence of a thymus, but thymus engraftment or infusion of thymic lymphocytes restores the total tissue, thereby indicating that local mesenchymal structures are also vitally important in the formation of lymphatic tissue microenvironments.

The earliest lymph node to develop in the newborn rat is the mesenteric. Immediately after birth this node is a lymphatic sac lying near the neurovascular bundle in the mesentery. Reticular cells are present, but the sac is not yet partitioned into lymphatic sinusoidal spaces and reticulum. Development of lymphatic channels occurs first; by the second day a subcapsular sinus and some medullary sinuses appear. Lymphocytes are present in the peripheral blood, but none have entered the lymph nodes. Between days 2 and 3, the small venous vessels in the developing lymph nodes undergo proliferation and segments of vessel appear, which have plump endothelial cells capable of reacting positively in nonspecific esterase histochemical preparations. Concomitant with the development of these new vessels, small lymphocytes begin to populate the mesenteric lymph node. Diffuse lymphoid tissue lacking germinal follicles and plasma cell cords is present by day 7; by 14 days the lymph node appears almost fully formed. A network of nonbranching HEV can be seen deployed vertically between the subcapscular sinus and the hilum in lymph nodes perfused intraarterially with alcian blue dye. By 12 weeks of age, most of the lymph nodes in the rat contain diffuse collections of lymphocytes, cords of plasma cells and germinal follicles.

The architecture of the rat spleen is apparently more developed than the lymph node at birth. The red pulp reticulum is a functional RE filter and also maintains some extramedullary hematopoiesis. The white pulp is fairly well developed. The periarteriolar lymphatic sheath (PALS) is delimited from the red pulp by a marginal zone rich in esterase-positive monocytic cells. There are clusters of small lymphocytes in the periarteriolar lymphatic sheath; some cells can be seen lining up in what appear to be networks of lymphatic capillaries which bridge PALS regions in a "chicken wire" network. Retrograde infusions of India ink from the thoracic duct to the spleen parenchyma reveal these lymphatics. The newborn rat spleen appears to be completely organized,

with respect to the partitioning of lymphoid and red pulp tissues, by the end of the first week of extrauterine life.

Peyer's patches do not appear to be morphologically recognizable in the intestines of late fetal and newborn rats. However, it has shown that an environment exists which subsequently becomes populated with lymphoid cells when these loops of intestine are grafted beneath the renal capsules of mature syngeneic rats. In addition, our unpublished studies indicate that an epithelial and connective tissue site which is predestined to become a Peyer's patch exists in these immature rats. By retrograde infusion of India ink into the mesenteric lymphatics, non-staining patches on the antimesenteric border of the intestine can be discerned because the ink blackens the subserosal lymphatic network up to, but not through, these sites. Plastic, 1-nm sections and ultrastructural examination of these patches reveals that they lack germinal follicles and small lymphocytes, but contain reticular cells, lymphatic channels and an overlying epithelium, which exhibits extensive endocytic activity. Again, small lymphocytes do not begin to populate Peyer's patches until extrauterine day 2, when esterase-positive HEV develop. Germinal follicles begin to appear in Peyer's patches by days 14-28, which is paralleled by the population of the interfollicular areas with recirculating T-cells.

The Work Unit was terminated due to transfer of the Principal Investigator.

Presentations:

1. Anderson, A.O. Lymphocytes and their functions: recirculation, homing clonal expansion, role in rejection of transplants and tumors. Presented, Lecture No. 3, Johns Hopkins Immunology Council course, "Injury, Inflammat'on and Repair," 5 Mar 1980.

2. Anderson, A.O. Role of chemotaxis in lymphocyte homing and recirculation. Presented, University of Maryland Dental School, Baltimore, MD, 18 Mar 1980.

3. Anderson, A.O., and J.T. Warren. Lymphocyte chemotaxis under agarose, cell interaction. Presented, FASEB, Anaheim, CA, 11-18 Apr 1980 (Fed. Proc. 39: 698, 1980).

4. Anderson, A.O. Structure and physiology of lymphoid tissues. Presented, Lecture No. 15 in the NIH Immunology-506 course, The Cell Biology of Immunity and Inflammation, Bethesda, MD, 22 Apr 1980.

5. Anderson, A.O. Lymphocyte chemotaxis in vivo and in vitro. Presented, Seminar of the Division of Biological Structure NIDR/NIH, Bethesda, MD, 23 Apr 1980.

6. Warren, J., and A.O. Anderson. Lymphocyte chemotaxis, cell orientation. Presented, Joint Immunology meeting between Johns Hopkins and University of Virginia, Hilltop House, Harpers Ferry, WV, 25 Apr 1980.

7. Anderson, A.O. Presence of inducible dendritic cells in thoracid duct lymph; their possible role in metastosizable immune responses. Presented, Joint Immunobiology meeting, between Johns Hopkins and University of Virginia, Hilltop House, Harpers Ferry, WV, 25 Apr 1980.

8. Anderson, A.O. Isolation of dendrite cells from thoracic duct lymph of normal and immunized rats. Presented, International Symposium of Alloantigenic Stimulation in the Rat, Philadelphia, PA, 23 Jun 1980.

9. Anderson, A.O. Cytoskeletal control of lymphoid cell traffic. Presented, 4th International Congress of Immunology, Paris, France, 22 Jul 1980.

10. Anderson, A.O. Effects of CP-20,961 on lymphoid cell traffic and viral immunity. Presented, 4th International Congress of Immunology, Paris, France, 24 July 1980.

Publications:

1. Anderson, A.O., and J.A. Reynolds. 1979. Adjuvant effects of the lipid amine CP-20,961 on lymphoid cell traffic and antiviral immunity. J. Reticuloendothel. Soc. 26(Suppl.):667-680.

2. Anderson, N.D., and A.O. Anderson. 1980. Lymphocytes, pp. 155-197. In Fundamentals of Clinical Hematology (J. Spivak, ed.). Harper & Row, Hagerstown, MD.

3. Anderson, A.O., and N.D. Anderson. 1980. Structure and physiology of lymphatic tissues. In The Cell Biology of Immunity and Inflammation (J.J. Oppenheim, D.A. Rosenstreich, and M. Potter, eds.). Elsevier/North Holland, Amsterdam (in press).

4. Anderson, A.O., N.D. Anderson, and J.D. White. 1980. Lymphocyte locomotion, lymphatic tissues and lymphocyte circulation in the rat. In Animal Models of Immunological Processes (J.B. Hay, ed.). Academic Press, New York (in press).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6420	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. SMSN ENTITY	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	011
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE: (U) Development of arbovirus vaccines for diseases of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
64 06	80 09	DA		C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 80	2.0	440
CURRENT FISCAL YEAR 81	0	0

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS: (U) Military medicine; (U) BW defense; (U) Arboviruses; (U) Vaccine development; (U) Dengue virus; (U) Chikungunya virus

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

23 (U) Develop and produce inactivated and attenuated arbovirus vaccines which may then be combined or administered sequentially to military forces for prophylaxis in geographically or BW oriented ways.

24 (U) Arboviruses are propagated in primary or certified diploid cell cultures and inactivated with formalin or selected for attenuation. Products are tested for safety and potency in animals. Efficacy is determined by subsequent challenge, or by determination of serological conversion.

25 (U) 79 10 - 80 09 - A dengue-1 (DEN-1) vaccine candidate, TP8-PL-2, which had been shown to be temperature sensitive (TS), small plaque (sp) and genetically stable in cell culture and after growth in mouse brain, failed to pass the monkey viremia test. Additional clones picked from this candidate, including 2 which grow well at 35 C and are sp and ts. A pilot test in rhesus monkeys on one of these vaccine candidates, TP 79-56, showed that it produced only low level viremia, was immunogenic, and was stable with regard to ts and plaque size during monkey passage. After additional tests in vitro, extensive tests in monkeys (including neurovirulence tests) will be made with the final DEN-1 vaccine candidate selected. Of the 4 candidate clones evaluated for possible use as a live attenuated Chikungunya virus vaccine, clone 181 was chosen for characterization. Results of exhaustive testing demonstrated that clone 181 was sp, ts, genetically stable, avirulent for suckling mice and monkeys, and induced antibody in both adult mice and monkeys. Both master and production seeds have been produced in certified DBS-103 (FRhL-2) cells; the production of the live attenuated Chikungunya vaccine and the planning of its subsequent safety testing are in progress.

Terminated for management efficiency. Continued in W.U. 871 BC 148.(DAOG1537)

*(Available to contractors upon originator's approval)

DD FORM 1 MAR 80 1493 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

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BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BE: Prevention of Viral Diseases of Potential BW Importance

Work Unit No. 871 BE 135: Development of Arbovirus Vaccines for Diseases of
(841 00 011) Military Importance

Background:

The need for attenuated Dengue (DEN) virus vaccines is self-evident in view of the health problems posed by these agents in many tropical and semitropical areas. As part of a joint effort coordinated and supported by USAMRDC, attenuated vaccines for all 4 DEN serotypes are being developed at WRAIR (DEN-2 and -3), at the University of Hawaii School of Medicine (DEN-4) and at USAMRIID (DEN-1). Three human isolates have been adapted in this Institute to acceptable vaccine substrate (DBS-103 cells) and subjected to plaque-to-plaque passage and terminal dilution techniques to obtain and purify avirulent, genetically stable vaccine candidate clones. The most promising clones are being examined for markers of presumed avirulence for man, including small plaque (sp) size, temperature sensitivity (ts) and inability to induce viremia in rhesus monkeys.

In a concurrent program, studies are in progress on the development of an attenuated Chikungunya (CHIK) vaccine. An earlier attempt to produce a killed product at WRAIR resulted in a moderately successful vaccine, supplies of which have since been depleted. In view of the severity of this disease and its active presence in both Africa and Asia, the need for an attenuated vaccine that may be expected to provide excellent protection after a single dose is obvious. Such a vaccine may be used to protect US military personnel and laboratory workers at risk to infection. CHIK virus strain 15561 has been subjected to massive plaque-to-plaque selection/purification schemes in MRC-5 culture, an acceptable vaccine substrate. Additional passes in DBS-103 (FRhL-2) culture have led to clones that are sp, ts and show markedly reduced viurlence for both suckling mice and monkeys.

Progress:

Part I. Attenuated DEN-1 Vaccine.

As indicated last year (1), 2 tests were in progress on the extremely promising TP8-PL-2 vaccine candidate. A comparative test in suckling mice inoculated IC with either "parent" (DEN-1 #1, 7th passage in DBS-103) or TP8-PL-2 showed virtually no difference in terms of virus growth by day 9. When titrated at 35 C on LLC-MK_2 plaque titers of brains pooled according to virus input showed a direct relationship between virus input and ultimate virus titers in the brain pools. As expected, only the parent virus produced progeny in the SMB that would plaque at 39.3 C. The value of this test as a "marker" is questionable.

A preliminary monkey viremia test was also completed. Two groups of 3 rhesus monkeys each were inoculated SC with 10^5 or 10^6 PFU of parent DEN-1 or TP8-PL-2, respectively on day 0. Serum specimens were obtained on days 1-14 for viremia determination by direct plaquing (1). Serum was also obtained on days 30 and 60 for determination of $PRSN_{80}$ titers. The mixed plaque sizes as well as the high viremia level seen in 1 of 3 monkeys (Table I) suggest that the TP8-PL-2 vaccine candidate either reverted during a single passage in rhesus monkeys or that perhaps some small, previously undetected subpopulation had been amplified as a result of the monkey passage. It should be noted that parent virus has in the past (1) produced results in monkeys similar to those seen in Table I. Plaque sizes were mixed from small (1-2 mm) to large ($\geq$3 mm) on LLC-MK_2 with parent virus.

TABLE I. VIREMIA RESPONSES OF RHESUS MONKEYS TO SC INOCULATION WITH DEN-1 PARENT OR TP8-PL-2 VACCINE CANDIDATE

VIRUS RECEIVED[a] (MONK NO.)	PFU/ml BY DAY								
	1-2	3	4	5	6	7	8	9	10-14
Parent									
4880[b]	0	0	10	46	310	1800	560	3.3	0
T-361	0	0	3.3	40	140	113	13	3.3	0
T-317	0	10	6.6	80	156	36	10	0	0
TP8-PL-2									
T-318	0	0	0	0	6.6 S	3.3 S	0	0	0
P-765	0	0	0	43 M	3.3 L	33 M	0	0	0
T-305	0	130 M	220 M	646 M	210 M	0	0	0	0

[a]Parent: 1.8×10^5 PFU/0.5 ml, SC (DEN-1 #1, P-7 in DBS-103); TP8-PL-2: 1.6×10^6 PFU/0.5 ml, SC

[b]Whole body rash on days 7-10; not due to measles.

[c]Subscripts: M, mixed plaque sizes; S, sp, 1-2 mm; L, large plaques, ≧3 mm.

With regard to serology on day 30 and 60, 5/6 monkeys exhibited PRN_{80} titers of 1:20 to 1:80 regardless of virus received. Monkey P-765 had no PRN_{80} titer and only a 1:20 titer in the PRN_{50} test. This was 1/2 monkeys that had a rash of unknown etiology on days 7-10.

Several of the plaques seen with monkeys that had received TP8-PL-2 were picked and amplified by 7-day outgrowth on DBS-103 culture. These outgrowths were then plaqued at 35 and 39.3 C for ts and plaque size determinations using parent and TP8-PL-2 virus preparations as controls. Typical results are shown in Table II. Of the TP8-PL-2 isolates that had been grown out in DBS-103, 5/6 exhibited characteristics that resembled parent virus, viz, mixed plaque sizes

and a loss in ts. This therefore, is the basis of our belief that reversion or amplification of a "hot" subpopulation took place during the rhesus monkey passage.

TABLE II. CHARACTERISTICS OF MONKEY VIREMIA ISOLATES AFTER AMPLIFICATION PASSAGE IN DBS-103 CULTURE

PLAQUE FROM MONKEY NO.	ORIGINAL VIRUS INOCULA	PFU ON LLC-MK_2[a] 35 C (size)	39.3 C
4880	Parent	9×10^5 (mixed)	$\geq 10^5$
T-318	TP-8-PL-2	$\geq 10^4$ (mixed)	Neg, Undil.
P-765	TP-8-PL-2	1×10^6 (mixed)	$\geq 10^5$
T-305	TP-8-PL-2	6×10^5 (mixed)	2.0×10^4
	Parent virus control	2×10^5 (mixed)	3.6×10^4
	TP8-PL-2 virus control	7×10^4 (1-2 mm)	Neg at 1:10

[a]Assay of 7-day outgrowth on DBS-103.

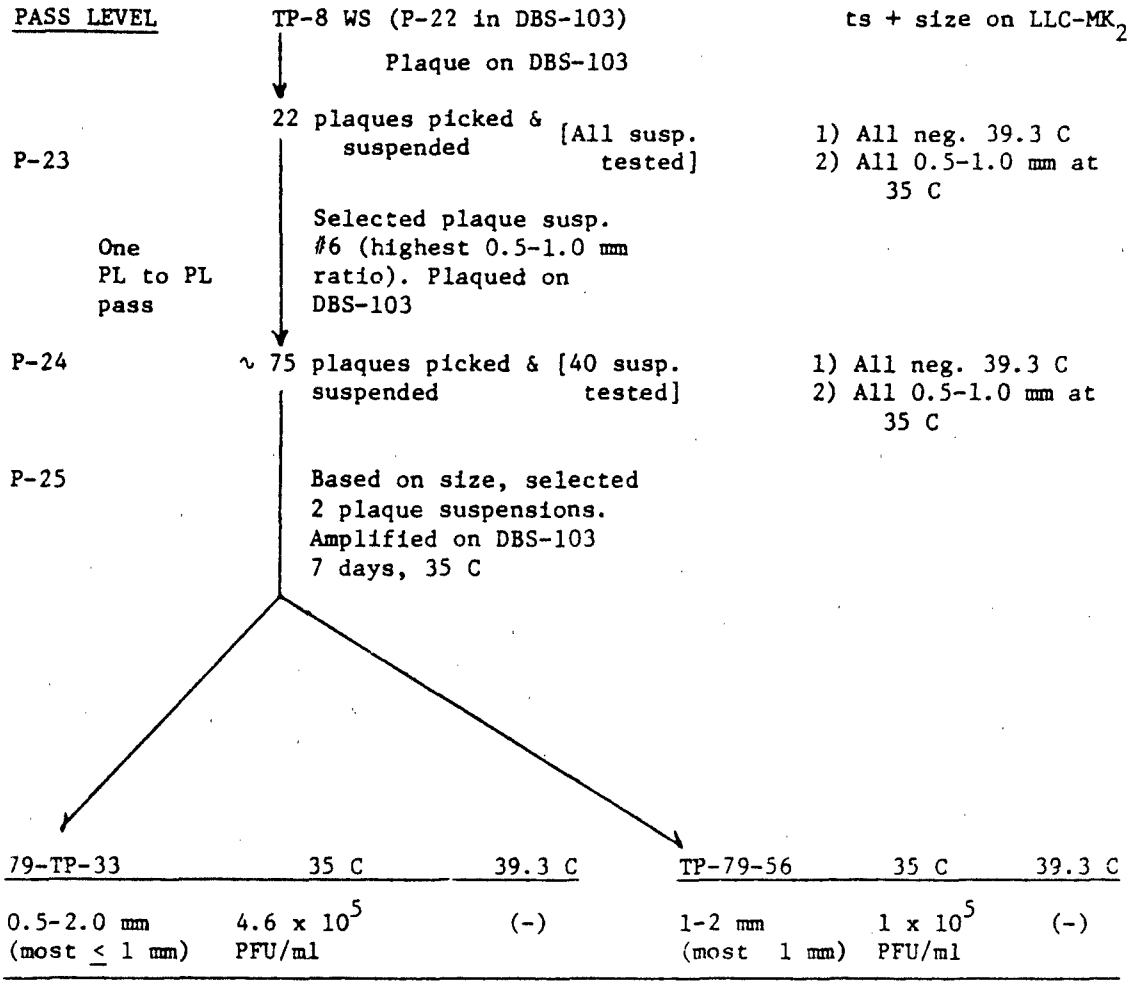
However, since the TP8-PL-2 candidate clone passed all tests except the monkey viremia test, studies were immediately initiated to obtain pure clones of the minute ($\leq$ 0.5 mm) plaque formers generally seen in this preparation. Fig. 1 depicts the 2 plaquing steps (1 PL to PL) used to obtain the 2 clones with which we are now working, TP-79-33 and TP-79-56. As shown in Fig. 1, the basis of our selection of clones at each step was (a) ts on direct plaquing at 39.3 C on LLC-MK_2 and (b) plaque size on LLC-MK_2 at 35 C not to exceed 1 mm. In all cases the majority of the populations exhibited plaque sizes of $\leq$ 1.0 mm.

As shown in Fig. 2, TP-79-33 was further cloned by 2 terminal dilution (TD) passages in DBS-103 culture, subsequently, using the 2nd TD pass harvest (flask #31), a passage was made at a low MOI. For this passage replicate cultures were incubated at 25 C and 35 C in an attempt to put additional environmental pressure on the clone, i.e., incubation at 25 C. The cultures incubated at 25 C exhibited good yields at day 8. Moreover, the predominant population in this 25 C series was minute (0.5 mm) in plaque size. All populations in the 25 and 35 C series were ts at 39.3 C. Additional TD passages at 25 C are in progress to try to obtain pure minute ts clones.

The 2nd clone selected for further purification was TP-79-56. This clone was handled in a manner identical to TP-79-33, as illustrated in Fig. 3. As with the latter clone, additional 25 C TD passes are being made in an attempt to further purify the minute, ts plaque population.

While the 25 C TD passes are in progress, other studies are being conducted with TP-79-33 and TP-79-56. Table III shows the results of direct plaquing on LLC-MK_2 of all four day 8 pools (Fig. 2 and 3). Four temperatures were used to compare these preliminary candidates with the parent. As indicated, parent virus plaqued well at 39.3 C; whereas the candidates failed to plaque. Of considerable interest is the fact that both 25 C and 35 C pools of TP-79-56 failed to plaque even at 38.5 C, in contrast to parent and TP-79-33. On the basis of these results, TP-79-56 appeared to be the more likely candidate.

FIG 1. FURTHER PURIFICATION OF DEN-1 #1 TP8-PL-2 WORKING SEED.



Controls: TP-8WS: 5×10^4 PFU; 11%, 2-3 mm, 55%, 1-2 mm, 35%, 0.5 mm at 35 C.

DEN-1 parent: 3×10^5 PFU; 26%, 3-4 mm, 57%, 1-2 mm, 17%, 0.5 mm at 35 C.

FIGURE 2. PURIFICATION OF DEN-1 CLONE TP-79-33.

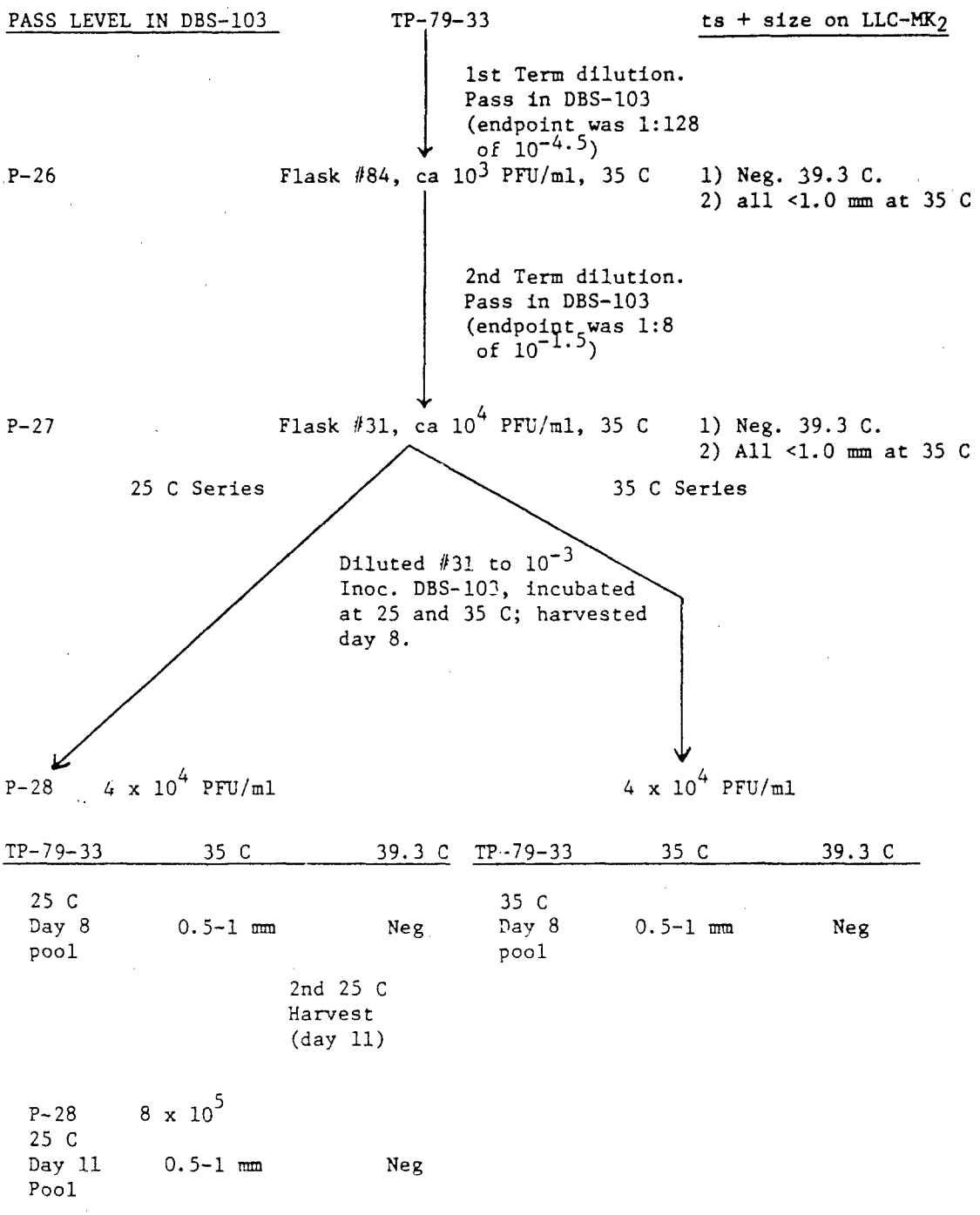


FIG. 3. PURIFICATION OF DEN-1 CLONE TP-79-56.

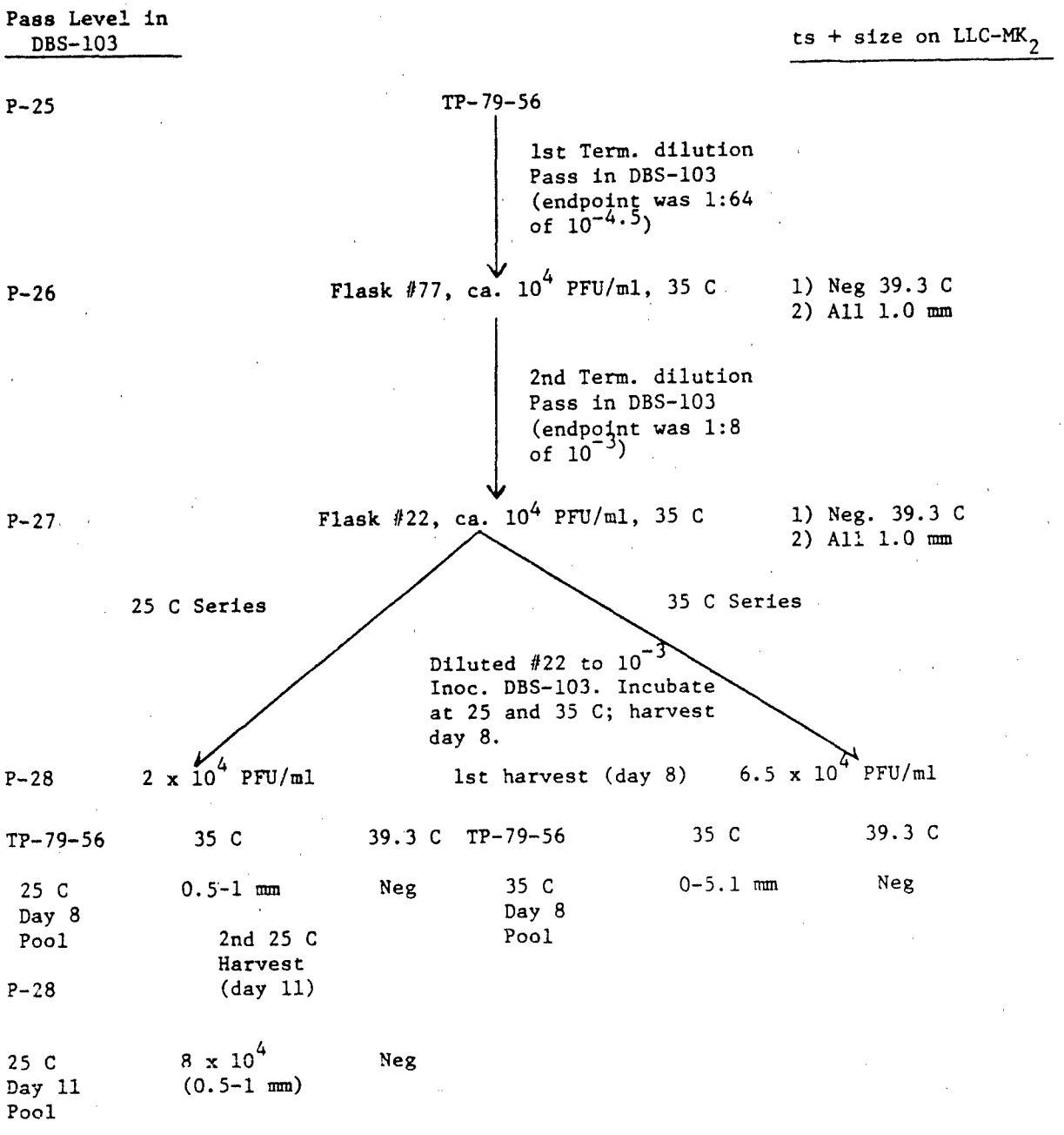


TABLE III. DIRECT PLAQUING OF DEN-1 CANDIDATES ON LLC-MK_2 AT 4 TEMPERATURES.

VIRUS	PFU/ml PLAQUED AT: 35 C	38.5 C	39.3 C	40 C
DEN-1 #1 Parent	2.3×10^5	2.9×10^4	8.0×10^3	-[a]
TP-79-33				
25 C Pool	4.0×10^4	7.8×10^3	-	-
35 C Pool	2.8×10^4	6.8×10^3	-	-
TP-79-56				
25 C Pool	2.2×10^4	-	-	-
35 C Pool	4.2×10^4	-	-	-

[a]No plaques observed.

To further evaluate the 4 candidates, abbreviated growth cruves were determined at various temperatures in LLC-MK_2 cultures which were inoculated at low virus input (Table IV) and maintained with liquid medium. Parent virus was included for comparison. Samples were taken on days 5, 7, and 10 and assayed at 35 C on LLC-MK_2 cultures by the plaque technique. Parent virus peaked early at all temperatures, producing plaquable virus even at 40 C. In contrast, neither 25- nor 35-C pools of TP-79-33 or TP-79-56 peaked early, except at lower temperatures. Even in these conditions, titers were generally maintained through day 7 or 10. As in the direct plaque ts study, TP-79-56 appeared to be the better prospect since it produced no plaquable virus even by day 10 at 39.3 C or 40 C. As indicated the TP-79-33, 35 C pool produced virus at 39.3 C through day 10, whereas the TO-79-33, 35 C pool did not.

Not shown in Table IV are the plaque sizes observed from titration of the growth curve samples. The TP-79-56, 35 C pool, was more uniform than the others in terms of plaque size, with no plaques larger than 1 mm emerging from any cultures incubated at 25 C or 35 C. Moreover, TP-79-56, 35 C pool was also stable when incubated at 25 C; however, a few scattered 2-mm plaques were seen on days 7 and 10, when cultures inoculated with this pool were incubated at 34 C. Thus TP-79-56, 25 C pool might be the better candidate. It should also be noted that no virus produced at any temperature by the 4 candidates was able to plaque at 39.3 C, in contrast to the parent virus.

In view of the promising results obtained with the TP-79-56 candidates in sizing and ts studies, a small-scale monkey viremia study was conducted. This study was also designed to compare the various viremia assay procedures on LLC-MK_2 cells that have been employed routinely here and at WRAIR. These include: (a) direct plaque assay using undiluted serum without removal of residual inoculum by washing (these were done here and by Dr. Eckles of WRAIR using coded serum specimens); (b) direct plaque assay of undiluted serum with subsequent washing; (c) direct plaque assay of a 1:2 dilution of serum; (d) plaque assay of the 9-day outgrowth of LLC-MK_2 cultures inoculated with 1:2 serum dilutions ("amplification") and (e) plaque assay of 14-day outgrowth in LLC-MK_2 and C6/36 mosquito cells by Dr. Eckles using coded specimens.

TABLE IV. GROWTH OF 4 DEN-1 CANDIDATES ON LIQUID OVERLAID LLC-MK_2 CULTURES AT 6 TEMPERATURES.

VIRUS (INPUT)	TEMP. OF INCUBATION (°C)	PFU/ml BY DAY 5	7	10
DEN-1 #1 Parent (8×10^2)	25	2×10^4	1×10^2	1×10^4
	35	9×10^5	5×10^3	1×10^3
	37	8×10^4	9×10^2	8×10^1
	38.5	2×10^4	2×10^2	2×10^1
	39.3	8×10^2	-	-
	40	2×10^2	-	-
TP-79-33 25 C Pool (9×10^2)	25	4×10^3	9×10^2	2×10^4
	35	8×10^4	2×10^5	6×10^4
	37	3×10^3	1×10^4	2×10^3
	38.5	1×10^3	4×10^2	5×10^3
	39.3 & 40	-	-	-
35 C Pool (8×10^2)	25	4×10^3	9×10^2	5×10^4
	35	2×10^5	1×10^5	5×10^4
	37	8×10^3	4×10^4	1×10^3
	38.5	2×10^3	1×10^3	6×10^3
	39.3	1×10^2	3×10^1	2×10^1
	40	-	-	-
TP-79-56 25 C Pool (8×10^2)	25	2×10^4	1×10^5	8×10^5
	35	2×10^4	3×10^5	5×10^4
	37	1×10^4	2×10^4	2×10^2
	38.5	2×10^3	3×10^3	1×10^3
	39.3 & 40	-	-	-
35 C Pool (8×10^2)	25	2×10^4	2×10^5	1×10^6
	35	1×10^4	3×10^5	9×10^4
	37	2×10^4	3×10^4	4×10^2
	38.5	5×10^3	3×10^3	2×10^3
	39.3 & 40	-	-	-

[a]Plaqued on LLC-MK2 by standard procedures at 35 C; only parent plaqued at 39.3 C.

TABLE V. QUANTITATIVE VIREMIA RESPONSE OF REHSUS MONKEYS TO SC INOCULATION WITH DEN-1 PARENT AND TP-79-56 VACCINE CANDIDATE CLONES MEASURED BY DIRECT PLAQUING TECHNIQUE ON LLC-MK_2.

VIRUS INOCULUM	MONKEY NO.	ASSAY METHOD	PFU/ml BY DAY										
			1	2	3	4	5	6	7	8	9	10	11-14
Parent	829A	Undil., unwashed	0	0	0	0	10	45	15	0	0	0	0
		Undil., unwashed (WR)[a]	0	0	0	2.5	0	5	0	0	0	0	0
		Undil., washed	0	0	0	0	0	25	0	2.5	0	0	NT
		1:2	0	0	6	48	153	207	135	0	0	0	0
	B7520	Undil, unwashed	0	0	0	0	0	15	30	0	0	0	0
		Undil, unwashed (WR)	0	0	0	0	0	2.5	7.5	0	0	0	0
		Undil, washed	0	0	0	0	0	0	2.5	0	0	0	NT
		1:2	0	0	4	4	34	115	226	80	8	0	0
TP-79-56 35 C Pool	B7508	Undil, unwashed	0	0	0	0	0	0	0	0	0	0	0
		Undil, unwashed (WR)	0	0	0	0	0	2.5	0	0	0	0	0
		Undil, washed	0	0	0	0	0	0	0	0	0	0	NT
		1:2	0	0	6	2	8	0	0	0	0	0	0
	B7462	Undil, unwashed	0	0	0	0	0	5	0	0	0	0	0
		Undil, unwashed (WR)	0	0	0	0	0	0	0	0	0	0	0
		Undil, washed	0	0	0	0	0	0	0	0	0	0	0
		1:2	0	0	4	42	68	36	0	0	0	0	0
TP-79-56 25 C Pool	B7465	Undil, unwashed	0	0	0	0	0	10	10	0	0	0	0
		Undil, unwashed (WR)	0	0	0	0	0	2.5	5	0	0	0	0
		Undil, washed	0	0	0	0	0	0	0	0	0	0	NT
		1:2	0	0	0	0	12	40	12	0	0	0	0
	B7523	Undil, unwashed	0	0	0	0	0	0	0	0	0	0	0
		Undil, unwashed (WR)	0	0	0	0	0	0	0	0	0	0	0
		Undil, washed	0	0	0	0	0	0	40	6	0	0	NT
		1:2	0	0	0	0	0	60	0	0	0	0	0

[a]Tests performed on coded serum specimen by WRAIR personnel; all others performed at USAMRIID.

In this study 2 monkeys received 10^4 PFU of TP-79-56 (day 8 pool) grown at 35 C, 2 monkeys received 10^4 PFU of TP-79-56 (day 8 pool) grown at 25 C, 2 monkeys received 10^5 PFU of DEN-1 #1 pass 7 parent: all inoculations were given SC. Serum samples obtained on days 0-14 were then tested for virus by the 5 methods. Results of the direct plaquing assay are shown in Table V; results of amplification studies are shown in Table VI.

Clearly, the results in Table V show the following: (a) the use of diluted serum as inoculum yields much higher titers and, in addition, detects virus in serum specimens that appear negative by direct plaquing with undiluted specimens; (b) there is a significant difference between the level of viremia elicited by parent virus and the 2 candidate vaccine clones; (c) there is only marginal agreement in the results of tests run here and at WRAIR using the "undiluted serum unwashed" inoculum system; and (d) the third type of assay (wherein the residual inoculum was washed after virus adsorption) generally resulted in insignificantly different results from those obtained with the "undiluted/unwashed" test system. Not shown in Table V is the fact that in these types of tests parent virus elicited mixed plaque sizes,whereas both candidate clones elicited uniformly small plaques.

Shown in Table VI are the results of amplification methods used for qualitative viremia determination. Diluted serum specimens were inoculated into LLC-MK_2 and incubated for either 9 days (USAMRIID) or 14 days (WRAIR-coded specimens). In addition, Dr. Eckles at WRAIR tried a mosquito cell line, C6/36, using a 14-day outgrowth period. Samples were titrated at the end of the amplification period using standard plaquing techniques on LLC-MK_2. The following generalities can be made from Table VI: (a) regardless of cell type used, or length of incubation, the amplification procedures were not as sensitive for detecting low level viremia as was direct plaquing of 1:2 serum dilutions, and (b) the mosquito cell line, C6/36, is somewhat more sensitive then LLC-MK_2 for the detection of DEN-1 virus by the amplification method.

One additional procedure was used to characterize the virus population seen in viremic monkeys: 30 plaques were picked from the direct plaque, 1:2 serum dilution test on sera from monkeys that received the 2 vaccine candidates (Table V). After outgrowth of the plaque picks in LLC-MK_2 cultures, the progeny were assayed at 35 C and 39.3 C. In all cases the candidates were uniformly sp and ts. Thus, although they induced a low level viremia in monkeys they retained the 2 *in vitro* markers of avirulence. No large plaque, non-ts revertants were observed as was the case with the TP8-PL-2 candidate discussed above.

With regard to serology, PRN_{50} tests run on sera taken on day 28 showed seroconversion of all monkeys, whereas only the parent virus and 25 C pool-recipients showed antibody by PRN_{80} tests. By day 60, titers for all monkeys for all monkeys were the same or slightly higher than at day 28.

Numerous ancillary studies have also been performed with DEN-1 candidates, some of which are still in progress due to their long-term nature. Studies with the mutagens (base analogues) 5 azacytidine and 5 fluorouracil yielded scores of plaques from TP-79-33 and TP-79-56 candidates, some of which have been partially characterized. In general, mutagenizing did not lead to strikingly different progeny. Plaque sizes observed were the same or in most cases larger than in pretreated viruses. Moreover, none showed increased ts and, indeed, as would be expected many were less ts than the pretreated viruses. Little additional study with these mutagens is warranted by the results thus far obtained.

TABLE VI. QUALITATIVE VIREMIA RESPONSES OF RHESUS MONKEYS TO SC INOCULATION WITH DEN-1 PARENT AND TP-79-56 VACCINE CANDIDATE CLONES MEASURED BY AMPLIFICATION TECHNIQUE.

VIRUS INOCULUM	MONKEY NO.	AMPLIFICATION METHOD	PFU/ML[a] IN OUTGROWTH BY DAY OF MONKEY SERUM SAMPLE									
			1	2	3	4	5	6	7	8	9	10
Parent	829A	9 days on LLC-MK_2	0	0	0	25	50	14,000	5	0	0	0
		14 days on LLC-MK_2 (WR)[b]	0	0	0	0	0	TNTC	0	0	0	0
		14 days on C 6/36[c] (WR)	0	25	45	65	0	90	0	25	0	0
	B 7520	9 days on LLC-MK_2	0	0	0	0	0	120	0	0	0	0
		14 days on LLC-MK_2 (WR)	0	0	0	0	300	0	70	TNTC	0	0
		14 days on C 6/36 (WR)	0	0	0	0	40	375	0	0	0	0
TP 79-56 35 C Pool	B 7508	9 days on LLC-MK_2	0	0	0	0	0	0	0	0	0	0
		14 days on LLC-MK_2 (WR)	0	0	0	0	0	0	0	0	0	0
		14 days on C 6/36 (WR)	0	0	0	0	0	0	0	0	0	0
	B 7462	9 days on LLC-MK_2	0	0	0	0	0	0	0	0	0	0
		14 days on LLC-MK_2 (WR)	0	0	0	0	0	0	0	0	0	0
		14 days on C 6/36 (WR)	0	0	0	0	20	50	70	0	0	0
TP 79-56 25 C Pool	B 7465	9 days on LLC-MK	0	0	0	0	0	0	0	0	0	0
		14 days on LLC-MK_2 (WR)	0	0	0	0	TNTC	0	0	0	0	0
		14 days on C 6/36 (WR)	0	0	0	0	105	55	25	65	0	0
	B 7523	9 days on LLC-MK_2	0	0	0	200	0	0	0	0	0	0
		14 days on LLC-MK_2 (WR)	0	0	0	0	0	0	0	0	0	0
		14 days on C 6/36 (WR)	0	0	35	0	0	0	0	0	0	0

[a] Plaqued on LLC-MK_2.

[b] Tested by WRAIR personnel on coded specimens.

[c] Mosquito cell line.

Some additional passages of the TP-79-33 (25 C + 35 C) and TP-79-56 (25 C + 35 C) candidates have also been made in certified DBS-103 cultures with a resulting increase in yield to $\sim 10^{5.8}$ $\log_{10}$ PFU/ml. Plaque size and ts markers were retained on passage. The TP-79-56 candidate remained the most ts, with a 38.5 C cut off in the direct plaquing assay. Additional evaluations in monkeys will provide a broader data base with regard to the monkey viremia marker. These studies may be restricted to a single candidate due to limitations in both monkey supply and available animal holding space.

Part II. Chikungunya: Development of an Attenuated Vaccine

After a series of plaque-to-plaque passages of CHIK strain 15561, 4 clones were obtained from the 74B (PL-2) series, and were designated 177, 178, 181, and 194. They were amplified in MRC-5 culture to provide working seeds. All had common qualities: (a) titers > 10^7 PFU/ml; (b) plaque size of 0.5-1.0 mm; and (c) positive identification as CHIK in PRN_{80} tests using NIH-certified antiserum. Further, much effort was expended to characterize these clones and thereby permit selection of the most promising for evaluation in rhesus monkeys. In an initial ts study, the 4 candidate clones, "parent virus" (pass 3 in PGMK) and pass 12 (original virus seed used to obtain clones) were titrated in replicate on both MRC-5 and Vero cells at several temperatures.

As indicated in Table VII, all 4 clones were restricted at 38-38.5 C in contrast to parent or pass-12 virus, which were restricted at higher temperatures. This was more evident on MRC-5 culture than on Vero; the former has been chosen for all subsequent ts studies due to greater sensitivity.

TABLE VII. EFFECT OF TEMPERATURE ON PLAQUING EFFICIENCY OF CHIK VACCINE CANDIDATES

	LOG_{10} PFU									
	MRC-5					Vero				
VIRUS	35°	37°	38°	38.5°	39°	35°	37°	38°	38.5°	39°
177	7.7	7.7	7.2	a		7.4	7.0			
178	7.9	7.8	7.3			7.5	6.9	4.2		
181	7.6	7.3	6.8			7.6	7.0			
194	7.5	7.4	6.9			7.1	6.2	4.4		
Pass-3	5.3	5.1	4.7	4.7	3.7	4.8	4.6	4.3		
Pass-12	7.8	ND	ND	6.5	6.3	7.1	ND	6.3	5.1	2.2

[a] $<10^{1.4}$ PFU/ml

Another marker examined was suckling mouse neurovirulence. SM (n=6) were inoculated IC with decimal dilutions of the parent and 4 clones and observed daily for 14 days for deaths. None of the candidate clones were neurovirulent.

In vitro reversion studies were also conducted with the 4 clones to determine whether they were genetically stable, i.e., would retain their nonvirulence for mice and ts marker after 4 serial passages in MRC-5 culture at high MOI levels. Shown in Table VIII are the results of studies performed on the 4th passage culture fluid of the candidates. Each passage was made at 35 C in MRC-5 culture with liquid medium; 2 virus inputs were used, 10^5 and 10^7 PFU. After incubation for 48 h fresh MRC-5 cultures were used to make the next passage at the same MOI. Only the results of tests with the 4th passage fluid appear in Table IX. It is clear that the candidates had reverted to some extent by the 4th high MOI MRC-5 passage. For example, they plaqued at 38.5 and 39 C. Not shown here is the fact that plaque size also increased from 0.5-1.0 mm to 2-4 mm. Moreover, #177 and #194 exhibited some slight SM neurovirulence not previously seen.

TABLE VIII. EFFECT OF 4 SERIAL MRC-5 CELL PASSAGES ON CHIK VACCINE CANDIDATES

VIRUS[a]	INPUT (LOG_{10}) INTO MRC-5 CULTURE	SUCKLING MICE DEAD/6		PFU BY DIRECT PLAQUING ON MRC-5 (LOG_{10})		
		10^{-1}	10^{-2}	35°	38.5°	39°
177	5	2	1	8.9	7.0	6.5
	7	0	0	ND	ND	ND
178	5	0	0	9.1	7.0	5.0
	7	0	0	ND	ND	ND
181	5	0	0	9.3	7.5	7.1
	7	0	0	ND	ND	ND
194	5	0	0	9.1	6.7	Bad cells
	7	1	0	ND	ND	ND
Pass-3 (Parent)		6	6	5.3	4.7	3.7

[a] 4th MRC-5 passage fluids.

The same 4 clones were evaluated for candidacy as attenuated vaccines. After careful consideration of results obtained from ts, mouse neurovirulence and genetic stability studies, clone 181 was chosen for further characterization.

Additional studies were performed to evaluate the genetic stability of CHIK 181 virus. This was accomplished by passing the virus for 4 serial 48-h passages in MRC-5 cells at various inputs, examining the 4th passage level at each input for observable changes in plaque size. The results of this experiment can be seen in Table IX.

It can be seen that inputs of $\geq$ 3 logs of CHIK 181 virus through 4 passes increases plaque size from 0.5-1 mm to 2-4 mm in diameter. In contrast, a 2-log input resulted in no observable change in plaque size. It is apparent from these results that genetic stability (i.e. sp) can be maintained by passages at low virus inputs, at least through 4 serial passages. In terms of applicability to practical, attenuated vaccine production, these results do not severely impair the program. Passage to produce seed virus for actual vaccine production runs would clearly be made at extremely low MOI.

TABLE IX. EFFECT OF VIRUS INPUT ON IN VITRO GENETIC STABILITY OF CHIK VIRUS 181, FOLLOWING 4 SERIAL 48-H PASSAGES IN MRC-5 CELLS

VIRUS INPUT (log_{10}PFU/ml	PLAQUE SIZE (mm)	TITER (Log_{10} PFU/ml)
2	0.5-1.0	6.5
3	2.0-3.0	8.6
4	2.0-4.0	8.6
5	2.0-4.0	8.6
7	2.0-4.0	8.0

Several studies were performed which examined the in vivo virulence of candidate 181 in both mice and monkeys. SM were inoculated with 0.03 ml IC of either parent, 181 or the 4th pass of 181. The mice were observed for 14 days and deaths recorded.

The parent virus had a characteristic high neurovirulence with a LD_{50} of $10^{8.1}$. No deaths were seen with 181 and 181 pass 4. In a separate experiment, we examined the growth and virulence of these viruses in SMB. Mice were inoculated IC with a high and low virus inoculum of each virus; the brains were harvested at 60 h and titrated for virus content. Results are seen in Table X.

TABLE X. GROWTH AND VIRULENCE OF ATTENUATED AND PARENT CHIK VIRUSES IN SMB.

INOCULUM	INPUT (Log_{10} PFU/0.03 ml)	VIRUS GROWTH[a] (Log_{10} PFU/ml)	DEATHS/6 AT TIME OF Harvest	DEATHS/6 AT TIME OF Day 6[b]
Parent	6.3	7.7	2	6
	3.3	6.9	0	6
181	6.4	5.5	0	0
	3.4	6.3	0	0
181 pass 4	6.3	7.3	0	0
	3.3	7.7	0	0

[a]Brains harvested at 60 h.
[b]Replicate titration, mice were held 14 days for observation.

All 3 viruses demonstrated significant growth at both inocula tested, with deaths occurring as seen previously, only in those mice inoculated with wild parent virus. These data indicate that candidate 181 grows significantly in vivo without neurovirulence.

One of the more critical markers of virus attenuation is the lack of monkey virulence. Although virulent CHIK virus is not lethal for rhesus monkeys, it does produce significant viremia. The 3 viruses, virulent parent, pool 181 and the partial revertant, 181 pass 4, were each inoculated into monkeys SC at a dose of 10^5 PFU/0.5 ml. For 10 days, the monkeys were observed for signs of illness and bled daily for viremia determinations. On days 14 and 21, sera were tested for neutralizing antibody. The results of the preliminary monkey virulence test are found in Table XI.

TABLE XI. PRELIMINARY VIRULENCE TESTS OF ATTENUATED STRAIN CHIK VIRUS IN RHESUS MONKEYS.

MONKEY NO.	INOCULUM (5 Logs, SC)	VIREMIA (Day)	VIREMIA (Peak titer/ml)	SN RESPONSE (Day 21)
4880	Virulent, pass 2 (Parent)	1-3	4.5	1:160
T-285		1-3	3.8	1:80
T-361	181	None	-	<1:10
T-317		None	-	<1:10
T-318	181, pass 4	3	1.5	1:10
P-765		None	-	1:10

Virulent virus produced a 3-day, 4-5 log viremia and antibody titers of 1:80 and 1:160. The highly attenuated 181 produced neither viremia nor seroconversion. The partial revertant produced little or no viremia and low titer antibody response. Upon challenge with heterologous, virulent CHIK strain on day 28, only the 181 recipients were susceptible. They exhibited a 3-day, 5-log viremia.

In an attempt to understand this apparent lack of immunogenicity of candidate 181 in rhesus monkeys, a second monkey virulence study was performed. Four rhesus monkeys were inoculated either by the IV or ID route with 6.8 and 5.8 logs of virus, respectively. In addition, 2 baboons, a natural host of CHIK virus in Africa were inoculated SC with 5 logs of virus. Results of this study are seen in Table XII. Both monkeys inoculated IV and one of the 2 inoculated ID seroconverted by day 14. Both baboons showed low, but detectable neutralizing titers by day 14.

Vaccine candidate virus CHIK 181, grown in certified MRC-5 cells, does not protect rhesus monkeys against challenge with virulent virus or significantly induce the production of neutralizing antibody in these animals at doses of approximately 10^5 PFU of virus. Higher virus doses ($10^{5.5-6.0}$) given by the ID or IV route did induce antibody production; however, since the titer of this preparation is only $10^{7.5}$ PFU/ml, the higher doses required would significantly limit its usage. It was important that we attempt to increase the titer of the 181 pool without altering its biological and genetic characteristics. This was accomplished by passing the virus at low multiplicities ($\leq$ 100 PFU) in FRhL 103 cells, certified for vaccine use. Results of this experiment are seen in Table XIII.

TABLE XII. SEROCONVERSION OF RHESUS MONKEYS AND BABOONS TO ATTENUATED CHIK 181 VIRUS.

SPECIES NO.	ROUTE (Log_{10} PFU)	TITER, DAY 14	
		PRN_{80}	PRN_{50}
Rhesus			
1	IV (6.0)	1:80	1:320
2		1:10	1:20
3	ID (5.0)	1:10	1:20
4		Neg	Neg
Baboon			
1	SC (5.0)	1:10	1:40
2		Neg	1:10

TABLE XIII. GROWTH, ts AND IMMUNOGENIC CHARACTERISTICS OF CHIK 181 VIRUS GROWN IN FRhL-2 and MRC-5 CELLS

181 VIRUS GROWN IN:	35°	39.3°	MPD_{50} [a]
MRC-5	7.5	0	4.2-5.0
FRhL-2 (103)	8.8	0	3.4

[a] Mouse protective $dose_{50}$ = vaccine virus dose that protects 50% of the mice upon challenge.

It can be seen that the growth of CHIK 181 virus was significantly higher in FRhL-103 cells ($10^{8.8}$ PFU/ml) than in MRC-5 cells ($10^{7.5}$ PFU/ml) when grown under similar conditions. It was necessary to determine if the enhanced growth in 103 cells altered the biological characteristics of the virus, making it unsuitable for vaccine use. Both plaque size (1-2 mm) and ts markers remained unchanged when tested concurrently with MRC-5-grown virus. Thus, it appears that the high-titered 103-grown virus has not changed biologically by passage in this more permissive cell.

To examine the immunogenicity of the 103-grown virus, weanling mice were inoculated IP with varying concentrations of the virus and challenged with virulent virus IC two weeks postinoculation. A dose of $10^{3.4}$ PFU protected 50% of the mice against lethal challenge, approximately a log of virus less than that required of the MRC-5-grown 181 virus. It appears that the passage in 103 cells not only enhanced the virus titer, but also its immunogenicity for mice.

Studies were conducted to examine the virulence of the new vaccine candidate for SM and rhesus monkeys. Two-day-old SM were inoculated IC with varying concentrations of CHIK 181 pass 1, pass 2 and pass 3. Virulent "parent" virus was examined

TABLE XIV. VIRUS ISOLATION AND ANTIBODY RESPONSE OF RHESUS MONKEYS INOCULATED WITH THE 3RD FRhL CELL PASSAGE OF CHIK 181.[a]

DOSE LOG_{10}	MONK NO.	VIREMIA - PFU/ml BY DAY											PRN_{80}
		1			2			3			4		
		UND	1:2	1:20	UND	1:2	1:20	UND	1:2	1:20	UND	1:2	Day 21
4.5	928B	0	0	0	0	0	0	0	0	0	0	0	40
	962B	0	0	0	0	0	0	0	0	0	0	0	40
5.5	540C	15	16	5	5	0	0	15	0	0	0	0	160
	906B	0	2	0	5	0	0	7	0	0	27	15	320
6.5	468C	15	5	2	2	5	2	0	0	0	0	0	320
	958A	2	7	0	0	2	0	2	0	0	2	2	1280

[a] Passaged at 2 log inputs.

concurrently as a positive control. Animals were observed daily and deaths recorded. All mice inoculated with FRhL-grown virus remained alive and healthy throughout the 3-week observation period. In contrast, ~ 1 PFU per inoculum of "parent" virus resulted in the death of 50% of the inoculated mice. The results of this in vivo study show that CHIK 181 virus grown in FRhL cells is not neurovirulent for suckling mice, thereby satisfying one of the markers for a live, attenuated vaccine.

A second and most critical in vivo marker of virus attenuation is the lack of or minimal virulence in subhuman primates as measured by levels of viremia.

Six rhesus monkeys were inoculated with varying concentrations of CHIK 181, pass 3 (FRhL cells), the equivalent of a vaccine passage level. The monkeys were bled daily on days 0-4; serum was tested for viremia. Additional bleedings were obtained on days 14, 21, and 28 for neutralizing antibody determations. No virus was detected by plaque assay in the serum of the 2 monkeys that received the lowest virus inoculum ($10^{4.5}$ PFU)(Table XIV.) Low titers of virus were isolated from 4 monkeys that received the 2 higher concentrations of virus. All virus isolates were found to be of sp and to be ts, comparable to that of the monkey inoculum. This observation shows that reversion to "parent" did not occur in vivo. In addition, all 6 monkeys demonstrated significant neutralizing antibody responses.

As a result of these studies done on CHIK 181 grown in FRhL cells, it was determined that the requirements for an attenuated live vaccine had been satisfied. Consequently both a Master Seed and a Production Seed were made. A live attenuated CHIK vaccine will be made and safety tested for its potential use in man.

Presentations:

1. Cole, F. E., Jr. Stability of DEN-1 vaccine candidace during passage in rhesus monkeys. Presented, Viral Vaccine Development Committee (VVDC), WRAIR, Washington, DC, Sep 79.

2. Levitt, N. H. Progress in CHIK vaccine studies. Presented, VVDC, WRAIR, Washington, DC, Sep 79.

3. Levitt, N. H. The attenuation of Chikungunya. Presented, VVDC, USAMRIID, Ft. Detrick, MD, Oct 79.

4. Cole, F. E., Jr. Selection of new clones of Dengue-1 vaccine candidates. Presented at VVDC, USAMRIID, Ft. Detrick, MD, Nov 79.

5. Cole, F. E., Jr., N. H. Levitt, S. E. Hasty, H. R. Ramsburg, G. A. Eddy. Studies on the development of a live, attenuated Chikungunya virus vaccine. Presented, Annu. Mtg., Am. Soc. Trop. Med. Hyg., Tucson, AZ, Nov 79.

6. Cole, F.E., Jr. Review of current status of DEN vaccine research. Presented at USAMRIID Advisory Committee, Fort Detrick, MD, Dec 79.

7. Levitt, N. H. Review of current status of CHIK vaccine studies. Presented at USAMRIID Advisory Committee, Fort Detrick, MD, Dec 79.

8. Levitt, N. H. Comparative markers of parent and vaccine clone of CHIK virus. Presented at VVDC, WRAIR, Mar 80.

9. Cole, F. E., Jr. Review of Dengue vaccine program - future goals. Presented at Seminar, USAMRIID, Ft. Detrick, MD, Mar 80.

Publications:

None

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases, 1 Oct 1979. Annual Progress Report, FT 1979, Fort Detrick, MD, in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6415	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCY	6. WORK SECURITY	7. REGRADING	8a. GRP'S INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M162776A841	00	012
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Include with Security Classification Code)
(U) Studies in immunization of the respiratory tract

12a. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE

		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 80	1.0	190
	CURRENT 81	0	0

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21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Provide EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Respiratory diseases; (U) Aerosols; (U) Particle size; (U) Immunoprophylaxis; (U) Animal models

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

23 (U) Investigate basic mechanisms of aerogenic immunization against respiratory infections. Most BW agents are transmissible in aerosols. Since this is the most feasible agent dissemination method for a large-scale covert BW operations, the need for establishing immune defenses in the respiratory tract make this work unit essential in a comprehensive BW defense program.

24 (U) Animals are immunized by respiratory or parenteral inoculation with inactivated or attenuated respiratory pathogens with and without adjuvants. Efficacy is determined by seroconversion, development of CMI responses, and protection against respiratory challenge.

25 (U) 79 10 - 80 09 - Aerobiological studies have demonstrated that Pseudomonas pseudomallei cells are capable of surviving aerosolization and remaining airborne long enough to constitute a potential hazard through transmission via aerosols under a variety of environmental conditions. Humoral and cellular immune responses in guinea pigs infected with P. pseudomallei were examined and compared with those of guinea pigs vaccinated with inactivated antigens. While both procedures stimulated antibody production, peripheral lymphocytes from infected guinea pigs demonstrated marked lymphocyte transformation to specific antigen that was not detectable in vaccinated guinea pigs. Prior sublethal infections, but not vaccination, significantly increased the resistance of guinea pigs to lethal infections as indicated by fewer postchallenge mortalities and a longer period of survival for those that died.

Terminated for management efficiency. Continued in W.U. S10 A0 199. (DAOG1522)

* Available to contractors upon originator's approval.

DD FORM 1 MAR 88 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.

BODY OF REPORT

Project No. 3M16102BS10: Military Disease, Injury and Health Hazards (U)
(3M162776A841)

Task No. 3M161102BS10 A0: Bacterial and Rickettsial Diseases of Potential BW Importance

Work Unit No. S10 A0 166: Studies in Immunization of the Respiratory Tract
(841 00 012)

Background:

The etiologic agent of respiratory melioidosis, Pseudomonas pseudomallei, in addition to producing natural infections of significant military importance, is considered a potential threat for biological operations against U. S. populations. Management of this disease depends almost entirely on antibiotic therapy. However, despite extensive search, no antibiotic has been found which is fully effective against all strains, nor is there an effective vaccine. Vaccines composed of nonliving P. pseudomallei antigens stimulate synthesis of specific antibody, but animals so immunized, while reflecting varying degrees of partial resistance to parenteral challenge, remain susceptible to lethal respiratory infections (1). By contrast, animals convalescing from previous sublethal infections enjoy significant resistance to reinfection. Accordingly, we have initiated studies to identify immune responses that are stimulated by infection, but which are lacking in animals vaccinated with nonliving antigens.

Studies have been completed which demonstrate that P. pseudomallei is sufficiently stable in aerosols to constitute a potential hazard through transmission via aerosols under several environmental conditions.

Progress:

Aerosol stability. P. pseudomallei, strain 23343, was cultured in trypticase soy broth containing 3% glycerol to a concentration of 2.5×10^9 colony forming units (CFU/ml). The stock culture was stored at -70°C for 5 months prior to aerosol testing. Little biological decay occurred during storage. When thawed, the culture contained 1.6×10^9 CFU/ml; 1 ml of the culture was disseminated using an FK-8 fluid nozzle into a 6,200-L aerosol chamber that had been preconditioned to either 30, 55, or 80% relative humidity (RH) at 24°C. Beginning 4 min after dissemination, the aerosols were sampled and assayed for viable cells at intervals over a period of 1 h. Four replicate aerosol trials at each RH were conducted. Estimates of the mean source strength and biological decay rate (BDR) obtained at each RH were recorded (Table I). Computation of these parameters utilized the exponential equation $C_t = c_o e^{-kt}$, where C_o represents source strength and is the percentage of viable, airborne P. pseudomallei cells at 0 time, and 100k is the estimated decay rate in %/min.

TABLE I. AEROSOL PROPERTIES OF P. PSEUDOMALLEI AT THREE RELATIVE HUMIDITIES

RELATIVE HUMIDITY (%)	Source Strength		BDR (%/min)	
	Mean, %	95% C.L.	Mean	95% C.L.
80	20.6	2.8-38.3	3.6	2.1-5.1
55	15.9	5.3-26.6	2.4	2.2-2.6
30	9.0	5.0-13.0	2.3	0.8-3.9

The variance among replicate trials was excessively high, for reasons not yet determined, and veiled any significant effects of humidity on either source strength or decay rate estimates.

Nevertheless, even though no effort was made to stabilize this culture prior to dissemination, the data indicated that sufficient numbers of infectious cells survived the stresses of aerosolization and persisted as airborne particles long enough to constitute a potential hazard through transmission via aerosols in all of the environmental conditions examined.

Immune response of guinea pigs. A series of experiments were conducted to examine the development of circulating antibodies in guinea pigs following either sublethal infection or vaccination with killed P. pseudomallei cells, and the association of these antibodies with resistance to infections A number of immunizing regimens were examined. Groups of Hartley strain, female guinea pigs were vaccinated with (a) a single dose of 10^9 killed cells, (b) 10^9 killed cells incorporated into complete Freund's adjuvant (CFA), or (c) 4 replicate doses administered at 4-day intervals by either the IM and IP routes. Two similar groups of guinea pigs were given sublethal infections by exposure to infectious aerosols or by IP injection.

At selected times after vaccination or infection, samples of sera from each group were collected and assayed for specific antibody by indirect immunofluorescence (IFA) procedures. At 2-4 weeks after vaccination or infection, animals from each immunized group, and an equal number of non-immunized guinea pigs were challenged with an LD_{90} of P. pseudomallei administered in small aerosol particles. Antibody titers for each group, measured at the time of challenge together with a measure of their resistance to lethal infection relative to non-immunized guinea pigs are summarized in Table II.

TABLE II. EFFECT OF VACCINATION OR PREVIOUS INFECTION ON PROTECTION OF GUINEA PIGS AGAINST RESPIRATORY MELIOIDOSIS

REGIMEN	MEAN IFA (n-4) TITER AT CHALLENGE	NO. DEAD/TOTAL	OF DEATH	P[a]
Killed Vaccine				
None, controls	0	20/27	10.7	-
Single IM dose	52	9/11	14.2	NS
Single IM dose in CFA	160	5/11	11.9	0.09
Replicate IM dose	64	11/16	13.0	NS
Replicate IP dose	78	13/20	12.4	NS
Sublethal Infection				
Aerosol route	320	4/22	8.4	0.05
IP route	160	5/12	7.2	0.09

[a]Fisher's exact test; mortality proportion for each group compared to that for the nonimmunized group.

A single vaccination with killed antigen stimulated only moderate levels of IFA. These peaked 3 weeks after vaccination but diminished thereafter and effective immunization was not achieved. Incorporation of the antigen in CFA resulted in somewhat higher antibody titers which persisted at peak levels for 38 days; 55% of the guinea pigs in this group survived a challenge infection that killed 74% of the nonimmunized animals. Antibody titers measured 2-3 weeks after the last of 4 repeated injections by the IM or IP routes were no higher than those stimulated by a single injection and did not result in increased protection. Apparently, a single injection of 10^9 cells was an adequate priming dose and no advantage was gained by increasing the antigenic mass administered through repeated injections at short intervals. Probably a more productive immunizing regimen would allow a longer rest period between injections so that antibody production has ceased prior to injection of additional antigen.

In an effort to establish immunizing sublethal infections in guinea pigs, the animals were either permitted to inhale or were injected with 10^3 P. pseudomallei cells. All of the animals exposed to the airborne bacteria became infected; bacterial concentrations in the lungs reached > 10^6 CFU within 14 days, but the lungs were clear of infection prior to challenge at 28 days; 27% died from this primary respiratory infection. The pattern of replication in lungs of IP-infected guinea pigs was similar, but slightly delayed, and peak bacterial concentrations in lung tissue were about 1/10 those in aerosol infected guinea pigs. None of the animals succumbed to the primary IP infection. Increased antibody titers were apparent in aerosol infected animals by day 16 and increased to 1:320 by 28 days; 82% of these guinea pigs survived an aerosol challenge that killed 74% of the previously uninfected guinea pigs. Antibody in IP infected animals increased more slowly to a titer of 1:160 by the time of challenge; their resistance to reinfection was not significantly altered

Results of these experiments suggest that the inactivated vaccine is only

weakly antigenic. Antibody stimulated by the vaccine alone was not longlasting and failed to protect guinea pigs against infection. Some immunoprotentiation was achieved through the use of Freund's adjuvant, but guinea pigs vaccinated with the killed antigen-adjuvant mixture were less resistant to lethal challenge than those convalescing from previous sublethal respiratory infections. Apparently, stimulation of the immune system by infection is more complete than in vaccinated animals and may result in cellular responses or other factors not seen in vaccinated animals. In an effort to identify some of these factors, a subsequent study was initiated to examine and compare both humoral and cellular immune responses in infected and vaccinated guinea pigs.

Groups of guinea pigs were either infected by exposure to aerosol doses of 700 viable P. pseudomallei cells, or were vaccinated IM with 10^{10} killed cells. A similar group was inoculated with sterile saline to serve as controls. At 2-week intervals, circulating lymphocytes from 5 animals in each group were tested for in vitro lymphoproliferative reactions in response to P. pseudomallei antigen and phytohemagglutinin (PHA). Serum samples from each animal were tested for anti-pseudomallei antibody by CPT Urbanski of Virology Division, using Purcell's (2) radioimmunodiffusion (RIA) procedures as well as IFA procedures. Four weeks after infection or vaccination, 5 guinea pigs from each group were tested for delay hypersensitivity (DHS) to P. pseudomallei antigen; 15 guinea pigs from each group were challenged with an LD_{50} respiratory dose of P. pseudomallei.

Infections following the primary "immunizing" aerosol exposure appeared limited to the respiratory tract; 10^5 CFU/lung were detected 2 weeks after the initial exposure and 36% of the guinea pigs died with a MTD of 19 days. No viable P. pseudomallei were found in the blood, liver, or spleens of infected guinea pigs. Unfortunately, the lung infections had not completely cleared at the time of challenge (4 weeks). There was no evidence of gross lung pathology at this time, but P. pseudomallei was isolated from the lungs of 1 of 2 guinea pigs examined.

Indications of the lymphoproliferative responses and antibody titers for each test group measured at the time of challenge are summaried, with survival data in Table III.

TABLE III. EFFECT OF VACCINATION OR INFECTION ON LYMPHOCYTE TRANSFORMATION, ANTIBODY, AND PROTECTION OF GUINEA PIGS AGAINST RESPIRATORY MELIOIDOSIS

TREATMENT	STIMULATION INDEX[a] Positive/ Total	Geom. Mean	GEOM. MEAN ANTIBODY TITER IFA	RIA	RESPONSE TO CHALLENGE[b] Dead/Total	of Death
Control	0/5	1.1	0	< 10	10/15	6.5
Vaccinated	1/5	1.6	27	640	10/15	8.2
Convalescent	4/5	6.8*	120	13511	3/15	10.2

[a]Positive responders were guinea pigs whose cultured lymphocytes yielded stimulation indices significantly greater than 1.0 ($P < 0.001$).

[b]1×10^5 CFU administered in small aerosol particles 29 days after vaccination or infection.

*$P < 0.01$ vs controls.

As observed in previous experiments, antibody titers stimulated by sublethal infection were significantly higher than those elicited through vaccination with killed P. pseudomallei antigen. Titers obtained using RIA procedures averaged 20 to 100 fold higher than titers obtained using IFA procedures. In spite of this dramatic difference in sensitivity, results obtained using the respective procedures were well correlated. The relative specificities of the 2 procedures remains to be determined.

P. pseudomallei antigen did not stimulate a significant proliferative response to lymphocytes from vaccinated animals, nor were they protected against a challenge infection. By contrast, lymphocytes from 4 of 5 previously infected guinea pigs responded with a mean stimulation index that was significantly ($P < 0.01$) higher than that for vaccinated or control guinea pigs.

These convalescent guinea pigs were better protected than vaccinated guinea pigs ($P < 0.05$) as indicated by longer MTD and lower postchallenge mortality. Increased skin reactivity to antigen was not detected. This lack of skin test response was unexpected, but there are diseases, such as candidiasis, in which disease activity is associated with loss of skin reactivity to antigens of the infecting organism. These guinea pigs will be tested again for DHS after complete clearance of the infecting organisms is demonstrated.

Since both humoral antibody titers and lymphocyte transformation indices were higher in previously infected than in vaccinated guinea pigs, the enhanced protection enjoyed by convalescing guinea pigs cannot be attributed exclusively to either the humoral or cellular components of the immune response. Both probably play an important role.

Combined vaccination and drug therapy. Since we have thus far been unable to protect effectively animals against respiratory P. pseudomallei infections

through active immunization with killed antigen alone, we considered the feasibility of managing the disease by a combination of vaccination and drug therapy. In vitro tests indicated that P. pseudomallei, strain 23343, was susceptible to chloramphenicol. Minimum in vitro inhibitory and bactericidal concentrations of 3 and 12 µg/ml, respectively, were estimated by the tube dilution technique. In vivo toxicity tests indicated that mice could not tolerate repeated IP injections of chloramphenicol. Three of 5 mice died after receiving 5 1-mg doses. However, 2 daily IM injections of doses up to 2 mg each for 5 consecutive days had no adverse effect on adult mice. We selected this dose level for use in the following experiment.

Four weeks after vaccination with 10^9 killed P. pseudomallei, a group of 40 mice were challenged with 4 LD_{50} of mouse virulent P. pseudomallei by the aerosol route. A similar number of nonvaccinated mice were challenged by the same procedure. Beginning 24 h after challenge, drug therapy was initiated in half of the mice in each group. Therapy consisted of 2 daily injections of 2 mg chloramphenicol (80 mg/kg BW) for 5 consecutive days. The remaining mice in each group received injections of sterile saline on the same schedule. Deaths were recorded over a period of 3 weeks following challenge. Data summarized in Table IV indicated that chloramphenicol treatment alone, vaccination alone, nor a combination reduced mortality compared to untreated controls. The first experiment was of an exploratory nature and not designed to produce definitive findings. Possibly, adjustment of the therapy-vaccination regimens would produce positive results.

TABLE IV. EFFECT OF VACCINATION COMBINED WITH DRUG THERAPY ON THE RESISTANCE OF MICE TO RESPIRATORY MELIOIDOSIS

TREATMENT	GEOM. MEAN (n=3) IFA TITER AT CHALLENGE [a]	DEAD/20	MEAN TIME-TO-DEATH (days)
Controls	0	20	4.7
Drug therapy only	0	20	4.7
Vaccination only	50.4	16	8.8
Vaccination & drug therapy	50.4	18	7.5

[a]Challenge dose of 600 CFU/mouse administered as small aerosol particles.

Publications: None.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 October 1979. Annual Progress Report FY 1979. Fort Detrick, MD, in press.

2. Purcell, R. H., D. C. Wong, Y. Moritsugu, J. L. Dienstag, J. A. Rontenberg, and J. D. Diggs. 1976. A microtiter solid-phase radioimmunoassay for hepatitis A antigen and antibody. J. Immunol. 116:349-356.

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. SMITH SMITH'S	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	N	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	013
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Enhancement of inactivated viral vaccines of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
73 04	80 09	DA		C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 80	1.0	195
FISCAL YEAR CURRENT 81	0	0

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21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Viruses; (U) Immune enhancement; (U) Adjuvant; (U) Vaccine; (U) Mice; (U) Monkeys

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

23 (U) Numerous inactivated vaccines have been developed to control infectious diseases; these vaccines often have marginal antigenic potency and cannot be used to stop a disease outbreak or in a BW situation. Immunological adjuvants plus inactivated vaccines frequently evoke more rapid and prolonged development of protective immunity. This work will develop new methods to enhance immunogenicity of presently available, marginally antigenic, inactivated viral vaccines for military personnel.

24 (U) Laboratory rodents and monkeys are inoculated with potential immunologic adjuvants combined with inactivated virus vaccines. Various immunologic responses and resistance against homologous virus challenge are determined to assess adjuvant efficacy and relative toxicity.

25 (U) 79 10 - 80 09 - CP 20,961 has been evaluated in monkeys and hamsters for adjuvanticity. In combination with inactivated RVF vaccine it helps elicit prolonged and significantly higher SN titers in monkeys. It has been shown to be an interferon inducer and an activator of cellular immune mechanisms which are presently under study in monkeys. A hamster model for adjuvant evaluation in combination with RVF vaccine, where antibody apparently is not the protective factor against survival to a lethal RVF infection, is also being evaluated. Liposomes are shown to have the most promise for future adjuvant evaluation and development because they can induce high antibody titers superior to vaccine prepared without them (demonstrated in the RVF-mouse system) and they are probably the only novel adjuvant at this time capable of approval for use in man. Terminated for management efficiency. Continued in W.U. 871 BC 148. (DAOG1537)

Publication: Infect. Immun. 28:937-943, 1980; Am. J. Vet. Res. 41, in press, 1980.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 80 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 69 (FOR ARMY USE) ARE OBSOLETE.

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BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M172776A841)

Task No. 3M162770A871 BC: Prevention of Viral Diseases of Potential BW Importance

Work Unit No. 871 BC 136: Enhancement of Inactivated Viral Vaccines of Military
(841 00 013) Importance

Background:

Adjuvants have been characterized as nonspecific immunostimulators which can enhance humoral and cellular responses to viral antigens. The depot effect of retaining a concentrated antigen at the injection site is best achieved with mineral oil or similar adjuvant components, but these often produce undesirable granulomas. Gels of $Al(OH)_3$ are now the only currently approved adjuvants for use in man. Lipid emulsion (LE) developed by us, has a potentially biodegradable peanut oil component, but leaves some deposited residue. A government patent is being sought for LE for eventual evaluation in human trials. LE is composed of lecithin, glycerol, and peanut oil, and is chemically defined, minimally reactogenic, and has been shown to potentiate the immune response to inactivated RVF virus in monkeys, VEE in mice, WEE in hamsters and RVF in sheep.

Other adjuvants of promise are CP 20,961, liposomal preparations, and Quil A saponin. Biochemically defined synthetic materials offer the most hope for use in man. The model adjuvant preparation is Freund's complete adjuvant (FCA) which is too reactogenic for use in man. CP 20,961 has been shown to potentiate the immune response to RVF vaccine in monkeys and mice, VEE vaccine in mice, and WEE vaccine in hamsters. A significant increase in PRN titer and protection against lethal challenge was demonstrated in each case except for the monkey experiment because of the monkey's natural resistance to RVF. But synthetic analogs of the active mycobacterial cell wall component in FCA (muramyl dipeptide, or MDP) have side effects too. MDP, poly A:U, and CP 20,961, all produce adjuvant arthritis in rats (This is a recent discovery for CP 20,961). Liposomes are the only adjuvants studied so far which are biodegradable, chemically defined, nonreactogenic, and immunopotentiating. Because they are partially composed of lecithin, low order concentrations of anti-phosphoryl choline antibodies are induced. Most importantly, an IM injection of liposomal vaccine mimics the antibody response to that with Freund's incomplete adjuvant (FIA). By varying the size and surface charge of the liposomes, preferential organ targeting can be obtained. Large multilamellar, negatively-charged liposomes targeted specifically to the liver were injected parenterally and shown to be an effective adjuvant for RVF vaccine in mice.

Emphasis has been placed on adjuvant evaluation in animal models which may have a general applicability for adjuvant evaluation in man. The criteria for a candidate adjuvant are the demonstrated ability to induce serum neutralizing (SN) antibodies superior to vaccine alone, the ability to significantly reduce mortality to a lethal homologous virus challenge and/or the ability to induce protective cell-mediated immunity (CMI) in animals not considered to be protected by SN antibody.

Progress:

SN antibody to inactivated RVF vaccine. Determinations of PRN_{80} titers were made in a long-term evaluation in cynomolgus monkeys of the adjuvanticity of CP 20,961 with RVF vaccine NDBR-103, Lot 2. Three groups of 4 monkeys each were vaccinated as detailed in Table I. Vaccine (0.5 ml) + 1.0 mg/kg of CP 20,961 induced significantly higher ($P < 0.05$, Student's t with the Bonferroni correction for multiple comparison) PRN_{80} antibody titers than vaccine without adjuvant on days 7-28 after primary immunization and on all days after booster immunization. The high dose of adjuvant produced a significantly greater peak antibody response and longer persistence than the low dose. A follow-up study on 2 other adjuvant-injected groups is underway to determine the persistence of SN titer after a single dose of adjuvant + vaccine.

TABLE I. PRN_{80} ANTIBODY TITERS IN MONKEYS ADMINISTERED RVF VACCINE WITH OR WITHOUT ADJUVANT CP 20,961

	RECIPROCAL GEOM. MEAN PRN_{80}		
DAY	Vaccine Controls	Vaccine + 0.1 mg/kg	Vaccine + 1.0 mg/kg
3	5	5	5
4	6	7	10
7	12	34	48*
10	24	80*	538*
14	34	113	761*
21	24	113	1810*
28	28	160	1810*
Booster, day 28			
3	34	269*	1810*
7	320	3620	17222*
10	320	12177	57926*
14	320	3153	10240*
21	226	3044	12177*
28	80	2560	12177*
35	56	640	3044
63	56	640	7241
94	24	538	4305
135	17	226	5120
152	12	135	905

* $P < 0.05$ vs. controls.

CP 20,961 as an interferon inducer in cynomolgus monkeys. PRN_{50} of vesicular stomatitis virus on Vero cell monolayers using a human interferon standard showed interferon (IF) induction in monkey plasma at 8, 24, 48, and 72 h after a single dose of RVF vaccine + adjuvant (Table II). The IF values were indistinguishable between vaccinates with and without adjuvant at 1.0 mg/kg. However, the IF levels were singificantly greater with adjuvant at 0.1 mg/kg for the 24-, 48-, and 72-h samples. Adjuvant dose apparently affects IF production, and titration is necessary for optimization of the response. A follow-up with a second group of monkeys is currently being examined for any correlation between plasma viremia and RVF challenge and IF production with adjuvant CP 20,961 with RVF vaccine. Other experiments, not shown here indicate that the natural infection of monkeys with RVF does not induce IF significantly.

TABLE II. PLASMA IF IN MONKEYS (n=4 2/GROUP) VACCINATED AGAINST RVF WITH NDBR-103 (0.4 ml, IM) AND THE ADJUVANT CP 20,961.

	LOG_{10} IF ± SD		
HOURS	Controls	0.1 mg/kg	1.0 mg/kg
8	2.41 ± 0.34	2.75 ± 0.30	2.39 ± 0.69
24	2.31 ± 0.22	2.75 ± 0.26	2.50 ± 0.46
48	2.36 ± 0.66	2.76 ± 0.32	2.50 ± 0.33
72	2.48 ± 0.19	2.74 ± 0.17	2.54 ± 0.18

CP 20,961 as an activator of CMI. Three general areas of evaluation were selected because of their applicability to study in man, these are: polyclonal mitogen stimulation, virus-specific stimulation, and lymphocyte subpopulation characterization. All tests were limited to peripheral blood. Polyclonal T-cell responsiveness to phytohemagglutinin (PHP-P) and RVF-specific stimulation of unspecified cell type were studied in conjunction with SN antibody titer and IF induction in the 3 groups of monkeys described previously. The optimal dose of PHA was found to be 20 µg/ml and 1:50 dilution of killed vaccine in whole blood culture of immunized monkeys for maximal tritiated thymidine incorporation at 3 and 5 days in microtiter culture, respectively. A significant increase ($P < 0.05$) in T-cell responsiveness was observed on days 2-4 after booster immunization in both adjuvant- and nonadjuvant-treated vaccine groups. This type of stimulation is notably antecedent to the detectable presence of SN antibody and is significantly present before an increase in titer observed by day 5 postbooster. Significant virus-specific lymphocyte transformation (LT) does not occur in the vaccine control group, but is observable by day 4 in the adjuvant-treated groups and is highly significant ($P < 0.05$, Table IV). The antigen-specific nature of this activation-suppression preceding antibody needs to be examined.

TABLE III. PHA-P STIMULATION OF T-LYMPHOCYTES IN WHOLE BLOOD CULTURE IN MONKEYS ACCINATED AGAINST RVF WITH OR WITHOUT CP 20,961.

	SI[a] ± SE (N=16)		
DAY	Controls	0.1 mg/kg	1.0 mg/kg
0	0.82 ± 0.11	1.28 ± 0.25	1.86 ± 0.34
1	1.65 ± 0.06	1.52 ± 0.13	2.63 ± 0.32
2	9.16 ± 1.86	2.47 ± 0.54	3.30 ± 0.55
3	6.59 ± 1.62	4.64 ± 0.72	2.10 ± 0.28
4	2.44 ± 0.43	3.84 ± 0.65	2.72 ± 0.09
7	1.30 ± 0.32	1.18 ± 0.08	1.24 ± 0.05
10	0.35 ± 0.05	0.76 ± 0.11	0.68 ± 0.09

a $SI = \frac{\text{Geo. mean } \log_{10} \text{ of test}}{\text{Geo. mean } \log_{10} \text{ of control}}$

Adjuvant induces effects unseen in vaccine-alone monkeys. Adjuvant significantly decreased PHA-P responsiveness in relation to dose; 1.0 mg/kg produced the greatest decrease. Follow-up studies on these monkeys and other monkeys are under way to determine the extent of the alteration of T-cell mitogenic responsiveness caused by CP 20,961.

TABLE IV. PHA AND RVF VACCINE MITOGENESIS IN WHOLE BLOOD CULTURE.

POSTBOOSTER DAY	GEO. MEAN ANTIBODY	0.1 mg/kg CP 20,961 + VACCINE (0.5 ml) RVF Antigen SI ± SE	PHA SI ± SE
0	160	0.91 ± 0.02	1.22 ± 0.18
1		0.91 ± 0.04	1.28 ± 0.25
2		0.96 ± 0.24	1.52 ± 0.13
3	269	0.95 ± 0.04	2.97 ± 0.54
4		0.97 ± 0.09	4.64 ± 0.72
5		1.46 ± 0.15	3.84 ± 0.65
7	3620	1.07 ± 0.01	1.18 ± 0.08
10	12177	1.25 ± 0.09	0.76 ± 0.11
14	2153	4.83 ± 1.00	-
21	3044	-	-
Vaccine (0.5 ml alone)			
0	28	1.02 ± 0.04	1.20 ± 0.07
1		1.08 ± 0.03	0.82 ± 0.11
2		1.48 ± 0.16	1.65 ± 1.06
3	34	1.42 ± 0.31	9.16 ± 1.86
4		1.05 ± 0.05	6.49 ± 1.62
5		0.95 ± 0.05	2.49 ± 0.43
7	320	1.03 ± 0.13	1.30 ± 0.32
10	320	0.82 ± 0.11	0.35 ± 0.05
14	320	0.86 ± 0.04	-
21	226	-	-

Surface markers of peripheral blood lymphocytes. Adjuvant CP 20,961 combined with RVF vaccine was found to produce altered lymphocyte populations in the peripheral blood of cynomolgus monkeys when compared to the vaccine alone. These differences were also observable in vaccinated and RVF-challenged monkeys and challenged controls. Table V gives the percent counts for E (active), EA and EAC rosettes. Using Freedman's 2-way analysis of variance, the 3 monkey test groups (n=4 each) were found to be significantly different 0-4 days for E rosettes ($P = .01047$); 0-10 days for EA rosettes ($P = 0.05$): and 0-10 days for EAC rosettes ($P = 0.005$). Using the appropriate multiple range test, the vaccine alone groups showed significantly lower percent E, EA and EAC rosettes ($P < 0.05$).

Table VI shows percentage counts for E, EA, and EAC rosettes where the 3 monkey groups are: controls, RVF vaccinated, and RVF + CP 20,961 vaccinated. Applying the same test on days 0-14, each rosette type comparison yields a significant difference among groups: E, $P = 0.079$; EA, $P = 0.0048$; and EAC, $P = 0.079$. Vaccine combined with adjuvant provides significantly lower counts for E and EA rosettes ($P < 0.05$), and vaccine alone produces a lower mean % EAC than the adjuvant and challenge control groups ($P < 0.05$). There are no significant differences among vaccinated and challenged groups compared to controls.

TABLE V. PERIPHERAL BLOOD B AND T CELL MARKERS DURING LIVE RVF VIRUS CHALLENGE[a] IN RVF PRIMED AND BOOSTED MONKEYS (n=4) WITH AND WITHOUT CP 20,961

	VACCINE CONTROL			1.0 mg/ml			0.1 mg/ml		
DAY	E%	EA%	EAC%	E%	EA%	EAC%	E%	EA%	EAC%
0	27.9	22.3	27.6	32.5	20.5	34.3	28.4	22.5	27.3
1	23.8	12.5	16.1	29.5	22.4	29.7	28.3	14.1	24.0
2	22.9	14.0	14.1	35.8	21.3	29.0	30.8	21.8	26.6
3	20.6	18.5	18.8	30.8	20.0	16.9	33.1	20.5	21.3
5	24.9	14.8	30.5	28.0	15.5	34.5	33.0	17.6	32.4
7	19.5	18.5	13.6	37.8	28.3	14.8	32.6	20.5	17.9
10	19.6	15.9	21.1	30.3	16.0	14.9	18.1	23.1	20.8
14	35.3	13.5	6.8	32.4	14.1	9.8	28.8	9.8	8.0
22	18.9	15.3	13.3	16.9	17.0	22.5	19.5	19.1	20.1
28	22.0	9.8	18.9	24.3	9.0	14.1	23.9	9.1	12.6
35	24.5	16.3	12.8	19.8	17.4	12.9	22.1	16.1	12.6

a Challenged with ZH-501 strain RVF 1.5×10^6 PFU, IV.

TABLE VI. PERIPHERAL BLOOD B AND T CELL MARKERS DURING LIVE RVF VIRUS CHALLENGE[a] IN MONKEYS (n=4) PRIMED WITH INACTIVATED VACCINE WITH AND WITHOUT ADJUVANT.

	CONTROLS			VACCINE ALONE			0.1 mg/ml + VACCINE		
DAY	E%	EA%	EAC%	E%	EA%	EAC%	E%	EA%	EAC%
0	18.2	20.1	27.0	18.3	14.5	17.4	18.0	16.3	26.9
1	32.4	42.1	35.8	34.1	23.3	18.5	29.8	25.4	19.9
2	29.8	8.5	24.1	20.9	31.0	20.5	25.1	27.4	23.4
3	21.8	25.7	21.3	17.1	24.1	20.4	20.0	23.1	24.1
4	30.5	21.3	18.2	-	-	-	-	-	-
5	34.2	22.0	26.5	22.8	17.8	11.9	20.8	16.1	20.0
7	38.6	6.7	5.1	17.0	15.1	9.5	19.8	15.2	15.3
10	24.6	22.6	14.8	18.6	14.8	8.0	19.5	14.4	11.3
14	22.9	19.9	21.3	23.6	9.9	11.8	22.6	10.9	11.1
15				25.5	16.9	15.9	27.0	20.9	17.0
16				21.1	6.9	8.1	22.5	9.3	8.8
17				20.9	10.8	10.3	27.9	15.0	12.9
19				34.3	13.9	12.3	24.6	10.8	10.4
21	15.7	15.9	12.6	25.8	12.9	14.6	19.9	10.4	12.6
24				21.8	14.3	15.4	25.1	17.8	16.5
28	21.8	15.5	17.8	9.0	14.0	10.8	11.9	10.3	17.3
36				30.1	9.3	13.4	28.4	8.0	13.5
43	21.1	20.7	16.1	25.4	8.4	8.5	22.9	11.4	12.0
58				32.9	25.1	18.9	25.4	24.1	12.1

[a]Control challenged day 0, adjuvant groups challenged IV with 1.5×10^6 PFU, ZH-501 14 days after vaccine.

The significance of these data is being reviewed for comparison with SN antibody, IF production, virus titer (in challenged groups), mitogen stimulation and antigen binding rosette assays.

Hamster model for RVF vaccine combined with adjuvants. The hamster is highly susceptible to RVF infection with high mortality. These experiments were intended to demonstrate the importance of cell-mediated mechanisms in resisting RVF infection. A previous experiment using 4 groups of 20 hamsters each injected with 1 mg/kg CP 20,961 + vaccine, 0.1 mg/kg CP 20,961 plus vaccine, vaccine alone, and a saline control group revealed cumulative mortalities after challenge at 21 days of 50, 79, 86, and 100%, respectively. None of these groups developed SN titers greater than 15. An experiment was designed to test the hypothesis of the observed reduction of mortality in the high-dose of adjuvant group as being attributable to enhancement of CMI to RVF virus.

Tables VII and VIII review the results of lymphocyte stimulation and migration inhibition factor (MIF) production from hamster spleen leukocytes. The high dose CP-20,961 group was solely able to withstand a challenge dose that vaccine alone and LE plus vaccine groups could not successfully resist ($P < 0.005$ for comparison with vaccine alone and LE plus vaccine, Chi-square test). Each data point represents the mean of 4 hamsters (See footnote for replication levels). A significant depression to both T cell mitogens[Concanavalin A (Con A), and poke weed mitogen (PWM)], PHA, RVF, and B cell mitogens (LPS, PWM, RVF) is noted on days 4-10; it is absent on day 14 (Table VII). It is assumed that the other groups could not demonstrate this rebound response because of inferior lymphocyte activation.

Similarly, MIF production is only convincingly demonstrated in the 1 mg/kg CP 20,961 + vaccine group. This phenomenon is demonstrated by significant reduction in macrophage migration radii on days 7 and 10, but not on day 14. None of the other groups demonstrate MIF production. Problems of RVF antigen purity are being worked out. Experiments have been partially completed which will characterize the portion of the LT and MIF assays being directed solely at virus-specific antigens.

Liposomes as adjuvants for RVF vaccine. Work was continued on adjuvant CP 20,961 in outbred Swiss mice in relation to SN antibody levels and protection against RVF virus challenge (Table IX). Adjuvant dosage in excess of 0.1 mg/kg significantly increased mortality and decreased antibody production compared to adjuvant dosage at $\leq$ 0.1 mg/kg. Adjuvant dosage in excess of 0.1 mg/kg was no better in reducing mortality (Groups 1-5). It was noted that 4.0 mg/kg adjuvant without vaccine had a slight protective effect (Group 8). Intralipid (R) solubilizing vehicle mixed with vaccine had a slight protective effect; it reduced mortality but produced little antibody (Group 11). Perhaps this was due to liposome formation which can occur during mixing. Vaccine alone at this dose and challenge level (1.5×10^6 PFU) produced marginal antibody which was not protective against challenge (Group 12).

Liposomal adjuvant induction of SN antibody and protection in Swiss mice using RVF vaccine was examined (Table X). The effect of 2 liposome preparations with and without CP 20,961 incorporated in combination with vaccine as both crude and washed vesicles was studied. Vaccine could be diluted 1:5 and produce significant results. The highest antibody titers were produced in groups 1 and 2. Adjuvant CP 20,961 did not enhance antibody titers when incorporated into this liposomal preparation. Lower antibody titers, but significant protection, resulted in groups 3 and 4; again CP 20,961 did not enhance this vesicle's effect.

TABLE VII. SI OF HAMSTER SPLEEN CELLS WITH MITOGENS IN RVF-CHALLENGED ANIMALS (n=5) PRIMED WITH INACTIVATED VACCINE WITH OR WITHOUT ADJUVANT

GROUP	DAY	MEAN SI ± SE					CUMUL. % MORTALITY
		Con A	PHA	PWM	LPS	RVF	
I	0	17.1 ± 3.8	7.6 ± 2.7	8.7 ± 2.9	2.0 ± 0.7	0.6 ± 0.4	
0.1 mg/kg	1	25.4 ± 5.0	8.6 ± 2.2	18.1 ± 2.1	1.3 ± 0.2	1.5 ± 0.3	
CP 20,961 +	2	7.8 ± 1.7	4.3 ± 1.9	8.5 ± 2.5	1.6 ± 0.1	1.4 ± 0.2	
vaccine	3	14.2 ± 7.6	2.5 ± 0.8	10.7 ± 5.1	0.8 ± 0.1	1.1 ± 0.2	
	4	1.2 ± 0.1	0.6 ± 0.1	1.3 ± 0.1	0.7 ± 0.1	0.9 ± 0.2	
	7	1.0 ± 0.1	0.5 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	
	10	1.5 ± 0.3	0.7 ± 0.1		0.7 ± 0.1	0.7 ± 0.1	
	14	8.5 ± 2.1	6.2 ± 1.3		1.1 ± 0.1	0.6 ± 0.1	
F Value		8.3*	6.5*	5.4*	4.3*	5.0*	50
II	0	16.5 ± 1.2	9.4 ± 0.8	9.3 ± 0.3	2.6 ± 0.5	0.5 ± 0.1	
LE + RVF	1	9.5 ± 1.4	2.4 ± 0.5	7.1 ± 1.0	1.3 ± 0.1	1.3 ± 0.1	
Vaccine	2	8.9 ± 4.3	2.2 ± 1.1	7.9 ± 3.0	1.0 ± 0.1	1.3 ± 0.4	
F Value		0.9	9.5*	0.3	3.5	1.8	79
III	0	17.6 ± 2.7	7.5 ± 2.1	9.4 ± 1.6	1.8 ± 0.4	0.5 ± 0.1	
RVF Vaccine	1	10.9 ± 3.4	5.5 ± 1.6	9.0 ± 2.4	1.2 ± 0.1	1.2 ± 0.2	
	2	13.9 ± 4.9	2.3 ± 1.1	10.4 ± 2.3	1.2 ± 0.1	1.1 ± 0.1	
	3	13.4 ± 4.1	4.6 ± 1.8	11.4 ± 2.0	1.1 ± 0.2	1.3 ± 0.3	
F Value		0.1	1.0	0.2	1.1	1.8	86
IV	0	15.9 ± 1.5	5.9 ± 0.3	7.8 ± 1.2	2.2 ± 0.3	0.7 ± 0.1	
Saline	1	10.9 ± 0.6	2.2 ± 0.2	6.0 ± 1.3	1.1 ± 0.2	1.1 ± 0.1	
Controls F Value		1.6	23.8*	0.2	1.5	2.7	100

[a] 381 LD_{50} ZH-501, IP, 14 days after vaccine; 4 replications/culture.

* One-way ANOVA, significant.

TABLE IX. EFFECT OF CP 20.961 ADJUVANT ON DAY-14 PRN_{80} TITERS OF CD-1 MICE VACCINATED SC WITH RVF (NDBR-103, LOt 2, 1:2 DILUTION) AND CHALLENGED

GROUP (n = 20)				PRN_{80}	28-DAY
No.	CP 20,961 (mg/kg)	Intralipid	Vaccine	TITER (on VERO)	MORTALITY %
1	4.0	+	+	< 10	20
2	2.0	+	+	< 10	50
3	1.0	+	+	< 10	60
4	0.5	+	+	< 10	55
5	0.25	+	+	11	40
6	0.1	+	+	1280	32(n=19)
7	0.01	-	-	1280	5
8	4.0	-	-	< 10	80
9	2.0	-	-	< 10	100
10	0.1	-	-	640	35
11	-	+	+	10	30
12	-	-	+	10	100

[a] ZH-501 380 LD_{50} IP, day 14 after vaccine.

TABLE X. EFFECT OF LIPOSOMAL ADJUVANT ENTRAPMENT ON SN TITERS OF VACCINATED SWISS MICE AND RESPONSE TO IP CHALLENGE[a] WITH ZH-501 STRAIN RVF

GROUP (n)	VACCINE	LIPOSOME PREP.[b] NO.	CP 20.961 (0.1 mg/kg)	MEAN TITER ON DAY 28	MORTALITY %
1 (33)	+	DPPC:Chol:DPPA	-	5120	10
2 (20	+	DPCC:Chol:DPPA	+	2560	0
3 (33)	+	DMPC:Chol:DCP	-	1280	0
4 (20)	+	DMPC:Chol:DCP	+	1280	0
5 (33)	+	DPPC:Chol:DPPA, washed	-	640	0
6 (33)	+	DMPC:Chol:DCP, washed	-	80	25
Controls					
7 (33)	+		-	1280	6
8 (33)	+	Intralipid	+	1280	15
9 (11)	·	Saline	-	<10	100

[a] 380 LD_{50} on day 18 after vaccination

[b] DPPC = L-α-dipalmitoyl phosphatidyl choline
Chol = Δ^5-cholesten-3-ol, 99% pure
DPPA = L-α-dipalmitoyl phosphatidic acid
DMPC = L-α-dimyristoyl phosphatidyl choline
DCP = Dicetyl phosphate

TABLE VIII. MIGRATION RADII OF INDICATOR MACROPHAGES IN AGAROSE DROPLET MIF ASSAY PERFORMED WITH HAMSTER SPLEEN CELL SUPERNATANTS (SAME GROUPS AS TABLE VII)

DAY	GROUP	MEAN MIGRATION RADII ± SE		P < 0.05[a]
		Control	Antigen	
0	CP 20,961	1.5 ± 0.2	3.4 ± 0.5[b]	0.0002
	LE + RVF	3.9 ± 0.4	3.2 ± 0.3	
	RVF	4.5 ± 0.4	4.0 ± 0.4	
	Saline	4.1 ± 0.4	3.6 ± 0.3	
1	CP 20,961	1.9 ± 0.1	1.8 ± 0.2[b]	0.000001
	LE + RVF	2.6 ± 0.2	2.8 ± 0.2	
	RVF	1.3 ± 0.2	1.0 ± 0.1	
	Saline	1.2 ± 0.1	2.0 ± 0.2	0.00007
2	CP 20,961	1.6 ± 0.4	1.3 ± 0.3	
	LE + RVF	1.2 ± 0.2	2.7 ± 0.8[b]	0.03
	RVF	1.4 ± 0.2	1.1 ± 0.2	
	Saline	-	-	
3	CP 20,961	1.9 ± 0.4	1.5 ± 0.3	
	LE + RVF	1.4 ± 0.2	4.0 ± 1.1[a]	0.01
	RVF	1.7 ± 0.2	1.4 ± 0.2	
	Saline	1.9 ± 0.2	1.7 ± 0.2	
4	CP 20,961	1.5 ± 0.2	1.4 ± 0.1	
	LE + RVF	1.5 ± 0.2	1.4 ± 0.2	
	RVF	1.9 ± 0.2	1.7 ± 0.2	
	Saline	-	-	
7	CP 20,961	1.5 ± 0.2	0.9 ± 0.1[c]	0.0003
	LE + RVF	0.5 ± 0.2	0.3 ± 0.2	
	RVF	1.5 ± 0.2	1.3 ± 0.2	
	Saline	-	-	
10	CP 20,961	1.2 ± 0.2	0.5 ± 0.1[c]	0.005
	LE + RVF	0.2 ± 0.1	0.2 ± 0.1	
	RVF	1.3 ± 0.2	1.3 ± 0.2	
	Saline	-	-	
14	CP 20,961	1.0 ± 0.2	0.9 ± 0.2	
	LE + RVF	1.8 ± 0.3	1.6 ± 0.3	
	RVF	2.5 ± 0.2	2.7 ± 0.2	
	Saline	-	-	

[a] Student's t test, exact probability.

[b] Enhancement.

[c] Inhibition

Washed liposomes were protective but since the total mass was the same as injected in groups 1 and 3 minus external vaccine, one would expect comparable antibody titers and mortality if the lipid mass (incorporating vaccine) were increased to augment the total vaccine dose similar to that given in groups 1 and 3. Liposomal preparations were comparable to vaccine (Group 7) alone and CP 20,961 plus vaccine (Group 8) in terms of mortality.

Experiments with other adjuvants. Formalin-inactivated Japanese encephalitis (JE) vaccine (prepared by LTC Harrington) was evaluated for immunogenicity in mice with the following adjuvants: CP 20,961, poly(ICLC), Saponin Quil A, $Al(OH)_3$, carbopol 934P, Freund's incomplete adjuvant (FIA), and LE. The vaccine was poorly immunogenic; the experiment was confounded by deaths after challenge due to hypersensitivity reactions. It was determined that hypersensitivity was caused by fetal calf serum (30%) in the vaccine. Subsequent removal of fetal calf serum from the tissue culture-propagated challenge virus was accomplished by unltracentrifugation with a resultant loss of titer. The challenge inoculum contained only 1 LD_{50} of JE virus, thus negating all challenge results. Investigation of JE vaccine will be terminated until there is: (a) an immunogenic vaccine, (b) stabilization of vaccine with a minimal quantity of foreign protein, and (c) establishment of a consistently lethal viral challenge inoculum.

Collaborative studies were conducted:

(a) With B. Osburn, University of California, Davis, Use of LE as an adjuvant for Blue Tongue virus; (b) with Plum Island, A sheep adjuvant study with inactivated Rift Valley fever vaccine; and (c) A patient is being sought for lipid emulsion use with Dr. Di Luzio of Tulane University and CPT Reynolds.

Presentations:

None

Publications:

1. Reynolds, J. A., D. G. Harrington, C. L. Crabbs, C. J. Peters, and N. R. Di Luzio. 1980. Adjuvant activity of a novel metabolizable lipid emulsion with inactivated viral vaccines. Infect. Immun. 28:937-943.

2. Harrington, D.G., H. W. Lupton, C. L. Crabbs, C. J. Peters, J. A. Reynolds, and T. W. Slone, Jr. Evaluation of a formalin-inactivated Rift Valley fever vaccine in sheep. Am. J. Vet. Res. 41: in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6420	80 10 01	UD-DRAE(AR)635

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'S INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	017
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Include with Security Classification Code)

(U) South American hemorrhagic fevers; pathogenesis, therapy and immunization

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 06	30 09	DA	C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE

	PRECEDING/CURRENT	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	80	1.5	400
	81	0	0

19a. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Provide KEY with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Argentine hemorrhagic fever; (U) Bolivian hemorrhagic fever; (U) Vaccine development

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

23 (U) Develop vaccine against Argentine hemorrhagic fever (AHF) that protects against AHF and Bolivian hemorrhagic fever. Develop test protocols for genetic stability, neurovirulence and peripheral virulence. Investigate cellular and possible autoimmune mechanisms of neurovirulence. Vaccines and methods of treatment are essential for these highly militarily important diseases.

24 (U) Develop methods of enrichment for possible attenuated strains of Junin virus. Isolate clones of virus and make virulence comparisons among candidate strains.

25 (U) 79 10 - 80 09 - Virus strains were isolated from 13 acute human sera and tested for virulence. One was clearly less virulent and is being passaged in cell culture for further attenuation. An already partially attenuated strain was further passaged and several clones isolated. Three vaccine candidate subpassages were developed and compared for virulence characteristics. All candidates were more attenuated than XJ clone 3, an experimental, but unsatisfactory, vaccine used in 500 persons in Argentina 10 years ago. Preliminary neurovirulence studies also suggest a more attenuated virus than XJ clone 3. More extensive neurovirulence, peripheral virulence and genetic stability studies are in progress. Clones are being isolated from the least virulent of the 13 human isolates.

Terminated for management efficiency. Continued in W.U. 871 BC 148. (DAOG1537)

* Available to contractors upon originator's approval

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BODY OF REPORT

Project No. 3M162770A871: (3M162776A841) Prevention of Military Disease Hazards (U)

Task No: 3M162770A871 BC: Prevention of Viral Diseases of Potential BW Importance (U)

Work Unit No. 871 BC 137: (841 00 017) South American Hemorrhagic Fever; Pathogenesis, Therapy and Immunization

Background:

Argentine hemorrhagic fever (AHF) and Bolivian hemorrhagic fever (BHF) are clinically indistinguishable diseases caused by closely related viruses, which can be differentiated only by a virus neutralization test. Although antisera against individual virus types will not cross-protect, we developed data in earlier reports that an attenuated Junin virus, the etiologic agent of AHF, protects monkeys against experimentally inoculated Machupo virus or BHF. On this basis a project was initiated to develop a vaccine against AHF suitable for us in humans, which hopefully would protect against both AHF and BHF. The Argentine Secretariat of Health with support from the United Nations Development Program and the Pan-American Health Organization established a collaborative program with USAMRIID to carry out the project. An Argentine scientist, Dr. Julio Barrera-Oro has worked at this Institute since April 1979 on this effort in collaboration with the principal investigator. This report summarizes the results through the reporting period.

Progress:

Two lines of endeavor were undertaken. The first was to identify an already attenuated strain of Junin virus that might be used as the parent virus for even further attenuation attempts. Since all such viruses have extensive passage experiences in laboratories, none would be as fully acceptable as a strain isolated from a human into certified cells and then attenuated. Nevertheless, it would provide a direct way to a highly attenuated virus that could be tested for acceptability by all of the appropriate methods.

An additional effort would be undertaken to develop a vaccine by more modern methods using virus isolated directly from man into certified cells. This would be passaged in a substrate that might enrich for attenuated variants and further attenuation attempts would be assessed. This second or alternate effort was intended to supplement the initial segment of the project in the event it was not successful.

Identification of an already attenuated Junin virus pool suitable for further attenuation and vaccine development. We determined that there were 4 possible virus pools to be considered for selection as the starting material. These are shown in Table I together with their characteristics or advantages for selection.

Although Clone 3 was inoculated experimentally into 600 persons 10 years ago without significant adverse reactions, its passage history was not known with certainty. There is a strong possibility that it was passaged in a

continuous cell line and would not be a suitable vaccine candidate. An equally attenuated strain with a known passage history would be better. For this reason the XJ-44 was selected. Data in Table II indicate that it is as attenuated as Clone 3 and that both are more attenuated than XJ-13. The pool, XJ-102, was not considered because of its even higher mouse passage level than XJ-44. Thus, insofar as guinea pig virulence is concerned, XJ-44 is as attenuated as the virus inoculated into 600 persons 10 years ago. Because of its better defined passage history, attempts at further attenuation were directed at XJ-44.

TABLE I. CHARACTERISTICS OF 4 JUNIN VIRUS POOLS FOR CONSIDERATION AS VACCINE "PARENT" VIRUS

DESIGNATION	PASSAGE HISTORY	ADVANTAGES/ DISADVANTAGES
Clone 3	Guinea pig, 11 Mouse, 13 Cell culture, unknown	Virus was given experimentally to 600 persons in Argentina 1969-1971
XJ-13	Guinea pig, 11	Relatively low passage but virulent
XJ-44	Guinea pig, 11	Attenuated virus at intermediate passage level
XJ-102	Guinea pig, 11 Mouse, 102	Attenuated virus at high passage level

Further attenuation. The pool XJ-44 was subpassaged 6 different ways in an attempt to enrich for more attenuation. The virus was first tested for adventitious mouse agents and found to be negative; it was then passaged in either MRC-5 cells (a diploid human cell) or FRhL cells (a diploid fetal rhesus lung cell line). One passage line in each cell was carried out at 36 C and

TABLE II. LETHALITY OF 3 JUNIN VIRUS POOLS FOR BABY GUINEA PIGS

VIRUS	DOSE (PFU)	DEAD/TOTAL	%
Clone 3	3000	7/20	35
XJ-13	9000	17/20	85
XJ-44	11,000	6/20	30

another at 32 C. The FRhL cell passages were carried out at either undilute (Hi MOI) or 1:1000 dilution passages (Lo MOI). The MRC-5 cell-passage series was passaged at a 1:10 dilution in most instances. Thus, the 6 passage series were as follows: MRC-5/36 C, MRC-5/32 C, FRhL Hi MOI/36 C, FRhL Lo MOI/36 C, FRhL Hi MOI/32C, and FRhL Lo MOI/32 C. After 8 - 12 passages these subpassages were assessed for virulence in 8-16-day-old baby outbred guinea pigs. The results shown in Table III suggested that little attenuation occurred with passage and FRhL passages were possibly more attenuated than MRC-5. Therefore, passage 12 in FRhL cells at a high multiplicity of infection (MOI) at 36 C was used for deriving clones.

TABLE III. LETHALITY OF VARIOUS SUBPASSAGES OF JUNIN VIRUS FOR BABY (8-16 DAY) OUTBRED GUINEA PIGS

EXP. NO.	VIRUS	DOSE (PFU)	DEAD/TOTAL	%
1	XJ-44	7,000	5/20	(25)
	MRC-5/32 C	40,000	12/20	(60)
	FRhL Hi MOI/36 C	40,000	3/20	(15)
	3551 (virulent)	40,000	10/10	(100)
2	XJ-44	20,000	4/17	(24)
	MRC-5/36 C	20,000	6/20	(30)
	FRhL Lo MOI 36 C	60,000	5/17	(29)
	3551 (virulent)	20,000	9/10	(90)
3.	XJ-44	6,000	1/19	(5)
	FRhL Lo MOI/32 C	20,000	5/17	(29)
	FRhL Hi MOI/32 C	6,000	6/19	(32)
	3551 (virulent)	20,000	10/10	(100)

Cloning of Junin virus. Different methods were used in an attempt to clone the virus after 8 - 12 passages in FRhL cells: terminal dilution, plaque isolation, and the pseudo-single burst (PSB) method. We found it impossible to form plaques on FRhL cells with Junin virus; therefore, we used only terminal dilution or PSB.

Terminal dilution involved making serial 2-fold dilutions near the infectivity endpoint of the virus pool using 20 flasks/dilution seeking the highest dilution infecting the fewest flasks. The PSB method was done by infecting cells in suspension with approximately 50 PFU, plating 20,000 cells on feeder layers of FRhL cells and harvesting the fluids on day 3 or 4.

We isolated 6 clones by terminal dilution and 13 by PSB; all were tested in baby guinea pigs with particular attention given to those not producing paralysis. The results were as follows: 5 of 6 terminal dilution clones induced paralysis and 2 of 13 PSB clones induced paralysis. We therefore concentrated our efforts on the PSB clones. Our attempts to assess virulence or attenuation are now

directed toward the use of suckling mice which permit a more quantitative assay. In particular we are interested in 3 PSB clones which produce no paralysis and relatively low mortality in baby guinea pigs in the several experiments. These are designated candidates 1, 2 and 3. They will be further assessed and reported.

Virulence comparisons of Junin virus isolates from AHF patients. Thirteen isolates from humans were passaged 2 times in FRhL cells; we inoculated each of the resulting pools into adult guinea pigs from the same source as the baby guinea pigs. Results in Table IV show that all of the human isolates were virulent for adult guinea pigs without regard to severity of the human illness. Virtually all of the low passage human isolates were uniformly virulent for adult guinea pigs; there were only occasional survivors. There were differences in virulence, however, which appeared to be reflected in mean time to death. For example, the Romero strain killed all guinea pigs tested with the mean day of death being approximately day 13.

One of the low-passage human isolates was relatively less virulent for guinea pigs. The Coronel strain killed only 55% of inoculated guinea pigs, and the mean day of death was later than for any other strain. This is encouraging in that it appears that this method may occasionally reveal a partially attenuated strain. The problem is that this strain was isolated from a patient who subsequently died following a severe, mixed, hemorrhagic and neurologic clinical course.

TABLE IV. VIRULENCE COMPARISONS IN ADULT (350-g) GUINEA PIGS OF VARIOUS ISOLATES OF JUNIN VIRUS FROM HUMANS ILL WITH AHF[a]

VIRUS STRAIN	CLINICAL FORM (OUTCOME)	DEAD/ TOTAL	MEAN DAY OF DEATH ± SD
Suarez	Mild (Recov.)	20/20	25.4 ± 6.4
Alberico	Mild (Recov.)	18/20	23.4 ± 6.8
Romero	Common (Recov.)	20/20	13.5 ± 1.8
Reina	Severe, Neuto (Recov.)	20/20	16.1 ± 2.4
Quinteros	Severe, Mixed (Recov.)	20/20	19.8 ± 3.9
Contreras	Severe, Neurol. (Fatal)	18/20	20.2 ± 3.0
Coronel	Severe, Mixed (Fatal)	11/20	26.2 ± 3.6
Portillo	Severe, Mixed (Fatal)	20/20	18.1 ± 7.9
Espindola	Severe, Hemorr. (Fatal)	Now on test	
Ledesma	Severe, Neurol. (Fatal)	20/20	16.3 ± 3.2
Posadas	Severe, Neurol. (Fatal)	20/20	19.4 ± 4.5
XJ-44	Attenuated Strain	2/20	25.5
3551	Severe, Hemorr. (Fatal)	20/20	20.5 ± 5.0
3551	Exp. 2	8/9	19.0 ± 2.6
3551	Exp. 3	8/10	22.4 ± 6.4
3551	Exp. 4	Now on test	
3551	Pass 8	17/20	19.1 ± 2.9

[a]Viruses were isolated from humans and passaged 2 times in FRhL cells except as noted.

This may be telling us that the severity of human illness is partly a function of host factors that play a role in ameliorating or aggravating an otherwise typical virus infection. Alternatively, it may be telling us that the guinea pig is not a precise model for assessing virulence of arenaviruses for humans. The Coronel strain was selected for attempted enrichment and attenuation. At the present time we are passaging this virus in chicken embryo cell cultures and plan to isolate clones after several passages and assess their virulence.

Publications:

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6428	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GROUP MGT'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	020
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Provide with Security Classification Code):

(U) Microbial toxins and their role in the pathogenesis of disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS:

003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 10	80 09	DA	C. In-house

17. CONTRACT/GRANT

a. DATE/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: NA d. AMOUNT:

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 80	3.0	211
	CURRENT 81	0	0

19a. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Provide KWOC with Security Classification Code):

(U) Military medicine; (U) BW defense; (U) Botulism, (U) Toxoid; (U) Clostridium botulinum

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

23 (U) Study production and purification and characterize microbial toxins. Use the purified toxins to produce toxoids which can then be tested for safety and efficacy as new immunizing agents. This work unit is an essential element in a comprehensive program for medical defense against BW agents because it is aimed at developing and testing new toxoids for use in military forces.

24 (U) Purify neurotoxins of Clostridium botulinum types A-C. Use these to develop a polyvalent toxoid.

25 (U) 79 10 - 80 09 - Various strains of C. Botulinum, Type G, were characterized as to their somatic antigens, optimum temperature for growth, and purification methods. This work unit is terminated due to retirement of the principal investigator. Further work will be reported under W.U. 871 BA 150 (TAOG3810).

*Available to contractors upon originator's approval.

DD FORM 1 MAR 88 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, NOV 85 AND 1498-1, 1 MAR 88 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871-BA: Prevention of BW Diseases Caused by Microbial Toxins

Work Unit No. 871-BA-121: Microbial Toxins and Their Role in the Pathogenesis
(841 00 020) of Disease

Background:

Bacterial products play a major role in the production of disease. It has been long recognized that the toxin of Corynebacterium diphtheriae produces all the symptoms of the clinical disease diphtheria and that antibody to the toxin will completely prevent the illness, even in the presence of the organism. Other toxins such as the enterotoxins of Staphylococcus aureus and the neurotoxins of Clostridium botulinum are taken into the host preformed. During the past year all efforts have been extended in the area of C. botulinum toxins.

A polyvalent toxoid was prepared in 1958 by Parke, Davis, and Co. under contract to Fort Detrick. This toxoid contains antigens to types A, B, C, D, and E neurotoxins. At the time of its preparation full knowledge of the neurotoxin was not available; the preparation contains less than 10% of the desired immunogens. Mild side reactions including tenderness, redness, heat, and swelling at the site of the injection are common. The basic course to produce satisfactory antibody titers requires 4 injections over a period of 1 year. In addition, little scientific investigation has been achieved to improve the production and purification of adequate amounts of pure neurotoxins to prepare a new toxoid.

Measurement of antibodies following immunization is accomplished by mouse neutralization test. This test has the inherent problems of an animal assay system and in addition requires large numbers of mice.

Treatment of C. botulinum intoxication consists of complete respiratory support and neutralization of circulating toxin. The antiserum currently in use for neutralization of toxin is of equine origin and has a high reaction rate due to sensitivity to horse proteins. (The only U.S. source of equine antiserum notified Center for Disease Control in July 1978 that they would no longer provide this product.)

Progress:

Studies on toxin production utilizing a fermentor system and purification investigations of types A and B are reported under work unit 871-BA-124. Studies on production of antitoxin (human and equine), toxoid evaluation, and the ELISA test are reported under work unit 871-BA-123.

C. botulinum, type G strains isolated from autopsy cases in Switzerland were compared with the original 2 isolates from soil samples in Argentina. Strain 714 and 1354 were from Argentina, while strains 2738-2742 were from Switzerland.

Strains 714 and 2740 had similar activation curves when the crude culture filtrate was treated with 100 µg/ml trypsin. The other strains showed a much lower degree of trypsin activation. Protease activity determined by casein digestion utilizing crude culture supernatants confirmed the high protease activity of strains 2738, 2739, 2741, and 2742, while strains 714 and 2740 were of low. Temperature and incubation time for optimum toxin production were similar in all strains.

Strain 714 was used as the prototype in most investigations. The crude culture supernatant was produced utilizing the dialysis sac method. The medium consists of 4% proteose peptone, 1% trypticase soy broth, 1% yeast extract and 1% dextrose. The frozen stock (2 ml) was utilized to inoculate 20 ml of cooked meat medium; after 24 h incubation at 37°C, this supernatant (10 ml) was used to inoculate the dialysis sac culture, which was incubated at 25°C for 10-14 days. Titers achieved varied between $10^{6.5}$ and 10^{7} LD_{50}/ml (after trypsin activation).

Two goats were immunized with formalin-toxoided alum-adsorbed crude culture supernatant. The initial injection for each goat included complete Freund's adjuvant. Antibodies were determined by mouse neutralization test. Unitage was established on the basis that 1 unit would neutralize 10,000 LD_{50} of toxin. After antibodies were detectable in the serum of the goats, repeated booster injections of toxin were administered s.c. Goat #19 was exsanguinated and all serum was lyophilized in suitable aliquots. The serum labeled Lot #2 is the standard antiserum for neutralization tests and Ouchterlony diffusion studies.

Antibodies to the somatic antigens of type G were elicited in a rabbit by injection of washed, boiled vegetative organisms. This antiserum agglutinates not only type G cells but also agglutinates proteolytic strains type A, B, and F. No agglutination occurs with type C, D, or E. Type G is weakly proteolytic, but represents the only proteolytic form which requires trypsin activation for full expression of toxicity. Type G has usually been classed as a fourth group of C. botulinum, but it appears that it may fall into the first group with the other proteolytic strains.

Antiserum to crude type G toxin (Goat #13), when added to blood agar base, forms a useful method for identification of type G organisms. After 48 h incubation under anaerobic conditions, type G colonies are surrounded by a halo of immunoprecipitate. All strains of type G tested respond in this manner, while all other types are negative. This immunoprecipitate was not identified as neurotoxin, but rather represents one of the other extracellular proteins unique to type G.

Repeated attempts to utilize standard chromatographic and precipitation methods developed for the other types of C. botulinum were unsuccessful with type G. Preliminary studies with dye-matrix gels (Amicon) revealed that type G toxin bound to Blue A, Green A and Red A gels. The toxin could be eluted from these gels by addition of 0.5 M NaCl to the eluting buffer. The procedure was standardized as follows:

1. Dialyze the crude culture supernatant against 0.05 M citrate, pH 5.5.

2. Pour a Red A dye-matrix gel column.

3. Elute unbound dye with 8 M urea and then equilibrate with 0.05 M citrate buffer.

4. Run the crude culture supernatant through the column at a flow rate of 0.5-1.0 ml/min.

5. Wash column with 0.05 M citrate buffer until OD = 0 at 280 nm.

6. Elute hemagglutinin and other impurities with 0.5 M NaCl in buffer.

7. Elute toxin with 1.5 M NaCl in buffer.

The 1.5 M NaCl fraction contained 2 distinct proteins. The specific toxicity with trypsin activation is $10^{6.3}$ LD_{50}/ml. This material, when chromatographed on Sephracyl S-300, revealed a single symmetrical peak at approximately 150,000 MW. Toxicity was distributed uniformly throughout the peak. Chromatography of the partially purified toxin (after Red A) on DEAE-Sepharose equilibrated with 0.15 M Tris-HCl, pH 8.0, was accomplished. Elution with 0.1 M NaCl resulted in the elution of an asymmetrical peak with an initial shoulder. The initial shoulder contained the neurotoxin, while the major peak contained no contaminating protein. Complete purification of toxin has not been accomplished.

The work unit is terminated because of retirement by the principal investigator.

Publications:

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

				DA 0B6423	80 10 01		
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	

	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	026
b.				
c.	STOG 80-7.2:2			

(U) Effectiveness of selected antiviral compounds against diseases of BW importance

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

14. START DATE	15. ESTIMATED COMPLETION DATE	16. PERFORMING AGENCY	17. PERFORMANCE METHOD
70 12	80 09	DA	C. In-house

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS ($ in thousands)
FISCAL YEAR 80	1.0	327
FISCAL YEAR 81	0	0

19. a. DATE EFFECTIVE: b. NUMBER: c. TYPE: NA d. AMOUNT: e. CUM. AMT.

20. PERFORMING ORGANIZATION
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21. PERFORMING ORGANIZATION
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24. GENERAL USE
Foreign intelligence considered

25. ASSOCIATE INVESTIGATORS
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26. KEYWORDS
(U) Military medicine; (U) BW defense; (U) Chemotherapy; (U) Togaviruses; (U) Arenaviruses; (U) Metabolism; (U) Bioavailability; (U) Monkeys

27. TECHNICAL OBJECTIVE, 28. APPROACH, 29. PROGRESS

23 (U) Evaluate potential antiviral compounds with significant antiviral activity against infectious diseases in animals. Experimental diseases selected for study are important human pathogens of military concern, and the information obtained will be invaluable in control and treatment of viral diseases in military personnel, emphasizing those of possible BW importance.
24 (U) Test candidate compounds against selected viruses in tissue culture, rodents and other subhuman primates.
25 (U) 79 10 - 80 09 - A complete pharmacokinetic evaluation of ribavirin was undertaken in monkeys and guinea pigs. This antiviral agent was shown to concentrate in red blood cells (RBC) but failed to saturate RBC even after multiple doses for 72 hr. Low levels of ribavirin were detected in the cerebrospinal fluid although in lower concentration than that of plasma. Ribavirin therapy at very high doses for 7 days caused a reduction in circulating RBC and the hematocrit. This was always followed by a significant rise of reticulocytes upon termination of treatment. Other hematological effects of ribavirin therapy were an increase in platelet count and decrease in hemoglobin. All effects were reversed to normal levels within 1-2 weeks after cessation of drug treatment.
Publication: In Ribavirin--A Broad Spectrum Antiviral Agent, pp 169-183, 1980.
Terminated for management efficiency. Continued in W.U. 871 BE 1.3 (DAOG3815).

30. AVAILABILITY

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

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162760A841)

Task No. 3M162770A871 BE: Exploratory Antiviral Drug Development

Work Unit No. 871 BE 144: Effectiveness of Selected Antiviral Compounds against
(A841 00 026) Diseases of BW Importance

Background:

Vaccines are not available for the prevention of most virus infections of military importance. Consequently, a program was undertaken to evaluate the potential for use of selected chemotherapeutic agents as an alternate or adjunct method to prevent or treat salient virus infections.

During the brief duration of this program, infection models have been established in rodent and subhuman primate species to simulate comparable disease entities seen in man. These models have been utilized successfully to demonstrate the great potential of selected antiviral drugs. The recent approval for use of adenine arabinoside for use against herpes encephalitis and the broadening of approval for use of amantadine against influenza virus in man increases the probability that new antiviral drugs will become clinically useful in the treatment of virus-induced diseases of military importance.

Progress:

Evaluation of the pharmacokinetics of ^{14}C-labeled ribavirin in rhesus monkeys was carried out in 2 groups of monkeys given 10 mg/kg 3X/day and 30 mg/kg daily for 3 days. Results revealed that at 24, 48 and 72 h. there was little difference in whole blood or blood cell radioactivity of ^{14}C-ribavirin between groups. The daily 1-dose regimen appears to be preferable since higher blood levels are reached sooner and remain high for at least 24 h. After 72 h of dosing (90 mg/kg) not only was there no indication of saturation of blood cells, but there was only a slight decrease in the rate of uptake of ^{14}C-ribavirin, indicating that the blood cell values were still well below saturation. Serial blood samples taken through day 30 and liver biopsies taken at days 3 and 30 revealed a constant rate of disappearance of ^{14}C-ribavirin (and metabolites) from the blood and a similar rate of disappearance from liver tissue. Further multiple dose experiments over a longer period will be required to determine if saturation of blood cells occurs and what the subsequent effects would be.

Previous experiments have shown that ribavirin effectively prevents the acute hepatitis associated with Rift Valley fever (RVF) virus infection of BALB/c mice, but that at certain doses mice die of late encephalitis. This effect is demonstrated in Table I. Only 20% of untreated control mice survived to day 5 with 4% survival on day 21. The lowest doses of ribavirin, 10 mg/kg, delayed the time to death with deaths occurring primarily due to encephalitis. The higher doses, 40 or 50 mg/kg, delayed the time to death even longer. Doses as high as 100 mg/kg are required to provide complete protection.

TABLE I. EFFECT OF RIBAVIRIN DOSE ON SURVIVAL OF RIFT VALLEY FEVER VIRUS-INFECTED BALB/c MICE (n = 25)

DAY AFTER VIRUS INOCULATION	% SURVIVED BY DOSE OF RIBAVIRIN (mg/kg)					
	0	10	20	30	40	50
5	20	92	100	96	96	
10	8	44	68	96	96	
21	4	16	44	48	68	

Ribavirin was evaluated further in mice infected with RVF by either the IC or SC route. Ribavirin was given SC at 50 mg/kg twice daily beginning 1 day prior to virus inoculation and continuing through day 14. No protection or delay in time to death was noted in mice challenged by the IC route. On the other hand, mice given 100 times as much virus by the SC route were protected by this regimen. Similar results were obtained in other studies using VEE virus infection of Syrian golden hamsters. In this experiment hamsters were not protected, thus confirming in another model that alphaviruses are resistant to the effects of ribavirin in vivo.

Experiments using ^{14}C-labeled ribavirin were continued in monkeys and guinea pigs. Multiple dose experiments in rhesus monkeys were extended to 6 days, since the preliminary experiment failed to show saturation of red blood cells by 72 h. In addition to periodic blood sampling, cerebrospinal fluid (CSF) and liver were sampled. Disappearance of the drug from the liver was similar to that previously reported. Further, low values of ribavirin were detected in the CSF. The concentration in CSF was somewhat lower than that of plasma.

A study was completed to evaluate the hematological response of monkeys given a high dose of ribavirin (100 mg/kg/day in divided doses every 12 h by the IM route). Significant reduction occurred in the red blood cell count and hematocrit by day 7 of treatment. Treatment was continued to day 14, at which time RBC counts and hematocrit had continued to fall and reticulocytes were detectable. Nucleated red cells were present in smears at this time as well. This increase in reticulocytes was visualized both in the mean corpuscular volume and in the size distribution as recorded by the use of the Coulter channelizer. The platelet count increased significantly within 1 week after initiation of drug treatment. Platelet counts continued to increase for 1 week following termination of drug treatment with individual platelet counts reaching 10^6/ml. Platelets increased in size with the largest platelets causing an aberrant peak in the red cell distribution curves of treated monkeys. This was seen as a small peak in the 5-μ range. By the 3rd week after the last dose there was a significant reticulocytosis reaching a mean peak of 7.8 ± 1.1. Red blood cell counts and hematocrits were not different from the saline control monkeys at this time. Platelet counts returned to the preexperimental values by week 8.

A study was performed to assess in monkeys the IV administration of ribavirin as proposed by CDC for use in human subjects with clinical signs of Lassa fever. A mg/kg comparison to monkeys was made using the human dosage regimen of 2 g IV initially, followed by 1 g IV every 6 h for 4 days, with the dosage then reduced to 0.5 g every 8 h for the remaining 6 days.

Drug-treated and saline control groups were used; each group consisted of 3 rhesus monkeys (2 female and 1 male). An 8 ml blood sample was drawn from the femoral vein of each monkey weekly beginning two weeks prior to initiation of the study and twice weekly upon initiation of therapy. Sampling was continued for 4 weeks after discontinuance of therapy.

This dosage regimen of ribavirin reduced red cell parameters but not to the extent anticipated. Drug treatment reduced the erythrocyte count of treated animals to 56% of that seen in controls; the packed red cell value for the former was 22% and 39% for the latter. Hemoglobin value for the treated group dropped from approximately 12 g/dl to slightly over 7 g/dl. These values reached their nadir between 3 and 7 days after conclusion of the treatment regimen and approached normal values in 2 to 3 weeks. Reticulocytes increased 5-fold in this latter time period. Total WBC was unchanged; however, the platelet count tripled The differential count and serum chemistries have not been completed.

Studies were initiated to develop a radioimmunoassay for detection of ribavirin in body fluids. Ribofuranosyl-triazole carboxylic acid, a ribavirin metabolite, was combined with poly-l-lysine, in an attempt to generate a molecule large enough to be antigenic and have specificity for the ribavirin molecule. In a preliminary study this preparation was administered to a rabbit at a regimen optimized to induce antibody production. Results failed to show antibody production to the preparation. The study was repeated using a lipid emulsion adjuvant with similarly negative results.

Little acute toxicity data exists in support of parenterally administered ribavirin. Consequently a study was conducted to establish the acute toxicity and dose-response curve of IP administered ribavirin in mice. Data on file at the FDA in support of ribavirin suggested the LD_{50} of the drug via this route in mice to be between 60 and 240 mg/kg. On this basis, our study was designed using groups of 10 male mice for each of 7 treatment groups (0, 40, 80, 120, 160, 200 and 240 mg/kg). Mice were observed for 10 days; none showed any signs of drug-induced toxicity. The LD_{50} in mice is apparently considerably higher than originally anticipated.

Presentation:

Stephen, E. L. Chemotherapeutic agents - review of compounds. Presented, Workshop on Development of Antiviral Drugs for the Prevention and Treatment of High Hazard Viruses held at USAMRIID, 14-15 Jan 1980.

Publication:

Stephen, E. L., D. E. Jones, C. J. Peters, G. A. Eddy, P. S. Loizeaux, and P. B. Jahrling. 1980. Ribavirin treatment of toga-,arena-,and bunyavirus infections in subhuman primates and other laboratory animal species, pp. 169-183. In Ribavirin - A Broad Spectrum Antiviral Agent (R. A. Smith and W. Kirkpatrick, eds). Academic Press, New York.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6427	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GROUP MEMB'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	029
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE: (U) Physiological aspects of drug therapy during infection of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 02300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
76 10	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS ($ in thousands)
PRECEDING 80	0.5	195
CURRENT 81	0	0

19a. RESPONSIBLE DOD ORGANIZATION
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20. GENERAL USE
Foreign intelligence considered

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

POC:DA

21. KEYWORDS: (U) Military medicine; (U) BW defense; interferon, ribavirin, diurnal body temperatures, blood volume, monkeys.

22. TECHNICAL OBJECTIVE, 23. APPROACH, 24. PROGRESS

23 (U) Determine and evaluate specific physiologic responses to new drug or chemical compounds of potential military application. Assess mechanisms for potentiating desirable and inhibiting undesirable actions of such drugs. Evaluate alterations in physiology or mechanism of pathogenesis induced by viruses and their modification through drug therapy.

24 (U) Develop techniques to measure various physiologic and biochemical changes in laboratory animals during selected viral infections. Evaluate toxicity of interferon and candidate antiviral compounds and their ability to prevent or modify the adverse virus-induced changes associated with infection.

25 (U) 79 10 - 80 09 - A single IV injection of human lymphoblastoid interferon in conscious, chair-restrained rhesus monkeys caused increases in rectal temperature and cardiac output; total peripheral resistance, hematocrit and plasma protein concentration decreased. Rectal temperature showed a biphasic change 6 hr post-interferon injection; all changes returned to preinjection levels within 24 hr. Using 14-C, pharmacokinetics of ribavirin were initiated in a Dutch rabbit. Ribavirin (10-40 micrograms/ml) did not alter red blood cell fragility after 2 or 24 hr incubation in vitro at room temperature. Diurnal changes in rectal and skin temperature were demonstrated in rhesus monkeys, suggesting that caution must be exercised concerning the consistent timing for recording and interpreting data. Techniques were developed to take a muscle sample (2-3 g) from anesthetized monkeys without loss of function. Blood, plasma and red blood cell volumes and capillary permeability were measured without net loss in rhesus monkeys.

Publications: Fed. Proc 39:989, 1980; Pharmacologist 22:306, 1980.

Terminated for management efficiency. Continued in W.U. 871 BC 148. (DAOG1537)

25. Available to contractors upon originator's approval

DD FORM 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.

BODY OF REPORT

Project No. 3M162770A 871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BE: Exploratory Antiviral Drug Development

Work Unit No. 871 BE 145: Physiological Aspects of Drug Therapy During Infection
(841 00 029) of Military Importance

Background:

Ribavirin has been shown to be a potential antiviral drug (1, 2). Since the evaluation of this compound is still in its experimental stage, the toxicity and side effects of ribavirin have not been thoroughly studied. However, ribavirin-induced anemia in rhesus monkeys has been demonstrated repeatedly by various investigators in the Institute.

Few studies reported normal values of rectal and skin temperatures in rhesus macaques. Comprehensive information on diurnal rectal and skin temperature changes in the nonhuman primate were also lacking. In a classical work, Galbraith and Simpson (3) measured the axillary temperature of monkeys (species unspecified) with clinical thermometers for recording diurnal changes. They found that when monkeys were kept continuously in the light, there was no regular diurnal change. The regular diurnal wave was observed only when monkeys were kept in 12 h of darkness and 12 h of constant light. The effect of light on diurnal changes in deep body temperatures was also demonstrated in caged capuchin monkeys (4). Since the rhesus macaque is commonly used as a primate model for the study of human fever, toxemia and infectious diseases, simple techniques for continuous measurements of rectal and skin temperatures are essential.

Using the dilution principle, Evans blue dye has been commonly used for the determination of plasma volume (5). Blood volume is calculated indirectly as the plasma volume/1 - hematocrit. Under these circumstances, only a limited number of indirect blood volume measurements may be made in small or hypovolemic animals. New techniques are needed for direct blood volume determinations without net blood loss.

Progress:

Part I. Interferon (IF)

The primary aim of this study was to establish a monkey model for studying certain physiological changes after an IV injection of human lymphoblastoid IF and determine its toxic effects.

Effects of a single IV injection of human lymphoblastoid IF (6.4×10^6 U/m^2 body surface area) were studied in 7 rhesus monkeys. Experimental results were compared with 7 control monkeys, injected IV with isotonic saline. The data revealed that the rectal and inner thigh skin temperatures and cardiac output increased significantly after IF injection; while hematocrit, plasma protein concentration and total peripheral resistance decreased (Tables I and II). However, these changes were transient and returned to baseline values within 24 h. No significant changes

were observed on blood pressures (systolic, diastolic, mean and pulse), heart rate, ECG, blood volume, hematologic variables, plasma glucose, plasma osmolality, and water and electrolyte changes in skeletal muscle. These findings suggest that caution must be exercised when IF is considered for the treatment of cancer or viral diseases.

TABLE I. EFFECT OF A SINGLE IV INJECTION OF INTERFERON (6.4×10^6 UNITS/m^2) ON RECTAL AND INNER THIGH SKIN TEMPERATURES IN CONSCIOUS, CHAIR-RESTRAINED RHESUS MONKEYS.

	TEMPERATURE (°C) ± SE			
	Rectum		Inner Thigh Skin	
HOURS	Control (n=7)	IF (n=6)	Control (n=7)	IF (n=7)
0	38.0 ± 0.2	39.1 ± 0.4	33.9 ± 0.4	33.2 ± 0.4
0.5	39.3 ± 0.3	39.5 ± 0.5	34.4 ± 0.5	34.7 ± 0.4
1	39.3 ± 0.3	39.7 ± 0.5	34.8 ± 0.3	34.1 ± 0.4
1.5	39.3 ± 0.3	39.9 ± 0.4	34.7 ± 0.4	34.4 ± 0.5
2	39.2 ± 0.3	40.2 ± 0.4*	34.8 ± 0.2	34.9 ± 0.5*
2.5	38.9 ± 0.3	39.8 ± 0.3*	34.7 ± 0.3	34.5 ± 0.6
2.75	38.7 ± 0.3	39.5 ± 0.4*	34.5 ± 0.3	34.4 ± 0.6
3	38.6 ± 0.4	39.5 ± 0.4	34.5 ± 0.3	34.2 ± 0.6
4	38.6 ± 0.5	38.8 ± 0.3	34.3 ± 0.3	33.6 ± 0.4
8	38.4 ± 0.6	38.0 ± 0.3	34.1 ± 0.3	33.6 ± 0.5
12	38.3 ± 0.3	37.4 ± 0.4	33.8 ± 0.5	32.7 ± 0.5
16	38.4 ± 0.4	37.1 ± 0.5*	33.2 ± 0.5	32.8 ± 0.6
20	38.8 ± 0.5	37.3 ± 0.6	33.1 ± 0.5	32.8 ± 0.6
24	38.9 ± 0.2	38.3 ± 1.0	33.7 ± 0.7	34.1 ± 0.6

*$p < 0.05$

Experiments on daily IV injection of IF interferon were started. Four control and 2 IF-treated monkeys were used. Due to technical and mechanical difficulties in keeping the monkeys chair-restrained, the longest period for chair restraint was 10 days with a minimum duration of 4 days. When leg edema, skin irriation at the sitting site, or hematoma occurred, or catheters came out of the blood vessels, the experiment had to be terminated. One of the treated monkeys died on day 5. Necropsy revealed that the monkey had enlarged lymph nodes throughout the body, particularly in the pelvis and the abdominal cavity. Nephritis, myocardial necrosis, hepatitis and enteritis were also observed. Because only one monkey died during interferon treatment, the cause of death may be coincidental and not to interferon toxicity.

Part II. Ribavirin

RBC fragility. As part of the study to investigate the mechanism by which ribavirin induced anemia, an in vitro test was performed to determine the effect of the drug on osmotic fragility of RBC. Rabbit RBC were incubated at room temperature in the presence of either 0, 10, or 40 µg/ml of ribavirin.

Osmotic fragility tests were performed at 2 and 24 h after incubation at 23 C on each of the 3 concentrations. In this single trial, there were no apparent differences in fragility between control and drug-exposed cells.

TABLE II. EFFECTS OF A SINGLE IV INJECTION OF INTERFERON (6.4×10^6 UNITS/m^2) OF CONSCIOUS CHAIR-RESTRAINED RHESUS MONKEYS (n = 7/GROUP)

GROUP	BASELINE	HOURS POSTINFECTION 0.5	3	6	24
Cardiac output (ml/min/kg)					
Control	160 ± 15	184 ± 16	142 ± 12	140 ± 12	145 ± 17
IF	177 ± 11	207 ± 17	170 ± 19	192 ± 15*	178 ± 17
Total peripheral resistance (Dyne·sec/cm^2 x 10^5)					
Control	.104 ± .006	.096 ± .010	.104 ± .005	.103 ± .008	.106 ± .011
IF	.091 ± .008	.076 ± .009	.086 ± .010	.074 ± .009**	.079 ± .011
Hematocrit (%)					
Control	39.3 ± 1.3	39.1 ± 1.6	36.6 ± 1.5	38.5 ± 1.3	36.2 ± 1.7
IF	37.0 ± 1.9	34.4 ± 2.1*	32.6 ± 2.5	28.3 ± 5.2	33.9 ± 1.6
Plasma protein (g/dl)					
Control	6.5 ± 0.1	6.2 ± 0.1	5.8 ± 0.1	not done	6.4 ± 0.2
IF	6.6 ± 0.2	5.9 ± 0.2*	5.7 ± 0.2	not done	6.1 ± 0.2

*P < 0.05
**P < 0.01

Pharmacokinetics. To study pharmacokinetics of any given drug in a biological system, the separation of a parent compound from its derived metabolites and the chemical identification of each major component are absoluteiy essential. Since we do not have the proper equipment and expert working experience in these areas, possible collaboration with Frederick Cancer Research Center (FCRC) has been explored. In preliminary discussions it was decided that plasma and tissue samples containing [^{14}C] ribavirin and its metabolites were needed in trial experiments for the separation and identification of various compounds of the drug.

To achieve this goal, 10 µCi of [^{14}C] ribavirin were injected IV into a Dutch rabbit. Plasma and RBC samples were taken from the common carotid artery through a cannula at 0, 0.5, 1, 2, 3, and 4 h after injection of the [^{14}C] ribavirin. At the end of 4 h, the rabbit was killed with an overdose of Innovar-Vet and 3 tissue samples, liver, left ventricular muscle and renal cortex were excised. RBC and all tissue samples were extracted by 10% TCA (1:4, w/v). All samples were counted and preliminary results are summarized as follows: (a) radioactivity disappeared rapidly from plasma; multiple components of the disappearance curve were obtained; (b) the exponential disappearance rate of ^{14}C in RBC was linear as a function of time, but slower than that of plasma. Furthermore, DPM/ml in RBC were always higher than in plasma at any given time interval; and (c) liver contained the highest radioactivity followed by kidney and heart.

To search for the best solvent for extracting ^{14}C-ribavirin and its metabolites from tissues, liver samples were homogenized in 10% TCA, methanol and acetonitrile. Counts of liver extracts in the various tissue solvents decreased according to the following sequence: 10% TCA, 5% TCA, methanol, and acetonitrile. Although a complete extraction can be achieved by using 10% TCA, it is unknown whether any further breakdown of the drug and its metabolites may be induced in vitro under the influence of strong acidity.

Part III. Diurnal Changes in Rectal and Skin Temperatures.

A rhesus monkey model was used to establish baseline diurnal rectal and skin temperature values. Under keatmine sedation calibrated disc electrodes were sutured to various skin sites (inner thigh, back, abdomen and top of head). Monkeys were restrained in primate chairs in an isolated room maintained at 23.3-24.4°C. Following recovery from sedation, skin temperatures were recorded with a Honeywell recorder connected via thermocouple wire to the disc electrodes. Rectal temperatures were determined with a stainless steel probe and recorded with a PDP-11 minicomputer connected via a telethermometer and Brush recorder. Results from continuous 48 h recordings of 11 monkeys indicated that: (a) rectal temperatures (36.7-37.6°C) varied less than skin temperatures (33.8-36.4 °C); (b) maximal rectal temperatures were recorded at 0800 hours and maximal skin temperatures at 1100 and 1400 hours; (c) minimal skin and rectal temperatures were measured at 2100 and 2400 hours; and (d) similar results were observed when monkeys were exposed to light all or part of the time (16 hours) during a 48 h period. Based on these diurnal changes in skin and rectal temperature, caution must be exercised concerning the constant timing for recording and interpreting temperature data under various experimental conditions which induce fever.

Part IV. Development of Techniques Using Rhesus Monkeys

Muscle sampling. To study biochemical changes in the muscle of rhesus monkeys after IV administration of IF, relatively large tissue samples were needed. A surgical technique was developed which involved splitting one gracilis muscle lengthwise and excising the medial half. Using this approach, 2.5-3.0 g of muscle could be obtained without apparent loss of function in the leg. Biceps muscle was also excised with satisfactory results.

Determination of blood volume and capillary permeability without net blood loss. Blood volume is usually measured by IV injection of Evans blue ^{51}Cr-labeled RBC. A series of blood samples must be taken for determining concentration or radioactivity of the injected indicator. Our recently developed technique requires only a single IV injection of Evans blue (2.26 mg/monkey) and allows withdrawal of blood without net loss. The technique was to establish baseline values with each monkey's own blood by constant withdrawal of arterial blood (6 ml/min) through a cuvette with a 630-mμ filter into a syringe; blood was returned to the animal via the vein as soon as possible. The whole procedure was repeated 6 min after IV dye injection. When a 2-3 min disappearance curve of Evans blue was obtained, both the blood volume and capillary permeability could be determined. If only blood volume is measured, a short duration of constant dye concentration curve should be obtained. With an established calibration curve for the dye, blood volume can be calculated as: Injected μg/blood dye concentration (μg/ml).

Two linear standard curves have been demonstrated for dye concentration in arterial blood and in saline. Since the "saline curve," shifted to the right compared to the "blood curve," a common conversion factor was found to be 1.35 under the present experimental conditions (e.g., fixed setting for the densitometer and preamplifier). Thus, any given dye concentration (5-15 mg/L) in saline can be used for calculation of blood values by applying the conversion factor of 1.35. Because this technique for measuring blood volume is so simple and reproducible, it will be applied to measure blood volume in small animals, including rats, guinea pigs, or rabbits. In 14 monkeys studied, a mean blood volume was determined to be 71.4 $\pm$ 8.9 ml, which agrees well with published data (74.1 $\pm$ 2.3 ml) using classical techniques (6).

Presentations:

1. Robbins, V. W., R. P. Sanders, and C. T. Liu. Diurnal changes in rectal and skin temperatures in restrained and conscious rhesus monkeys. Presented, Annu. Mtg. FASEB, Anaheim, CA, 13-18 Apr 80 (Fed. Proc. 39:989, 1980).

2. Liu, C.T., V. W. Robbins, R. P. Sanders, M. J. Griffin, E. L. Stephen, and H. B. Levy. Effects of interferon on cardiohepatic functions in rhesus monkeys. Presented, Am. Soc. Pharmacol. Therapeutics, Rochester, MN, 17-21 Aug 1980 (Pharmacologist 22:206, 1980).

LITERATURE CITED

1. Becker, Y. 1975. Trends in research and development of antiviral substances. Isr. J. Med. Sci. 11:1135-1167.

2. Sidwell, R. W., R. K. Robins, and I. W. Hillyard. 1979. Ribavirin: an antiviral agent. Pharmacol. Therapeut. 6:123-146.

3. Galbraith, J. J., and S. Simpson. 1903. Conditions influencing the diurnal wave in the temperature of the monkey. Proc. Physiol. Soc. (London) xx-xxii.

4. Winget, C. M., D. H. Card, and N. B. Hetherington. 1968. Circadian oscillations of deep body temperature and heart rate in a primate (Cebus albafrons). Aerospace Med. 39:350-353.

5. Gibson, J. G., W. A. Evans, Jr. 1937. Clinical studies of the blood volume. I. Clinical application of a method employing the azo dye "Evans blue" and the spectrophotometer. J. Clin. Invest. 16:301-316.

6. Liu, C. T., M. K. Griffin and R. T. Faulkner. 1976. Effect of staphylococcal enterotoxin B on body fluid compartments in conscious rhesus monkeys. J. Med. Primatol. 5:336-344.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6420	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GROUP EXEMPTION	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10a. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
b. PRIMARY	62776A	3M162776A841	00	030
c. [illegible] /				
d. [illegible]	STOG 80-7.2:2			

11. TITLE: (U) Physiologically directed treatment of biological toxemias of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 08	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR: PRECEDING 80	0.5	103
FISCAL YEAR: CURRENT 81	0	0

19a. RESPONSIBLE DOD ORGANIZATION
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20. GENERAL USE
Foreign intelligence considered

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

POC:DA

21. KEYWORDS: (U) Military medicine; (U) BW defense; (U) Electrocardiogram; (U) Toxemias; (U) Cyclic AMP; (U) Chemoperfusion

22. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide brief, clear paragraphs identified by number. Precede text of each with security Classification Code.)

23 (U) Study physiological and biochemical responses in animals of selected bacterial military importance. Evaluate pharmacologic and/or physiologic means for toxemias.
24 (U) Evaluate effects of an IV injection of purified cholera enterotoxin on plasma and tissue levels of cyclic AMP. Treat SEB toxemia with hemoperfusion of activated charcoal, bicarbonate-induced alkalosis or tannic acid.
25 (U) 79 10 - 80 09 - Techniques for measuring electrocardiograms in sedated rhesus monkeys and a rat model for drug evaluation and toxin studies have been developed. Methods of inducing anesthesia of Dutch rabbits have been improved by injection of Innovar-Vet IV (0.08 ml/kg). A hemoperfusion system with activated charcoal was also developed for detoxification of drugs or chemicals in Dutch rabbits. No significant changes were observed in tissue concentrations of cAMP after an IV lethal dose of cholera enterotoxin (100 micrograms/kg) compared to control rabbits. A higher dose of cholera toxin increased cAMP levels in skeletal muscle, liver, lung, renal cortex, renal medulla, and spinal cord. Tannic acid (5 mg/kg) or bicarbonate-induced alkalosis (blood pH 7.5-7.6) provided no protection against SEB toxemia in Dutch rabbits. No SEB was bound to leukocytes in vitro at room temperature.
Due to organizational changes this work unit was terminated.
Publications: Fed. Proc. 39:315, 1980; Am. J. Vet. Res. 41:399-404, 836-839, 1980; Toxicon 18:309-314, 502-504, 1980.

* Available to contractors upon originator's approval.

DD FORM 1498 (1 MAR 79) PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M172776A841)

Task No. 3M162770A871 BE: Exploratory Antiviral Drug Development

Work Unit No. 871 BE 146: Physiologically Directed Treatment of Biological
(841 00 030) Toxins of Military Importance

Background:

Overdoses of drugs and intoxications with chemicals or toxins are important medical problems. Although standard procedures of supportive management have traditionally been established, positive manipulative methods often are needed for rapid removal of circulating toxic substances. Since hemoperfusion through activated charcoal for treatment of drug intoxication and poisoning recently has been reported for animals (1) as well as for man (2), it appears necessary to develop a similar hemoperfusion system with activated charcoal using the Dutch rabbit as an animal model in lieu of the subhuman primate which is in short supply.

Cholera enterotoxin causes death when administered to animals either orally or intravenously. Increases of hepatic adenyl cyclase and serum alkaline phosphatase were demonstrated, respectively in rats (3) and dogs (4) after IV injection. Furthermore, recent studies showed that IV cholera enterotoxin produced diffuse hemorrhage and death in monkeys (5). The most striking changes were found in heart. Alterations in tissue water and electrolytes were also seen in rabbits given cholera enterotoxin by the IV route.

Intestinal losses of water and electrolytes during cholera were shown to be a result of an activation of intestinal mucosal adenyl cyclase, which increases the conversion rate of intracellular ATP to cAMP (6). Since cAMP has been proposed as a "second messenger," which mediates the effects of a variety of hormones, and causes vasodilation and hypotension (at pharmacologic dosage IV), the present experiments were designed to test the hypothesis that universal increases in plasma and tissue cAMP may be associated with cholera enterotoxin-induced death after IV administration.

Progress:

Responses to hemoperfusion with activated charcoal. Techniques for a hemoperfusion system with activated charcoal were developed for studying circulatory detoxification of toxins or drugs in 5 Dutch rabbits. During a period of 8 h of hemoperfusion, mean arterial blood pressure, heart rate, hematocrit, RBC fragility, plasma protein concentration, plasma osmolality, leukocyte and platelet counts, and rectal temperature did not show significant changes compared to control values. All rabbits survived prolonged hemoperfusion, indicating that the presently established system is safe.

Effects of IV cholers toxin on tissue cAMP. Death occurs after cholera enterotoxin is administered either orally or IV to animals. After oral ingestion, death results from intestinal losses of water and electrolytes. The probable mechanism is via adenyl cyclase activation and increased cAMP concentration in intestinal mucosa. Although IV cholera enterotoxin (50 µg/kg) in rhesus monkeys produced diffuse hemorrhage (Toxicon, 18:309, 1980) and alterations in tissue water and electrolytes of rabbits (Physiologist, 22:77, 1979), the possible causes of these changes have not been identified. In order to test the hypothesis that a generalized increase in tissue cAMP concentrations may play a role in the development of biochemical and pathologic changes leading to death, 13 Dutch rabbits were injected IV with cholera enterotoxin at a dose of 100 or 200 µg/kg. Nine control rabbits received normal saline IV. When the low dose was given, no significant differences were observed in plasma, urine and 13 tissue cAMP concentrations between control and experimental groups. The higher dose caused significant increases in cAMP values of renal cortex, renal medulla, skeletal muscle, liver, spinal cord, and lung (Table I). The selective elevation in tissue cAMP concentrations suggests differences in tissue sensitivity to cholera toxin. The experimental results fail to support in toto the proposed hypothesis.

TABLE I. CYCLIC AMP CONCENTRATIONS IN PLASMA, URINE AND TISSUE OF CONTROL AND CHOLERA-INTOXICATED RABBITS.

SAMPLE	MEAN pMole/g or ml		
	Control (n=9)	100 µg/kg (n=7)	200 µg/kg (n=6)
Skin	262.5 ± 49.8	208.0 ± 52.2	566.4 ± 233.1
Diaphragm	338.1 ± 104.4	248.0 ± 65.6	605.6 ± 189.3
Heart (left ventricle)	819.2 ± 239.9	333.9 ± 92.7	1060.8 ± 345.2
Lung	391.8 ± 80.1	441.7 ± 68.2	1699.8 ± 703.4*
Renal Medulla	378.5 ± 61.9	342.1 ± 91.6	781.6 ± 188.6*
Stomach	303.5 ± 79.1	277.8 ± 86.8	503.8 ± 180.6
Jejunum	194.6 ± 29.7	211.3 ± 35.7	1155.9 ± 699.9
Cerebellum	1097.0 ± 371.9	419.6 ± 131.1	622.8 ± 117.9
Thalamus-hypothalamus	647.9 ± 90.2	617.5 ± 132.0	1307.4 ± 511.7
Spinal cord	225.6 ± 24.8	380.7 ± 140.4	1201.0 ± 469.8*
Liver	117.8 ± 20.2	218.8 ± 59.6	506.3 ± 127.7**
Renal cortex	245.0 ± 44.8	152.6 ± 28.3	641.4 ± 146.5
Muscle (rectus abdominis)	136.3 ± 26.1	179.5 ± 48.7	512.9 ± 65.3***
Plasma (ml)	35.5 ± 6.3	55.5 ± 16.4	50.8 ± 6.7
Urine (ml)	21815 ± 16505	67551 ± 60080	77410 ± 36427

*P = < 0.05
**P = < 0.01
***P = < 0.001

Methods of anesthesia. To anesthetize a Dutch rabbit for surgical operations associated with intestinal flux studies under the influence of SEB have been unsatisfactory. According to routine practices, an IM dose of Innovar-Vet (0.14-0.17 ml/kg) is unpredictable, frequently resulting in death or a failure to anesthetize. The IV approach proved to be superior. When 0.08 ml/kg of Innovar-Vet was injected into the marginal ear vein, the rabbit was anesthetized within 1 min. A surgical level of anesthesia could subsequently be maintained with additional Innovar-Vet, 0.04 ml/kg, IV.

Treatment of SEB Toxemia. After reviewing the work of Okonogi, et at (Toxicon 17:524-527, 1979), concerning the use of persimmon tannin for detoxifying snake venums and bacterial toxins, Fisher certified grade tannic acid was used to determine its toxicity and its possible effectiveness in treatment of SEB toxemia. Rabbits were administered 5-150 mg/kg of tannic acid by IV injection of solutions with a concentration of 40 or 80 mg/ml. Rabbits died within a few minutes after administration of high doses of tannic acid (50 or 150 mg/kg). Neurological syndromes were induced with lower doses of tannic acid (7-10 mg/kg). Since toxic signs were not observed after injection of 5 mg/kg of tannic acid, this dosage was selected for the treatment of SEB toxemia. Tannic acid treatment of SEB toxemia was performed as follows: 2 rabbits were pretreated with 6 mg/kg of tannic acid (commercial grade) 15 min before administration of 50 µg/kg SEB; 2 rabbits were given 5 mg/kg of tannic acid 10 min after IV administration of 50 µg/kg SEB; and 2 untreated control rabbits were given 50 µg/kg of SEB. All rabbits died within 12 h, indicating that the commerically available tannic acid exerted no protection against SEB toxemia in Dutch rabbits.

Previous work in our laboratory demonstrated that daily SC injections of 0.5 ml of 0.02 N NaOH for 6 days prevented death after an IM lethal dose of SEB (0.1 mg/kg). An increased blood pH may have been responsible for the survival. Techniques were developed to induce metabolic alkalosis (pH 7.6-7.65) by constant IV infusion of 7.5% $NaHCO_3$ (0.71 ml/h) for 6 h after a 2-ml primer dose of 7.5% $NaHCO_3$ was injected.

To test the hypothesis that acid-base imbalance modified SEB toxicity and cell membrane functions, $NaHCO_3$-induced alkalosis was used for the treatment of SEB toxemia in Dutch rabbits. Fifteen minutes after SEB (50 µg/kg was injected into the external jugular vein via a cannula, a loading dose of $NaHCO_3$ was given IV to increase blood pH to 7.5-7.6 and then the higher pH level was maintained by constant infusion of $NaHCO_3$ for a period of 5 h. Although the alkalotic rabbit died 10 h after administration of SEB, $NaHCO_3$ altered the SEB-induced signs (hyperventilation, struggling, weakness). In fact, the treated rabbit showed signs of recovery from SEB after $NaHCO_3$ infusion. More experiments are needed to determine whether $NaHCO_3$-induced alkalosis has any benefits in modifying SEB toxicity.

Many projects in this laboratory include cardiovascular studies in monkeys. In the past, the study primarily involved the measurements of hemodynamic alterations. With the addition of the ECG machine (Burdick EX-11 electrocardiograph), these studies can now be expanded to include electrophysiologic considerations. The rates, amplitudes and duration of individual ECG wave forms and complexes can be measured and cardiac vectors calculated (Table II).

A rat model was established for studying effects of certain drugs or toxins. Measurements of O_2 consumption and rectal temperature, as well as techniques for taking a series of small blood samples to perform biochemical analyses were achieved. The use of a rat model is largely based upon its low price, sufficient supply and minimal amounts of a drug or toxin required in terms of body weight.

In vitro studies on SEB binding. Pretreatment with a nonlethal dose of total body x-irradiation (400-500 r) has been shown to prolong survival in rhesus monkeys (Am. J. Vet. Res. 39: 1213, 1978) or to prevent death in Dutch rabbits (Radiation Res. 76:402, 1978) with SEB toxemia. A possible mechanism for this protection against SEB was based upon the belief that SEB molecules bind to leukocytes in the circulation.

Since x-irradiation produced leukopenia, we postulated that less SEB was transported to the lung to cause pulmonary capillary damage and eventual edema. Although Crawley, et al. (J. Infect. Dis. 116:48, 1966) reported that 5 and 20% of ^{131}I-labeled SEB was found in the "buffy coat" layer of the blood *in vivo* and *in vitro*, respectively, the quantity of SEB to the number of luekocytes was not given. Furthermore, their technique of incubating radioactive SEB with whole blood and separating each fraction for counting after centrifugation are questionable.

TABLE II. BASELINE ECG VALUES OF NORMAL KEATMINE-SEDATED RHESUS MONKEYS (n=15).

	MEAN ± SE	
WAVE	Duration (sec)	Amplitude (mv)
P	0.04 ± 0	0.15 ± 0.01
P-R	0.09 ± 0.01	-0.06 ± 0.01
QRS	0.04 ± 0	0.79 ± 0.01
QT	0.22 ± 0.01	0.14 ± 0.02
T	0.1 ± 0.02	0.06 ± 0.02
Angle of mean electrical axis (°)	75.7 ± 2.5	
Total voltage of mean QRS complex (mV)	0.765 ± 0.11	

In our studies, leukocytes in plasma were obtained from donkey blood (40 ml) after 0.5-1.0 h sedimentation (Proc. Soc. Exp. Biol. Med.84:54, 1953). The binding of SEB to leukocytes was determined by reading the difference in optical density (at 280 nm) between 2 samples: SEB in saline with and without leukocytes. Saline alone or saline containing leukocytes was used as a blank to set zero on the Beckman DB spectrophotometer. With a direct approach of incubating SEB (75 µg) to 1 x 10^9 leukocytes in 2 ml of isotonic saline for 2 hr at room temperature, no binding was found between SEB and leukocytes.

As a result of institutional reorganization, this work unit has been officially terminated.

Presentations:

Liu, C.T. and E. J. Galloway. Effects of an IV lethal dose of cholera enterotoxin on tissue cAMP concentrations in rabbits. Presented, FASEB, Anaheim, CA, 11-18 Apr 1980 (Fed. Proc. 39:315, 1980).

Publications:

1. Liu, C. T., and R. P. Sanders. 1980. Modification of lethality induced by staphylococcal enterotoxin B in Dutch rabbits. Am. J. Vet. Res. 41:399-404.

2. Liu, C. T., R. P. Sanders, J. W. Dominik, and S. B. Formal. 1980. Effects of intravenous and aerosol administration of crude *Shigella* toxin to rhesus monkeys: preliminary study. Am. J. Vet. Res. 41:836-839.

3. Liu, C. T., E. J. Galloway, and P. S. Loizeaux. 1980. Cardiohepatic and gross pathological changes in rhesus monkeys after intravenous injection of purified cholera enterotoxin. Toxicon 18:309-314.

4. Liu, C. T., R. P. Sanders, E. W. Larson, and P. S. Loizeaux. 1980. Resistance of monkeys to aerosol administration of purified cholera enterotoxin. Toxicon 18:502-504.

LITERATURE CITED

1. Hill, J. B., F. L. Palaia, J. L. McAdams, J. L. Palmer and S. M. Maret. 1976. Efficacy of activated charcoal hemoperfusion in removing lethal doses of barbiturates and salicylate from the blood of rats and dogs. Clin. Chem. 22:754-760.

2. Vale, J. A., A. J. Rees, B. Widdop, and R. Goulding. 1975. Use of charcoal hemoperfusion in the management o severely poisoned patients. Br. Med. J. 1:5-9.

3. Baker, A., M. Kaplan, and D. V. Kimberg. 1973. Alklaine phosphatase. Possible induction by cyclic AMP after cholera enterotoxin administration. J. Clin. Invest. 52:2928-2934.

4. Pierce, N. F., J. R. Graybill, M. M. Kaplan, and D. L. Bouwman. 972. Systemic effects of parenteral cholera enterotoxin in dogs. J. Lab. Clin. Med. 79:145-156.

5. Liu, C. T., E. J. Galloway and P. S. Loizeaux. 1980. Cardiohepatic and gross pathological changes in rhesus monkeys after intravenous injection of purified cholera enterotoxin. Toxicon 18:309-314.

6. Field, M. 1971. Intestinal secretion: Effect of cyclic AMP and its role in cholera. New England J. Med. 284:1137-1144.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6411	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. USER'S ENTER	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	K. COMPLETION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	031
b. CONTRIBUTING /				
c. CONTRIBUTING /	STOG 80-7,2:2			

11. TITLE: (U) Mathematical and computer applications in medical BW defense research

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 009700 Mathematics and statistics

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 11	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR 80 (PRECEDING)	1.0	120
FISCAL YEAR 81 (CURRENT)	0	0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Barquist, R.F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Physical Sciences Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if required.)
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TELEPHONE: 301 663-7514
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Oland, D.D.
NAME: POC:DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS: (U) Military medicine; (U) BW defense; (U) Diagnosis; (U) Computers; (U) Medicine; (U) Statistics

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

23 (U) Develop and apply computer programming, biostatistical, mathematical, biomedical engineering, and information management techniques to gather, store, process and interpret biomedical information in a research program for medical defense against BW agents, emphasizing diagnostic, therapeutic and immunoprophylactic studies.
24 (U) Theories and disciplines of numerical analysis, statistical tests of hypotheses, experimental design, differential equations, simulation, information storage and retrieval, and pattern recognition are utilized to analyze and interpret data gathered by investigators.
25 (U) 79 10 - 80 09 - USAMRIID and WRAIR have coordinated their ADP and biostatistical efforts to avoid unnecessary duplication and to provide support for each other when possible.

Significant steps have been taken to place terminals in the hands of end-users so they can perform their own data entry and analysis.

Research efforts have been concentrated in the areas of biomedical indices of infection, information management, and experimental design, biostatistical and computational consultation. Objectives have been accomplished; the work unit is completed.

* Available to contractors upon originator's approval

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

BODY OF REPORT

Project No. 3M162770A870: Risk Assessment of Military Disease Hazards (U).
(3M762776A841)

Task No. 3M162770A870 BC: Prevention of Viral Diseases of Potential BW Importance

Work Unit No. 870 BC 044: Mathematical and Computer Applications in Medical BW
(841 00 031) Defense Research

Background:

The research mission of the Computer Science Office (CSO) is to develop and apply computer, biostatistical, mathematical, biomedical engineering, and information management techniques to gather, process, and interpret biomedical data generated within the Institute. The 3 general areas of support provided by CSO are: (a) Biostatistics and Applied Math; (b) Data Processing, and (c) Research Support (implementation of minicomputers for control of experiments, data acquisition and analysis). The CSO is tasked with providing support to all 10 divisions of USAMRIID.

The CSO has recently undergone a significant expansion in the scope of work required and performed. The acquisition of the PDP 11/34 minicomputer for USAMRIID and the recent availability of an interactive file management system (WYLBUR) on the IBM computer at Fort Detrick have greatly expanded the number of new systems (scientific and business systems) to be developed and maintained.

The long-range goals for ADP support at USAMRIID are: (a) Provide ADP equipment and programs that will best fill the needs of investigators. (b) Assure that all ADP equipment is hardware- and software-compatible. The long-term solution to the ADP equipment needs of USAMRIID calls for a mix of real-time, interactive, and batch processing to fill various needs. This can be accomplished with an appropriate combination of minicomputers, microcomputers, terminals and calculators, but it is essential that compatibility between ADP equipment be preserved as much as possible, and (c) Obtain the most cost-effective ADP systems.

The basic direction in which the CSO is attempting to move is to develop programs and then provide programs and terminals to the non-ADP user so he can process his own data. This puts the data analysis closer to the user and also frees CSO personnel to spend more time developing and maintaining programs rather than running routine programs. With the addition of new ADP equipment during the past year, the number of researchers using terminals for data analysis has increased. A more detailed discussion of the equipment and software that has become available, and the possibilities which such equipment and software provide, is contained in TSP-21.

Progress:

Indices of infection. In this continuing project to develop profiles of biochemical values for early detection of infection in man, further analyses have been done on the USAMRIID control study of 81 blood parameters measured on each of 130 males and 80 females. The objective of analyzing the control group is to

define a normal control population for each of the parameters, so that a probability of occurrence can be computed for each blood parameter measured on a patient.

All data files and computer programs used for the Immunization System were on the Univac computer system at the National Bureau of Standards (NBS). During the past year, NBS discontinued the availability of their computer to users from outside NBS. This resulted in a major project of transfer and conversion of data files and programs from NBS to the IBM 360 computer at Management Information Systems Directorate (MISD), Fort Detrick. This has been accomplished.

Work on this project is continuing on 3 fronts: (a) Generation of polynomial equations and appropriate normalizing transformations and confidence intervals for each parameter measured on the USAMRIID controls. This has been completed for the 38 blood parameters that showed male/female differences and for 25 of the 43 parameter distributions which did not show sex-related differences, (b) Development of an interactive program on the PDP 11/34 minicomputer which will allow a test blood sample to be compared to the control populations for calculation of individual probabilities for each biochemical parameter and calculation of a combined probability for independent parameters. This program is operational for the calculation of some individual probabilities, but not for combined probabilities; and (c) Use of multivariate analysis techniques (discriminant and cluster analyses) to identify independent parameters among the controls and to separate groups of control and ill patients. The most recent version (1979) of the BMDP Biomedical Computer Programs have been received and installed on the IBM 360. The multivariate analysis programs in BMDP will be used for this effort.

Because of other demands on CSO, work now being done on this project is limited.

Immunization System. Three aspects of the computerized USAMRIID Immunization System should be noted: (a) In November 1979 an emergency request was made by the Chief, Medical Division to CSO for a list of individuals with a specific blood type and titer response. This list was needed too quickly to find suitable volunteers to donate blood for a medical emergency unique to the Institute. Despite the fact that the request was made at 1630 hours and despite serious computer failures of the IBM computer at MISD, CSO was able to do computer searches of all active and inactive individuals on the immunization data base and deliver the requested list by 1900 hours that evening, in time to be used by the Chief, Medical Division. This is a good example of one of the uses of the computerized Immunization System. (b) In response to a request from the USAMRIID Immunization Committee, the CSO participated in gathering the data, and stored the entire current USAMRIID smallpox data set for 547 USAMRIID individuals on computer files. The smallpox files contain information gathered from May 1979 to 13 June 1980. No procedure has been established to provide CSO with updates to this file. Programs have been written to print division lists of individuals not yet vaccinated. The computerization of this information has saved many man-hours of record-keeping, sorting, and preparing lists of unvaccinated employees by personnel in Medical Division and (c) There are over 320 individuals active in the computerized immunization program at USAMRIID, and over 210 individuals in an inactive status. At the present time, the computerized Immunization System contains partial medical records (started in 1972) with information on 6 agents: VEE, EEE, WEE, Q Fever, tularemia, and yellow fever. Limited information is on file for 2 agents: smallpox (May 1979 - June

1980) and Rift Valley fever (started July 1980). Incorporation onto computer files of other human vaccine results (botulism, anthrax, dengue, RMSF, chikungunya, and typhus) as requested by the Immunization Committee cannot be accomplished until: additional personnel are available; a terminal and printer are installed in Medical Division; a Data Base Management System is installed on the post IBM computer and CSO personnel are trained in the use of the DBMS; and a complete, comprehensive review of all vaccination procedures and policies associated with each agent is completed. A detailed plan has been prepared which itemizes the resources required for the Immunization System update.

Primate Registry. Daily accounting and management of 500 primates at USAMRIID is required because of the numerous projects using primates, and the need for rapid and frequent screening of primate records to identify those which have or have not been used in specific protocols. USAMRIID has been participating in the computerized WRAIR Primate Registry by supplying information to WRAIR (Primate Registry Worksheets) and receiving quarterly printed reports. The frequency and nature of these reports makes them totally inadequate for USAMRIID's needs. Efforts are now in process by CSO to gain update and interactive access to the Primate Registry via a terminal at USAMRIID. This will allow an assigned individual in AR Division to enter, update, and query the data files of the Institute's primates in an interactive mode using a computer terminal connected to WRAIR's CDC 3500 computer via telephone. This will be a great improvement over the current manual procedures at the Institute, but is viewed as a partial and temporary solution until a more complete primate registry program is developed for USAMRIID's specific needs.

SMAC System Blood Analysis. USAMRIID routinely sends patient's blood samples to WRAMC via courier for analysis in the SMAC lab. Printed results are then returned to USAMRIID via courier. Current medical care practices require faster turnaround time than the current 5-7 days for SMAC results. Equipment has been tested and evaluated, and is being requested which will be added to an HP 9830 microcomputer system at WRAMC so that the HP system will be able to communicate, as if it were a terminal, with the PDP 11/34 minicomputer at USAMRIID. SMAC results will then be transmitted to the USAMRIID minicomputer on the afternoon of the day the blood samples are analyzed. A terminal and printer in the USAMRIID Clinical Lab will then access the SMAC results from the Institute's minicomputer that same day.

Consultations. Numerous biostatistical/computer consultations were held with investigators of all divisions of the Institute to develop solutions to experimental design, statistical or computational problems. Considerable expenditures of time and resources are required to meet these data analysis problems.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6413	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GROUP MARK'G	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10a. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	036
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE: (U) Spontaneous diseases in laboratory animals used for developing medical anti-BW defense

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
64 08	80 09	DA			C. In-house

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

18. RESOURCES ESTIMATE

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING 80	1.0	54
CURRENT 81	0	0

19a. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Barquist, R. F.
TELEPHONE: 301 663-2833

20. GENERAL USE
Foreign intelligence considered

19b. PERFORMING ORGANIZATION
NAME: Pathology Division
ADDRESS: USAMRIID
Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic participation)
NAME: DePaoli, A.
TELEPHONE: 301 663-7211
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Rozmiarek, H.
NAME: POC:DA

21. KEYWORDS (Provide KEY WORDS with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Laboratory animals; (U) Spontaneous diseases

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

23 (U) To evaluate and monitor the health status of laboratory animals on arrival in the Institute and to identify and characterize spontaneous diseases which develop in laboratory animals while in the colony or on research projects. This information is necessary to preclude or minimize the experimental variable of natural diseases, allow selection of adequate animal suppliers, control zoonoses and ultimately the successful completion of the laboratory's BW research defense research mission.

24 (U) Predetermined numbers of animals from the various suppliers will be sacrificed on a monthly basis and monitored histopathologically. In addition, diagnostic techniques to include clinical pathology, histopathology, animal inoculation, etc. will be employed to investigate all natural animal deaths.

25 (U) 79 10 - 80 08 - The continuing histopathological monitoring program of rodents entering the Institute revealed that most animals supplied to the laboratory during the past year were free of significant disease. This represents an improvement in the health status and quality of animals received over the previous year. Deaths in the animal colony were sporadic; in the rodent population these were generally the result of bacterial infections. A total of 74 nonhuman primates died during this reporting period. A significant number (47) died as a result of complications attributed to experimental manipulations.

Publication: Vet. Pathol. 17; in press, 1980.

Terminated for management efficiency. Continued in W.U. 871 BB 149. (DAOG3813)

* Available to contractors upon originator's approval

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

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BB: Prevention of Bacterial and Rickettsial Diseases of Potential BW Importance

Work Unit No. 871 BB 126: Spontaneous Diseases in Laboratory Animals Used for
(841 00 036) Developing Medical Anti-BW Defense

Background:

The usefulness of laboratory animals in medical research is dependent to a large degree on the reproducibility of experimental procedures. The single most important factor determining data reproducibility in a given laboratory animal species is the variable of concurrent animal disease. The severity of such diseases varies from clinically silent or chronic enzootic conditions to acute highly lethal states. All, however, may interfere with animal experimentation, compromise experimental data, or worse, preclude planned studies. It is apparent, therefore, that the variables of clinically silent diseases in the experimental animal must be characterized and if possible eliminated. Likewise, disease outbreaks or unexplained deaths of animals during an experiment must be investigated and their impact on the ongoing studies evaluated.

Progress:

The continuing histopathological monitoring program of rodents entering the Institute revealed that most animals supplied to the laboratory during the past year were free of significant disease. Subclinical endemic diseases such as chronic respiratory disease of rats and mice and intestinal nematodiasis were encountered but these generally were mild in degree and did not affect the animal's utilization in experimental studies.

Two diseases encountered with the potential for disrupting experimental studies were Sendai virus infection in mice and encephalitozoonosis in guinea pigs. Both were detected in only one shipment of animals early in the year and have not been noted in subsequent deliveries. This is a considerable improvement over the previous year when both entities were not uncommon in shipments of animals, particularly those received during the latter part of the year.

Colony deaths investigated as part of the disease surveillance program included a number of species and causes. Deaths in the rodent population were sporadic and most commonly the result of bacterial diseases. Viral diseases, sialodacryoadenitis caused by a coronavirus in rats and Sendai virus infection in mice, were responsible for two discrete outbreaks of clinical disease and mortality.

A total of 74 nonhuman primates were lost during the reporting period; these included 44 cynomolgus, 16 macaques, 8 African greens, 3 marmosets, 2 squirrel monkeys and 1 vervet monkey. Death in 47 of these animals was attributed directly

or indirectly to complications associated with experimental manipulations, most commonly vascular catheterization. Causes of death in this group of animals included exsanguination from site of catheterization, septic thrombosis and embolism, myocardial necrosis, necrotizing arteritis, thromboembolic encephalitis, cerebral infarction, and postsurgical shock. Other primate losses were the result of bacterial pneumonia, enterocolitis, acute gastric dilatation, intestinal intussusception, ketosis, and Gentacin toxic nephrosis.

Monitoring of laboratory animals entering the Institute revealed that the health status of rodents supplied was acceptable. Losses of colony animals to natural disease were minimal. The only significant losses were in monkeys and these were associated with experimental manipulations.

Presentation:

DePaoli, A. Pathology of gastrointestinal diseases of nonhuman primates. Presented, Course of Pathology of Laboratory Animals, AFIP, Washington, DC, 11-15 Aug 1980.

Publication:

Elwell, M.R., A. DePaoli, and G.D. Whitney. 1980. Cytoplasmic crystalliods in the ovary of a Woolly monkey. Vet. Pathol. 17: in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6429	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9A. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	040
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE: (U) Hazards and variables associated with research animals used in medical defense against BW

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology; 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
76 10	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE	PROFESSIONAL MAN YRS	FUNDS ($ thousands)
FISCAL YEAR PRECEDING	80	1.0	42
FISCAL YEAR CURRENT	81	0	0

19a. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Barquist, R. F.
TELEPHONE: 301 663-2833

20. GENERAL USE
Foreign intelligence considered

19b. PERFORMING ORGANIZATION
NAME: Animal Resources Division
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Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic Institution)
NAME: Rozmiarek, H.
TELEPHONE: 301 663-7221
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Stokes, W. S.
NAME: Hall, W. C. POC:DA

21. KEYWORDS: (U) Military medicine; (U) BW defense; (U) Parasites; (U) Laboratory animals; (U) Microbiology; (U) Infectious diseases

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

23 (U) Monitor clinical health of newly arrived and in-house research animals. Investigate any deviation from normal and evaluate the effect on proposed use in ongoing research at USAMRIID; institute corrective and preventive measures when indicated. This work is essential to assure that the best quality of animal possible is used in all critical infectious disease research of military significance in medical defense against biological attacks.

24 (U) Conduct a quality control program for newly received animals to include evaluation of their viral, bacterial, parasitic, hematologic, metabolic and neurologic status. Histopathologic evaluation will be done where indicated. All primates will be examined upon receipt and semiannually thereafter for symptoms of zoonotic and other diseases, to include testing for tuberculosis. The effects of any deviations from normal on their proposed use in ongoing research will be investigated in detail and evaluated.

25 (U) 79 10 - 80 09 - Significant findings this year included pasteurellosis in rabbits, mite infestation and coccidiosis in chickens, pinworms in rats and mice, chronic respiratory disease in rats, severe dysentery caused by Shigella flexneri in African green monkeys, strongyloidiasis in African green and squirrel monkeys, and Prosthenorchis elegans in newly received marmosets. No cases of herpesvirus, measles or tuberculosis were detected in nonhuman primates. The cynomolgus monkey was evaluated as an animal model for the study of diarrhea caused by enterotoxigenic Escherichia coli. If preliminary work now being completed indicates that this monkey is a suitable model, further studies will be directed at the evaluation of hydrophobic gels as prophylactic or therapeutic treatment entities.

Terminated for management efficiency. Continued in W.U. 871 BB 149. (DAOG3813)

*Available to contractors upon originator's approval.

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BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BB: Prevention of Bacterial and Rickettsial Diseases of Potential BW Importance

Work Unit No. 871 BB 127: Hazards and Variables Associated with Research Animals
(841 00 040) Used in Medical Defense Against BW

Background:

Even in well-managed, efficient research institutes, the constant hazard exists for contamination of normal conventional animals with undesired unusual disease entities. This is especially a problem for an infectious disease research institute, as the contamination problem may be masked by the effects of the conditions under study, and may, in turn, change the results of the research study. This potential for contamination is amplified at USAMRIID where space limitations generally preclude in-house breeding programs, and all conventional animals are purchased from commercial sources whenever available. Animals subjected to the stresses of shipping and handling are especially susceptible to a wide variety of bacterial and viral diseases. When received, they are often latent carriers of disease. Symptoms may not show up until the animals are stressed in research. An effective system of identifying these latent conditions, characterizing them, and eliminating them whenever possible is essential in maintaining the integrity and reproducibility of research.

Progress:

The animal disease surveillance program began at a restricted level due to staff shortages, but with the addition of new personnel was able to achieve a more desirable level within a few months. The quality control monitoring of incoming rodents involved the examination of 245 rats, mice, guinea pigs, gerbils and hamsters from 11 different commercial sources as well as the screening for internal parasites from each shipment of rodents. Collection of hematologic data continued to establish normal parameters for the "USAMRIID rodent." These data are useful as a standard for comparison in the early identification of animals showing abnormal hematologic values. Rabbits, poultry, sheep, and primates were received from vendors throughout the year and examined by the veterinary staff; several rejections were due to various disease conditions.

A chronic recurring problem with upper respiratory disease (pasteurellosis) in newly received rabbits from a single vendor led to a number of animals being rejected and subsequent cancellation of the contract with that vendor. This disease, caused by Pasteurella multocida, is a serious threat to those rabbits already in USAMRIID because it is difficult to cure affected animals and to eradicate, once established. Several partial shipments of rabbits were rejected due to active ear mite (Psoroptes cuniculi) infestation. Control of ear mites for in-house rabbits has been accomplished using a routine scheduled treatment program.

One shipment of 8 chickens from a commercial vendor showed that all were infested with northern feather mites (Ornithonyssus sylviarum). The entire shipment was rejected and the birds replaced. Another shipment required the rejection of several chickens due to coccidiosis (Eimeria sp.).

The following diagnostic specimens were examined during the past year from incoming and in-house animals:

Examination	Number
Hematology	1939
Bacteriology	554
Parasitology	523
Histopathology	1003
Serology	178
Serum bichemistry	40
Urinalyses	2

The most significant problem encountered in incoming rodents was pinworms (Syphacia obvelata) in rats and mice. Groups of infested rats were rejected and alternate orders placed with other vendors. Each shipment of rats and mice received is being screened for pinworms. Control of this parasite is especially important in infectious disease research because it has been shown to depress T-cell-dependent immune responses.

Chronic respiratory disease (CRD) continues to be the most common finding in incoming rats. Due to Mycoplasma pulmonis, it is found in differing degrees of severity in nearly all populations of conventional rats. Of 130 rats examined, 67 (52%) had CRD lesions. Of these, 87% were classified as mild or minimal lesions, while 12% were classified as moderate and 1%, as severe. A summary of the number of newly arrived rodents necropsied and a distribution of their lesions is shown in Table I

Bacteriologic cultures were made from lung and stool samples of the quality control rodents. Salmonella, which has been a problem in the past, was not isolated this year. Citrobacter freundii (mouse) and Pseudomonas sp. (rat) were the only other intestinal pathogens isolated. Four positive lung cultures yielded Escherichia coli (2), Proteus sp. (1), and Staphylococcus aureus (1). No ectoparasites were detected during this period.

TABLE I. QUALITY CONTROL EXAMINATION OF NEWLY RECEIVED RODENTS: SUMMARY OF PATHOLOGIC FINDINGS, 1979-80

		NO. WITH PATHOLOGIC CHANGE			
Species	Number Examined	Pulmonary	Gastro-Intestinal	Genito-Urinary	Misc.
Rat	130	67	32	11	11
Mice	79	12	10	6	4
Hamster	10	2	1	0	1
Gerbil	11	1	4	1	1
Guinea pig	15	5	0	2	1
TOTAL	245	87	47	20	18

A severe outbreak of dysentery due to Shigella flexneri occurred in a group of newly arrived African green monkeys (Cercopithecus aethiops) and resulted in the rejection of 4 that eventually died. Illness in the group persisted and resulted in a 1-month delay in the completion of their quarantine. Intestinal parasitism due to Strongyloides sp. was detected in 2 groups of African green monkeys and one group of squirrel monkeys (Saimiri sciureus) received. This is a common intestinal nematode which was successfully eliminated with antihelminthic therapy before the animals were removed from quarantine and issued for research. Six marmosets (Callothrix jacchus) were received and quarantined in Bldg 1412. Two deaths were attributed to parasitism by the acanthocephalan Prosthenorchis elegans. There is no effective treatment for this parasite; this problem will have to be further evaluated if this animal proves suitable for use by Virology Division.

Persistently elevated eosinophil counts were observed in cynomolgus monkeys (Macaca fascicularis) due to unknown etiology; antigenic stimulation due to parasitism is a common cause in animals, as well as allergic reactions. Examination procedures were instituted for microfilaria and malaria but none have been detected.

No significant zoonotic problems were identified during this period. No positive cases of measles, Herpesvirus simiae, or tuberculosis were diagnosed. Two cynomolgus monkeys had palpebral skin tests that were suspect for tuberculosis. These were negative on retest and after an additional quarantine were released for issue. Tuberculosis testing continues to be performed quarterly on all primates as well as during quarantine of new arrivals.

Evaluation of the cynomolgus monkey as an animal model for enterotoxigenic E. coli (ETEC) infections in man. CPT Stokes collaborated with Dr. Torkel Wadstrom, a visiting Swedish scientist, on the evaluation of the cynomolgus monkey as an animal model for human diarrhea caused by enterotoxigenic E. coli. In vitro studies indicate that the intestinal cells from these monkeys are susceptible to ETEC. However, no animal model presently exists for the clinical evaluation of this disease entity. Dr. Wadstrom has shown that hydrophobic gels in vitro could block the effects of the ETEC and wanted to test this in a suitable animal model.

Experiments were performed with 3 ETEC strains which were isolated from diarrhea outbreaks in people: strain H1049b (Evans) which has colonizing factor antigen/I (CFA/I) and heat-stable (ST) and heat-labile (LT) enterotoxins, and strains 80a and c922, which contain the other colonizing factor antigen found in human strains of ETEC, DFA/II, as well as both ST and LT enterotoxins. During the first experiment with the CFA/I strain, cultures taken after 3 days showed that colonization had occurred, but no diarrhea was observed. Metronidazole, a bactericidal drug which kills only anaerobic bacteria, was consequently administered to see if this would allow overgrowth of the aerobic ETEC with subsequent diarrhea. Acute watery diarrhea resembling the traveller's diarrhea in people caused by ETEC was then seen in 3 of 4 of the monkeys. This same acute diarrhea was seen in 3 of 4 other monkeys pretreated with metronidazole and then given the ETEC strains. Four monkeys given the CFA/II strain developed no diarrhea. ETEC strains were isolated from all monkeys during the diarrheal episodes.

Control studies are planned to determine what effect the subclinical dosages of metronidazole used alone would produce on these monkeys. Serum samples taken before and after infection are being examined for titers to the CFA antigens and

LT-enterotoxin by Dr. Wadstrom in Sweden. Completion of the serologic tests and the outcome of the control studies will determine if the cynomolgus monkey should be further investigated as a model for human ETEC diarrhea. If these results indicate that this monkey is a suitable model, studies could then be directed at the evaluation of the hydrophobic gels, as well as chlorpromazine (recently shown to inhibit the cholera-like LT-enterotoxin), as prophylactic or treatment entities. The clinical differences between ST, LT and ST-LT strains could also be further evaluated in this model.

Presentations:

1. Rozmiarek, H. Handling hazardous experiments in the laboratory animal facility, Presented, 9th Annu. Session, National Capital Area Branch, American Association for Laboratory Animal Science, Hunt Valley, MD, 25-26 Oct 1979.

2. Rozmiarek, H. Biocontainment in the animal colony. Presented, Operational Laboratory Animal Medical Problems Course, USAF School of Aerospace Medicine, Brooks Air Force Base, TX, 5-9 May 1980.

Publications:

1. Heisey, G.B., H.C. Hughes, C.M. Lang, and H. Rozmiarek. 1980. The guinea pig as a model for isoniazid-induced reactions. Lab. Anim. Sci. 30: 42-50.

2. Bryant, J.M.. 1980. Vest and tethering system to accommodate catheters and a temperature monitor for nonhuman primates. Lab. Anim. Sci. 30: 706-708.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

79 10 01	H. TERMINATION	U	U	DA 0E6411	80 10 01	NA	NL	☒ YES ☐ NO	DD-DR&E(AR)636

	Program Element	Project Number	Task Area Number	Work Unit Number
a. Primary	62776A	3H162776A841	00	043
b.				
c.	STOG 80-7.2:2			

(U) Respiratory disease mechanisms, pathogenesis and therapy of airborne infections

003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)

Start Date	Est. Completion Date	Funding Agency	Performance Method
73 02	80 09	DA	C. In-house

Contract/Grant: NA

Fiscal Year	Professional Man Yrs	Funds
80	2.0	113
81	0	0

Responsible DOD Organization: USA Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21701.

Responsible Individual: Barquist, R. F. — 301 663-2833

Performing Organization: Aerobiology Division, USAMRIID, Fort Detrick, MD 21701

Principal Investigator: Larson, E. W. — 301 663-7453

Associate Investigators: Dominik, J. W.

POC:DA

Keywords: (U) Military medicine; (U) BW defense; (U) Prophylaxis; (U) Viral disease; (U) Aerosols; (U) Respiratory infections; (U) Pathogenesis

23 (U) Examine respiratory disease mechanisms, including penetration, retention, clearance and replication of pathogens introduced in the host by enemy attack via the respiratory route. The research is essential for determining the pathogenesis and developing promising approaches to prophylaxis and therapy of infections, both respiratory and systemic, acquired by host inhalation, the most likely means of exposure in BW operations.

24 (U) Assess aerosol properties of infectious microorganisms and challenge experimental animals by respiratory route; assess postexposure effects using microbiological, histopathological and immunological methods.

25 (U) 79 10 - 80 09 - A lowest aerosol dose of 2.7 log PFU of Lassa fever virus was uniformly lethal for the cynomolgus monkey as was a medium dose of 3.9 log PFU. One of 4 monkeys survived a high dose of 4.8 log PFU. New World squirrel monkeys were resistant to all levels of aerosol challenge including a high dose of 3.9 log PFU. Serial sacrifices of guinea pigs, after respiratory challenge with Lassa virus, supported earlier observations that the lung is a primary site for virus replication with transport via the circulation to a wide range of organs also capable of supporting virus growth. Virus was recovered from numerous organs of surviving guinea pigs as late as 33 days after challenge. Ribavirin, when administered either intramuscularly or by aerosol, failed to protect strain-13 guinea pigs from death after aerosol challenge with Lassa fever virus. However, mean times-to-death were significantly extended by all regimens of therapy. Terminated for management efficiency. Continued in W.U. 870 BB 069 DAOG3814

Publications: Abstracts, Annu. Mtg. ASM - 1980, T107, p. 253; Infect. Immun. 30: in press, 1980.

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BODY OF REPORT

Project No. 3A162770A870: Risk Assessment of Military Disease Hazards (U)
(3M162776A841)

Task No. 3A162770A870 BB: Assessment of Airborne Microbial Agents of Potential BW Threat

Work Unit No. 870 BB 042: Respiratory Disease Mechanisms, Pathogenesis and Therapy of Airborne Infections

Background:

Under this continuing program of research for medical defense, we have previously reported on the aerosol stability and respiratory infectivity properties of Japanese B encephalitis (JBE) virus, Bolivian hemorrhagic fever (BHF) (Machupo) virus and Lassa (LAS) virus (1,2). Extensions of the work have included investigations on the pathogenesis, immunogenesis and therapy of the infections produced in experimental animals after respiratory challenges. The object of the research has been to assess the potential threat of these viruses as BW agents and to determine the possible hazards of their airborne transmission in nature as well as among research personnel.

The continuing efforts in this report period were directed to studies on (i.) the respiratory infectivity of LAS virus in nonhuman primates, (ii.) the completion of work to quantitate the virus population dynamics in guinea pig tissues after respiratory exposure, and (iii.) the efficacy of ribavirin administered both IM and by aerosol for the treatment of Lassa fever in guinea pigs. Significant effort during this report period, also was directed to supporting the Biological Detection and Warning Program of the Chemical Systems Laboratory, Aberdeen Proving Ground.

Progress:

Respiratory infectivity of LAS virus in primates. We reported previously that 2 of 3 cynomolgus monkeys challenged by the aerosol route with $10^{4.8}$ PFU of LAS virus succumbed to infection 12 days after exposure. Both monkeys were viremic at the time of death (ca. $10^{3.0}$ PFU/ml serum). In this report period, 9 additional cynomolgus monkeys weighing from 2.0-2.9 kg each were challenged with graded doses of the virus to attempt to quantitate the lethal respiratory dose responses.

Four monkeys were challenged with a low dose of $10^{2.7}$ PFU virus; 4 additional monkeys received a medium dose of $10^{3.9}$ PFU of virus and one monkey was challenged at the previously used high dose ($10^{4.4}$ PFU). The monkeys were challenged by established methods in a modified Henderson, dynamic aerosol apparatus contained within total containment biological safety cabinets. A DeVilbiss #40 refluxing nebulizer, operated at 20 psi compressed air was employed as the aerosol generator. The monkeys were sedated, chaired, and exposed to aerosol individually for 10 min by means of head helmets.

Table I summarizes the mortalities and day-to-death observations. All 9 monkeys succumbed to the infections, indicating that the respiratory LD_{50} was below the lowest exposure dose of $10^{2.7}$ PFU. All were viremic when first bled 7

days after exposure. The geometric mean concentration of virus/ml of serum was $10^{3.0}$ PFU. Viremia levels were not different among challenge doses. At 13 days after challenge, the geometric mean concentration of virus in the sera of 5 monkeys remaining alive was $10^{6.1}$ PFU/ml, a level markedly higher than that seen at 7 days. Body temperature profiles from measurements taken twice weekly were unremarkable except that hyperthermia was frequently observed in the period from 5-2 days prior to death; while hypothermia was a frequent observation immediately (1-2 days) before death.

TABLE I. MORTALITIES AND DAYS-TO-DEATH OF CYNOMOLGUS MONKEYS CHALLENGED WITH GRADED DOSES OF LAS VIRUS AEROSOLS

EXPOSURE DOSE (Log_{10} PFU)	MONKEY MORTALITIES	
	Dead/4	Day of death
2.7	4	13, 14, 16, 18
3.9	4	12, 12, 13, 16
4.8	3	12, 12, 14

Complete histopathologic examination by Pathology Division was performed on 2 monkeys which died after challenge at the medium dose. One or both of these revealed lesions generally seen in monkeys dying with LAS, including: interstitial pneumonia and multifocal hepatitis, splenitis, myocarditis, epicarditis, choroiditis, and meningoencephalitis.

The susceptibility of New World squirrel monkeys to respiratory infection with LAS virus was examined incidentally because of the availability of animals after their use in studies on Legionnaires' disease. Three monkeys were challenged with a low aerosol dose of $10^{1.6}$ PFU of LAS; 3 animals received a medium dose of $10^{2.6}$ PFU of virus and 2 a high dose of $10^{3.6}$ PFU. Aerosol exposure methods were identical with those employed in the cynomolgus monkey challenges except that the squirrel monkeys were not sedated and were exposed in exposure boses (whole body). None of the squirrel monkeys succumbed to these challenges and only one animal receiving the medium dose showed minimal signs of illness consisting of lethargy on days 6 and 7 after exposure. These results were not unexpected, since Virology Division (Dr. Jahrling, personal communication) has reported a similar lack of susceptibility in the squirrel monkey when challenged SC.

LAS virus concentrations in guinea pig tissues after aerosol exposure. We reported previously (2) on the results, through 24 days, of serial sacrifice studies designed to examined LAS virus concentrations in selected tissues of Hartley strain guinea pigs (Buckberg) after aerosol exposure. Those studies were completed during this report period with the assay of samples of tissues collected after 27, 30, and 33 days. Table II summarizes the results of the experimentation including, for completeness, the information through 24 days reported earlier.

TABLE II. LOG_{10} PFU OF LAS VIRUS PER GRAM OF GUINEA PIG TISSUE AS A FUNCTION OF TIME AFTER AEROSOL CHALLENGE WITH $10^{4.1}$ PFU OF VIRUS

DAYS POSTCHALLENGE	MEAN LOG_{10} PFU/g TISSUE (n=2, except as noted)					
	Blood	Liver	Spleen	Brain	Lung	URT
3	Neg	2.55	Neg	Neg	3.50	Neg
6	2.37	2.48	6.03	Neg	6.53	4.85
10	4.30	4.55	8.08	3.17	7.35	6.52
13	4.69	5.30	8.51	3.67	7.41	6.99
18	4.24	4.32	7.77	6.79	7.20	6.71
20	4.31	4.58	7.76	4.45	7.30	6.75
24	3.30 (1)	3.98 (1)	5.79 (1)	4.76 (1)	4.60 (1)	4.19 (1)
27	3.37	3.54	5.76	3.54 (1)	4.26	4.86
30	2.34 (1)	Neg	5.36 (1)	3.78 (1)	3.95 (1)	Neg
33	3.77 (1)	5.40	5.50	4.74	3.00	4.86

Virus was present, generally throughout the entire period of examination, in all of the tissues on which measurements were made. In the period through 20 days after exposure, measures of virus concentrations were highly reproducible and generally consistent between animals within a period. High concentrations were measured from lungs with peak concentrations exceeding $10^{7.0}$ PFU/g. Only the pleen yielded high concentrations, starting at 6 days, and peaking between $10^{8.0}$ and $10^{8.5}$ PFU/g at 10-13 days. The blood, brain, liver, and upper respiratory tract (URT) contained modestly lower concentrations, although even in these tissues the virus levels were very significant and consistent through 20 days.

In contrast to the consistent virus recoveries from animal-to-animal and tissue-to-tissue through 20 days, virus recoveries from later samples were sporadic and generally lower than seen earlier. Nonetheless, at least one of 2 guinea pigs was positive for virus at levels of $10^{3.0}$-$10^{5.5}$ PFU/g of tissue at 33 days.

These data suggest that the lung represents a primary site for virus replication in respiratory LAS in guinea pigs; hematogenous transport of the virus to a wide range of organs also capable of supporting virus growth is further suggested.

Ribavirin therapy of respiratory LAS in guinea pigs. Jahrling, et al. (3) have reported that IM administration of ribavirin is efficacious in preventing death of rhesus monkeys after SC challenge with LAS virus. Results with ribavirin in strain-13 guinea pigs have been equivocal after challenge with either LAS of Pichinde (PIC) virus. We have completed 2 studies to compare IM with aerosol administration of ribavirin for treating respiratory LAS in strain-13 guinea pigs. Our working hypothesis was that since the lung is a primary organ for virus replication in LAS, aerosol administration of the drug may be advantageous.

Three groups of 8 guinea pigs were challenged with ca. $10^{3.0}$ PFU of virus by the aerosol route in both sutdies. In the first study, the guinea pigs were treated for 11 days after exposure. One group was shamtreated twice daily by exposure for 32 min to aerosols of distilled water; a second group was treated in the same manner

with aerosols of ribavirin in distilled water (100 mg/ml). The third group received ribavirin IM twice daily. With both IM and aerosol administration, the animals received 30 mg ribavirin/kg/day. The treatments in the second study were similar except that the daily dosages of ribavirin were doubled (60 mg/kg/day) and the period of treatment was extended to 18 days.

All of the guinea pigs in all treatment groups in both experiments succumbed to the LAS infections. Administration of ribavirin influenced results only to the extent of altering the mean times-to-death. Table III summarizes the effects of ribavirin therapy on the geometric mean days to death in each treatment.

TABLE III. EFFECTS OF RIBAVIRIN THERAPY ON TIME-TO-DEATH OF STRAIN-13 GUINEA PIGS WITH RESPIRATORY LAS

	GEOM. MEAN DAY OF DEATH		
EXPERIMENT	Sham (Aerosol)	Ribavirin Aerosol)	Ribavirin (IM)
1	18.5	20.5	25.6
2	15.5	23.3	27.4

Compared to the sham-treated guinea pigs, IM treatment with ribavirin extended the mean time-to-death somewhat more than did aerosol administration in both experiments. Moreover, the differences were somewhat greater in the second experiment, wherein dosages were increased and the treatment period was extended.

These ribavirin therapy investigations will be continued. Treatment efficacies will be compared after aerosol and SC challenges. The resulting infection after aerosol challenge may in some way be unique. Other variables which are considered worthy of investigation include the combination of IM and aerosol administration of the drug, the administration of initial loading doses and possibly the source of strain 13 guinea pigs. Initial studies by Virology Division employed guinea pigs procured from outside sources, while the recent studies, guinea pigs from the USAMRIID colony were used.

Support for biological detection and warning. The XM2 Biological Sampler is a component of the Biological Detection and Warning System (Aberdeen Proving Ground). Another component, the XM19 Alarm, is designed to detect the presence of a biological aerosol, signal an alarm and activate the XM2 to sample the aerosol into a liquid collection medium. The collection fluid consists of physiological saline, phosphate buffered to pH 7.2, with 0.05% Tween-80 added. The required operational characteristics and practical requirements of the XM2 sampler are that at least 1% of any pathogen of interest collected by the sampler shall survive after 4 h of sampler storage. The objectives of the testing performed under this work unit were to determine a practical temperature for storing these collected samples to achieve optimal survival and to determine the 4-h survival properties of several pathogens in the sampler when stored at this temperature.

It is important to investigate two variables of temperature. These included the temperature of the liquid collecting medium during sampler operation (the

period when the sampler would have access to system support utilities), and the temperature of the sample after collection when the sampler would be in transit to a medical laboratory. SCHU-4 strain, Francisella tularensis was employed, initially, to study the effects of these temperature variables on organism survival on the basis that this susceptible organism would provide a sensitive test.

Aerosols of F. tularensis were disseminated into one of the Aerobiology Division 6,200-L aerosol chambers with conditions set at 100°F temperature and 80% relative humidity. This temperature was selected to correspond to a high temperature that might be encountered operationally. Three XM2 samplers with collection media stabilized and maintained at 40, 80, and 90°F were employed to collect 1-min samples of the aerosols. Baseline assays were performed before continuing with sampler operation in clean air at 100°F for 44 min. The samples were then reassayed and subdivided for storage at each of 3 temperatures (40, 80, and 90°F) for 4 h. All samples were reassayed after storage. Four replicates of the experiment were completed.

Table IV presents the mean survival rates of the F. tularensis as a function of both collection medium temperature during operation and the temperature during 4 H storage. The survival rates of the F. tularensis before storage and after 44 min of sampler operation were high and between 85 and 95% regardless of the collecting medium temperature. Survival rates after storage were clearly related to storage temperature. Excellent survival was achieved with sampler storage at 40°F and the survival rates were less than 5% when stored at 90°F.

TABLE IV. EFFECT OF COLLECTING MEDIUM TEMPERATURE DURING OPERATION AND SUBSEQUENT STORAGE TEMPERATURE ON THE SURVIVAL PROPERTIES OF F. TULARENSIS, SCHU-4, IN THE XM2 BIOLOGICAL SAMPLER

	SURVIVAL INDEX[a]		
STORAGE TEMPERATURE FOR 4 h	40°F	80°F	90°F
No storage[b]	91.6	84.5	94.8
40°F	92.2	66.0	74.0
80°F	26.8	34.0	45.3
90°F	3.4	4.8	6.4

[a]All organism concentrations normalized to the concentrations measured in the XM2 immediately after collecting a 1-min aerosol sample.

[b]Immediately after 44 min operation in clean air.

The results of two studies conducted with TC-83, VEE virus are summarized in Table V. Survival rates at 40, and 80°F for both the collecting medium and storage air were examined in both experiments. An added comparison to determine the importance of the Tween-80 additive was included in the second study.

TABLE V. EFFECTS OF STORAGE TEMPERATURE AND TWEEN-80 ADDITIVE ON THE SURVIVAL PROPERTIES OF VEE (TC 83) VIRUS IN THE XM2 BIOLOGICAL SAMPLER

	SURVIVAL INDEX[a] (%)					
	Experiment 1 with Tween		Experiment 2			
			with Tween		without Tween	
TREATMENT	40°F	80°F	40°F	80°F	40°F	80°F
No storage[b]	67	48	82	51	80	6
Stored 4 h	8	0.13	26	0.3	0.5	Nil

[a]Virus concentrations normalized to the concentrations measured in the XM2 immediately after collecting a 1-min aerosol sample.

[b]Immediately after 44-min operation in clean air.

Sampler operation and storage at 40°F were clearly superior to 80°F. Additionally, the presence of Tween in the collecting medium provided for markedly better survival during storage, and at 80°F, produced better survival after sampler operation.

While Tween tested superior to no Tween in terms of storageability in the sampler, the Tween tended to interfere in direct antigen recognition systems such as ELISA and chemiluminescence. On the theory that the VEE losses in the sampler in the absence of Tween may be due to surface adherence phenomena rather than inactivation, efforts were made to recover the VEE by after-storage treatment with either Tween or bovine serum albumen (BSA). Neither treatment achieved an increase in the secondary recovery of virus. On the other hand, when 1% BSA was added to PBS (without Tween) as a sampling medium, excellent VEE recoveries after sampler operation (161% of the standard) and after 4 h storage (120%) were achieved. This BSA study was conducted, however, only to indicate levels of optimal VEE recoveries. Based on practical considerations and operational limitations, the developers of the XM2 are committed to the present collecting medium

The survival properties of Pseudomonas pseudomallei, Legionella pneumophila and the virus of Rift Valley fever (RVF) after storage were examined in additional experiments. The testing was simplified by using only the PBS with Tween-80 added and testing only the 40°F treatment of both the collecting medium and storage temperature. In addition, the organisms were not disseminated as aerosol but were pipetted into the collecting medium prior to operating the sampler in clean air (100°F) for 45 min. All the organisms survived operation and storage reasonably well. The survival rates after treatment were 98, 74, and 54% for L. pneumophila, P. pseudomallei and RVF virus, respectively.

Presentations:

1. Larson, E. W., The aerobiological threat. Presented at meeting of USAF representatives at USAMRIID, 11 Jan 80.

2. Larson, E. W., Biological threat and U. S. vulnerability. Presented at seminar. Chemical Systems Laboratory, Aberdeen Proving Ground, 4 Apr 80.

3. Larson, E. W., Aerosol stability and respiratory infectivity of Lassa fever virus. Presented at Annual Meeting, ASM, Miami Beach, FL, 11-16 May 1980 (abstracts, 1980, T107, p. 253).

4. Larson, E. W., Survival of airborne organisms: session chairman's introduction. Presented at A Godeon Conference on Aerobiology. Meriden, NH, 10-15 Aug 80.

Publication:

Larson, E. W., J. W. Dominik, and T. W. Slone. 1980. Aerosol stability and respiratory infectivity of Japanese B encephalitis virus. Infect. Immun. 30: in press.

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3. Jahrling, P. B., R. A. Hesse, G. A. Eddy, K. M. Johnson, R. T. Callis, and E. L. Stephen. 1980. Lassa virus infection of rhesus monkeys: pathogenesis and treatment with ribavirin. J. Infect. Dis. 141:580-589

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6412	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. GRP'S SRSTY'S	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM / A. WORK UNIT
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	045
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE: (U) Animal models and animal resources for medical defense studies of diseases of BW importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 002600 Biology; 001700 Animal Husbandry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
76 10	80 09	DA		C. In-house

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

18. RESOURCES ESTIMATE

	PRECEDING	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	80	1.0	63
	CURRENT 81	0	0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Jaax, G. P.
NAME: Stokes, W. S. POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Provide KEYWORDS with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Primates; (U) Animal models; (U) Animal resources

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

23 (U) Evaluate animals as models for the study of infectious and other disease processes under study at USAMRIID: establish such models when appropriate animal species do not exist. Develop facilities and expertise necessary to raise and produce these animals in-house when not available commercially. Establish expertise to raise rhesus monkeys in-house in the event that present scarce supplies become nonexistent or economically prohibitive. This work is essential to assure an adequate supply of appropriate animal models for the study of infectious diseases of potential BW importance.

24 (U) Establish breeding, maternity, obstetric and pediatric techniques for rhesus monkeys. Establish a viable, productive inbred strain 13 colony of guinea pigs to meet critical needs of the Institute. Establish breeding colonies of other laboratory animals as needs arise for specific entities dealt with by USAMRIID investigators.

25 (U) 79 10 - 80 09 - Breeding colonies of Calomys and Sigmodon have stabilized and continue to provide vesper mice and cotton rats as necessary for investigator use. A breeding colony of inbred strain 13 guinea pigs has been established, using NIH-retired breeders in addition to brother-sister mating pairs. The colony has progressed rapidly, undergoing several profound changes in configuration and intent. At present, all offspring produced suitable for breeding are being reintroduced into the colony until the desired population is reached. Twenty live births and 18 weaned juveniles have added to the rhesus colony. Several colony-reared females have begun sexual cycling and to date have shown none of the abnormal behavioral patterns commonly reported in individually housed colony-reared rhesus monkeys. Observations will continue on these animals.

Terminated for management efficiency. Continued in W.U. 871 BB 149.(DAOG3813)

(Available to contractors upon originator's approval)

DD, FORM 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.

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards
(3M162776A841)

Task No. 3M162770A871 BB: Prevention of Bacterial and Rickettsial Diseases of Potential BW Importance

Work Unit No. 871 BB 128: Animal Models and Animal Resources for Medical Defense
(841 00 045) Studies of Diseases of BW Importance

Background:

With the many varied and unusual organisms used at USAMRIID, occasional diseases or organisms are encountered for which no animal model or natural reservoir is known. In some instances a model is known to exist, but no reliable commercial source for that particular animal species exists. If no animal model is known, investigations must be carried out to determine what species would be acceptable. If a model is known but availability is a problem, in-house colonies must be established to meet investigators needs.

Two rodent species not available commercially have been identified as models for diseases under investigation at the Institute. A Calomys callosus (vesper mice) is the natural reservoir host for many arenaviruses, including Machupo virus, and is the only known species colonized which allows Korean hemorrhagic fever virus to grow and replicate. Sigmodon hispidus (cotton rat), is a natural reservoir for VEE and Tamiami viruses.

Preliminary studies with inbred strain 13 guinea pigs have shown them to be the model of choice for research on a number of arthropod-borne arboviruses currently under investigation at USAMRIID. As this strain is not available commercially in the numbers required, a breeding colony in-house is necessary to meet Institute needs. More husbandry problems occur in this strain than in the conventional Hartley strain guinea pig and must be investigated and overcome to assure a viable productive colony.

The distinction of being the most widely used nonhuman primate in research belongs to the rhesus monkey. Because of political developments and population dynamics in the countries of origin, this valuable research asset is in short supply. The large data-base and volumes of experimental results available on the rhesus require that efforts be made to somehow maintain supplies of this research animal. To this end, a program was initiated to develop the expertise necessary to produce and raise the species domestically.

Progress:

Rodent colonies. The primary function of the hispidus and callosus colonies is to meet the documented demands of the Institute for these commercially non-available animals for investigative needs. We have achieved this goal and are

continuing to provide vesper mice and cotton rats to interested investigators. A summary on the 2 species shows that from approximately 28-30 breeding pairs of S. hispidus, 296 were weaned, 190 were issued, and 92 were sacrificed. Of 35 breeding pairs of C. callosus, 311 were weaned, 70, issued, and 121, sacrificed. No major problems associated with husbandry or reproduction were encountered during the reporting period.

A USAMRIID breeding colony of strain 13 guinea pigs, Cavia porcellus has been established, using NIH retired strain 13 breeders in addition to brother-sister mating pairs. Since its inception, the colony has progressed rapidly, undergoing several profound changes in configuration and intent. Allocation of additional space has increased the production potential of the colony considerably and maximum production of offspring should be reached in the fall of 1980. Breeding is proceeding in a harem configuration utilizing standard guinea pig chow supplemented by vitamin C in the drinking water. The breeding colony will eventually be comprised of harems with 660 breeding females. At the present time, all available pigs produced suitable for breeding are being reintroduced into the colony until the desired breeding population is reached.

Macaca mulatta. The rhesus monkey breeding colony has remained stable during the reporting period with 26 female and 5 male rhesus monkeys in residence. Five females and one male breeder were replaced during the period for various reasons. Twenty live births were recorded with 18 juveniles weaned. Four juveniles and one adult female monkey died during the period. The current colony status includes 4 females cycling, 5 females nursing, 8 females pregnant, and the remaining 6 females either bred or pending normal cycle after parturition. There have been 46 surviving offspring since the colony was founded. Growth and maturation data from a physical, radiological and hematological standpoint continue to be collected from neonatal and juvenile rhesus monkeys born in the colony.

Several colony-reared females have begun to cycle and are within normal limits on the basis of physiological parameters. Up to this point, we have not observed the abnormal behavior patterns, e.g., spinning, etc., that have consistently been reported in nonhuman primates individually housed at an early age. This may be attributable to our limited gang housing system for newly weaned monkeys which allows for additional socialization after separation from the mother. Observations on these animals will continue as they approach sexual maturity

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6421	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. ESTR'S ENTRY	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M162776A841	00	047
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Procede with Security Classification Code)

(U) Physiochemical and biological characterization of components of Coxiella burnetii

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
72 09	80 09	DA			C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE

	PREVIOUS/CURRENT	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	80	1.0	133
	CURRENT/ESTIMATE 81	0	0

19a. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
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TELEPHONE: 301 663-2833

20a. PERFORMING ORGANIZATION

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ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

23. KEYWORDS (Provide Each with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Vaccines; (U) Q fever; (U) Rickettsia

24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Provide each with appropriate paragraph identification by number. Precede text of each with security classification code.)

23 (U) Demonstrate vaccine potential of components of Coxiella burnetii to protect troops against Q fever from natural contact or from potential employment as a biological warfare agent.
24 (U) Isolate purified components of C. burnetii, determine antigenic, immunogenic, allergenic and physiocochemical properties; investigate effectiveness for aerosol immunization. Examine pathophysiology of disease in appropriate animal models.
25 (U) 79 10 - 80 09 - Pretreatment of guinea pigs with microgram amounts of lysozyme prior to vaccination with the soluble phase I antigen of C. burnetii enhanced antibody response and protection against challenge. An observed effect on macrophage migration suggested that the role of lysozyme includes stimulation of cell-mediated immunity. Initial steps were taken to focus effort on physical and chemical characterization of phase I antigen in order to meet possible requirements before the antigen could be used as a subunit vaccine. As one approach towards obtaining highly purified antigen, gel filtration chromatography on Sepharose 2B has given definitive separation of the antigen from other components of partially purified preparations. The solubility characteristics of the antigen are being studied in conjunction with the use of centrifugation for concentration of the purified antigen.
Publication: Abstracts, Annu. Mtg. Am. Soc. Microbiol. 1980, p. 61.
Terminated for management efficiency. Continued in W.U. S10 A0 199. (DAOG1522)

*Available to contractors upon originator's approval

DD FORM 1498 1 MAR 80 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 80 (FOR ARMY USE) ARE OBSOLETE.

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BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M162776A841)

Task No. 3M161102BS10 AO: Bacterial and Rickettsial Diseases of Potential BW Importance

Work Unit No. S10 AO 167: Physicochemical and Biological Characterization of Components of Coxiella burnetii

Background:

The main objective of this work unit has been to evaluate the potential of the soluble phase I antigen of Coxiella burnetii for use as a vaccine. The antigen, which has been tested as a vaccine in human volunteers in Czechoslovakia and Romania is much less reactogenic than either cell-wall or whole organism preparations (1). We reported earlier that (a) the antigen is stable to lyopphilization, to storage at 4°C in liquid or dry state, and even to autoclaving; (b) that it has the capacity to induce a cellular immune response; (c) that it is 100X less skin-reactogenic than the Merrell National Laboratories particulate, phase I Q fever vaccine (NDBR-105). The antigen has usually been employed as the dialyzed trichloroacetic acid (TCA) extract of the phase I organism; we have shown that TCA-extracted preparations can be separated, by passage through Sephadex G-200 columns, into 2 major components, only one of which is antigenic and immunogenic.

We found that the immunogenicity of the phase I antigen could be enhznced by the complex of polyriboinosinic-polyribocytidylic acid, poly-l-lysine, and carboxymethylcellulose (poly(ICLC)) if the complex were administered prior to the antigen. In experiments to modify the antigen enzymatically, we determined that an increase in antigenicity and immunogenicity observed with lysozyme was attributable to an adjuvant effect of lysozyme. The results obtained with lysozyme are summarized here, with information relative to its mode of action. Also, during this report period, it was recommended that the main thrust of this work unit be in the direction of characterizing the phase I antigen, physically and chemically, with the objective of meeting possible future requirements for characterization that would be necessary before the antigen could be approved for use as a human subunit vaccine. This report will therefore include results of initial steps to obtain the antigen in purified form from TCA-extracted preparations.

Progress:

Effect of lysozyme on the immune response of guinea pigs to the soluble phase I antigen of Coxiella burnetii. In the enzyme experiments mentioned above, we observed that guinea pigs vaccinated with lysozyme-treated antigen had higher antibody titers and were more resistant to Q fever challenge than those given untreated antigen (2). Continued investigation indicated that lysozyme increased protection by adjuvant action, rather than enzymatically. Results presented here suggest that lysozyme may influence both humoral and cellular immune responses.

In a series of 5 similar experiments, guinea pigs (8 or 10/group, for a total of 92) were inoculated SC with 2 doses, 14 days apart, of antigen only or lysozyme

followed by antigen 4 to 5 h later. Saline and lysozyme control groups (an additional 64 guinea pigs) were included. A dialyzed TCA extract of concentrated, partially purified phase I C. burnetii, Henzerling strain, was employed as antigen. Lysozyme (3X crystalline egg white, Sigma Chemical Co.) was administered at both first and second dose intervals. (Doses of antigen and lysozyme used in each test are presented in the footnotes to Table I). Serum samples obtained 14 days after the second dose were assayed for microagglutinating (MA) and complement-fixing (CF) antibodies.

TABLE I. EFFECT OF PRETREATMENT OF GUINEA PIGS WITH LYSOZYME ON PROTECTION AGAINST Q FEVER BY PHASE I ANTIGEN OF C. BURNETII (MEAN OF 5 TESTS)

TREATMENT	NO. FEBRILE/TOTAL	MEAN FEVER DAYS
Saline	40/41	4.3
Lysozyme[a]	17/22	3.0
Antigen[b]	19/47	1.4
Lysozyme[a] prior to antigen	8/46	0.78

[a]Lysozyme (μg), 1st and 2nd doses per respective test: 12.5, 12.5; 25,25; 12.5, 25; 50, 250.

[b]Antigen (μg protein), 1st and 2nd doses per respective test: 3.5, 3.5; 3.5, 7.0; 3.5, 14.0; 7.0, 7.0; 2.0, 6.0.

Guinea pigs were challenged 28-45 days after the second dose with 5×10^5 MID_{50} of phase I C. burnetii. Temperatures were recorded daily for 10 days; animals with temperatures > 40°C for ≥ 2 consecutive days were considered unprotected.

The effect of pretreatment of guinea pigs with lysozyme on protection against C. burnetii challenge is summarized in Table I. The doses of antigen and lysozyme employed are shown in the footnotes. No optimal dosage combination was found; lower dose levels were as effective as higher levels. Of 46 guinea pigs that received antigen 59% were protected compared to 83% of 46 that received lysozyme prior to antigen ($P < 0.02$). In the same experiments, the effect of lysozyme on antibody response was determined on sera obtained 14 days after the second dose. Table II shows the geometric mean titers and % animals responding for phase I and II MA antibodies and phase II CF antibody (phase CF antibody is not produced at detectable levels by immunization with the phase I antigen, or with killed whole organism vaccines).

TABLE II. EFFECT OF PRETREATMENT OF GUINEA PIGS WITH LYSOZYME ON ANTIBODY RESPONSE TO PHASE I ANTIGEN OF C. BURNETII

ANTIBODY	TREATMENT		GEOM. MEAN TITER	% POSITIVE
	Antigen	Lysozyme		
CF-phase II	+		4.5	42
	+	+	12.2	78
MA-phase I	+		2.9	63
	+	+	6.3	82
MA-phase II	+		28.6	74
	+	+	39.8	98

The most pronounced difference was seen with the phase II CF antibody ($P < 0.001$); differences for MA-1 and MA-11 antibody titers were also significant, $P < 0.05$ and $P < 0.001$, respectively.

The time of administration of lysozyme relative to antigen may be important. In a single experiment, lysozyme injected 24 or 48 h before antigen reduced the level of protection. Administration of lysozyme and antigen at the same time, or of a mixture of lysozyme and antigen, gave no consistent results in several trials.

To investigate the possibility that lysozyme increased protection by stimulation of cellular immune mechanisms, we applied the macrophage migration-inhibition (MMI) technique to peritoneal cells from groups of guinea pigs (4/group). One group that received 2 doses, 2.0 and 6.0 µg (protein) of antigen only, 14 days apart, was compared with a group that received lysozyme, 50 and 250 µg, 5 h before each dose of antigen. Saline and lysozyme control groups were included.

Peritoneal exudate calls were harvested, processed, and employed in the agarose droplet method as described by Kishimoto and Burger (3) to detect direct MMI. Cells were collected 4 days after IP injection of 25 ml of sterile sodium caseinate. Half of the animals were tested, i.e., given caseinate 1 week, and half 2 weeks after the second dose of antigen. In the absence of apparent differences, results from the 2 time periods were combined for purposes of analysis and presentation. Twenty replicate agarose droplets containing exudate cells were prepared from cells harvested from each guinea pig. Subsets of 5 droplets each were overlaid with 0.2 ml of medium 199 (with calf serum) alone, or 0.2 ml of medium containing: (a) 100 µg/ml lysozyme, (b) 20 µg/ml antigen, or (c) 100 µg/ml lysozyme and 20 µg/ml antigen. Cultures were incubated, droplets examined and MMI calculated as described by Kishimoto and Burger (3). As shown in Table III, MMI from guinea pigs that received antigen only was much less than the inhibition of macrophages from animals that received lysozyme prior to antigen. Also, inhibition observed in subsets of droplets in the test system where lysozyme + antigen were used as additives was substantially greater than in subsets with antigen alone; this was especially pronounced with macrophages from guinea pigs that received the lysozyme-antigen regimen. Also in this group, lysozyme itself produced limited inhibition.

TABLE III. MMI OF GUINEA PIGS VACCINATED WITH PHASE I ANTIGEN OF C. BURNETII WITH AND WITHOUT PRIOR ADMINISTRATION OF LYSOZYME (n=4)

ADDED TO TEST SYSTEM (µg/ml)	% MMI	
	Antigen Only	Lysozyme Prior to Antigen
Lysozyme, 100	0	13
Antigen, 20	9	22
Lysozyme, 100 - Antigen, 20	20	57

Active immunity to Q fever has been reported to depend on both humoral and cellular responses. Other recent research has indicated that cellular immune mechanisms are exclusively responsible for protection against Q fever. The increase in antibody titer and the effect on macrophage-migration, which we have observed, suggest that the role of lysozyme could include a stimulation of both humoral response and cell-mediated immunity.

Purification and characterization of the phase I antigen of C. burnetii. Initial steps were taken to direct the research towards physical and chemical characterization of the soluble phase I antigen of C. burnetii, with the objective of meeting possible future requirements for characterization that would be necessary before the the antigen could be approved for use as a vaccine.

Gel filtration. Physicochemical characterization requires available of the purified antigen in amounts sufficient to permit valid analyses. We have initiated approaches to isolate the antigen as a single entity from a TCA extract of phase I C. burnetii. We have shown previously that dialyzed TCA extracts could be separated by filtration through Sephadex G-200 columns into 2 major components, only 1 of which was antigenic and immunogenic. However, since the active component was eluted from Sephadex G-200 with, or near, the void volume, it was difficult to assess the degree of resolution obtained with this column.

In our current investigation of purification techniques, we found that gel filtration on Sepharose 4B was not satisfactory in that the active component eluted near the void volume, as it had done on Sephadex G-200. However, with the more porous sepharose 2B, we have obtained good separation of the active peak from the void volume as well as from a second (inactive) peak. Using descending chromatography at a flow rate of 5 ml/h on a colum 9 x 120 mm, 1-ml fractions were collected; 0.09 M NaCl was used as eluant. Fractions were examined for UV adsorption at 224, 233, 254, and 280 nm. With 0.3- or 0.4-ml samples applied to this column, the active component was eluted in 4 fractions with a peak at fraction 8; the second component eluted in 6 fractions with a peak at fraction 28. Activity of peaks was established by measuring the CF titers of individual fractions. In earlier sephadex G-200 experiments, we had shown that CF-negative fractions did not. Also, we found in skin tests with guinea pigs that CF-negative fractions possessed some reactogenicity; therefore, a purified antigen would be even less reactogenic than TCA extracts of the antigen.

It will be necessary to scale up filtration on Sepharose 2B to larger columns and to confirm immunogenic and reactogenic properties of the eluted components.

Centrifugation. Kazar et al. (4) reported that high-speed centrifugation (105,000 x g for 4 h) of TCA extracts of phase I C. burnetii sedimented a lipopolysaccharide-protein complex (LPS-PC) which induced in mice and rabbits "fair" levels of antibody if high doses (up to 1,000 µg) were administered. Repeated attempts to obtain antibody response in guinea pigs were unsuccessful. Mice were partially protected against challenge by 100-µg doses of LPS-PC; partial protection of guinea pigs by 2 400-µg doses was obtained, but only by lowering the fever cut-off temperature to 39.5°C, instead of the usual 40.0°C. The lipopolysaccharide (LPS) which Baca and Paretsky (5) obtained by high-speed centrifugation (105,000 x g for 3 h) of the aqueous phase of hot phenol extracts of phase I C. burnetii was shown to produce physiological and biochemical effects in the guinea pig characteristic of Q fever. No mention was made of antigenicity; it is assumed that this LPS was nonantigenic since phenol extraction of TCA extracts of the phase I antigen yields nonimmunogenic haptenes.

We were interested in determining whether high speed centrifugation of our TCA extracts of phase I C. burnetii would produce a sedimentwith the very low antigenicity described by Kazar for the LPS-PC and, if so, in determining its immunogenic and reactogenic properties. For evaluation in a guinea pig protection test, high-speed sediment and supernatant samples were prepared by centrifugation of a TCA extract of the phase I antigen at 105,000 g for 4 h. The small translucent sediments were resuspended in water, recentrifuged and resuspended in water to 5X original volume. Protein contents of starting TCA extract, and of sediment and supernatant were determined, and these preparations were diluted with physiological saline to 4 µg/ml protein. Guinea pigs (10/group) were inoculated SC with 0.5 ml (2 µg protein). Freshly prepared samples were injected at the same dose level 16 days after the first dose. Postvaccination sera, obtained 11 days after the second dose, were assayed for phase I and II MA antibodies and phase II CF antibody. Geometric mean titers and the percentage of animals responding are shown in Table IV.

TABLE IV. COMPARISON OF ANTIGENICITY OF HIGH-SPEED SEDIMENT AND SUPERNATANTS

	CF-II		MA-1		MA-II	
PREPARATION	Titer[a]	% Pos.	Titer	% Pos.	Titer	% Pos.
TCA extract	4.0	60	1.2	10	7.0	60
Supernatant	2.1	40	1.2	10	2.1	30
Sediment	97.0	100	2.8	80	222.7	100

[a]Geometric mean titer.

Contrary to expectations, the group of guinea pigs that received the sediment preparation had much higher antibody levels, specifically phase II MA and CF, than the other groups. Guinea pigs were challenged IP 32 days after the second dose with 1 x 10^6 ID_{50} of phase I C. burnetii. Temperatures were recorded daily for 10 days; animals with temperatures > 40.0°C for ≥ consecutive days were considered unportected. As indicated in Table V, the group of 10 guinea pigs that received the sediment sample had only 3 days of fever for a fever day/animal ratio of 0.3, much lower than those of the other groups. Based on these results, it appears that the high-speed sediment obtained here is not the same, at least biologically, as that reported by Kazar.

TABLE V. COMPARISON OF THE IMMUNOGENICITY OF HIGH-SPEED SEDIMENT AND SUPERNATANTS OF EXTRACTS OF THE PHASE I ANTIGEN OF C. BURNETII

PREPARATION	NO. FEBRILE/TOTAL	TOTAL FEVER DAYS	MEAN FEVER DAYS
Saline	6/7	18	2.3
TCA extract	4/10	14	1.4
Supernatant	1/7	7	1.0
Sediment	1/10	3	0.3

We plan to reassess the solubility characteristics of the phase I antigen, considering the possible application of this information to the objective of obtained purified antigen.

Presentations:

1. Wachter, R. F. Q fever, How big is the threat? Presented at Symposium on Military Veterinary Medicine, Walter Reed Army Institute of Research, Washington, DC, 19 May 1980.

2. Wachter, R. F., and G. P. Briggs. Effect of lysozyme on the immunogenicity of the soluble phase I antigen of Coxiella burnetii. Present at Ann. Mtd. ASM, 1980. (Abstract Am. Soc. Microbiol., p 61).

LITERATURE CITED

1. Anacker, R. L., D. B. Lackman, E. G. Pickens, and E. Ribi. 1962. Antigenic and skin-reactive properties of fractions of Coxiella burnetii. J. Immunol. 89:145-153.

2. Wachter, R. F., and G. P. Briggs. 1979. Enzymatic modification of the antigenicity and reactogenicity of the soluble phase I antigen of Coxiella burnetii.

3. Kishimoto, R. A., and G. T. Burger. 1977. Appearance of cellular and humoral immunity in guinea pigs after infection with Coxiella burnetii administered in small particle aerosols. Infect. Immun. 16:518-521.

4. Kazar, J., S. Schramek, and R. Brezina. 1978. Immunological properties of the lipopolysaccharide-protein complex of Coxiella burnetii. Acta Virol. 22:309-315.

5. Baca, O. G., and D. Paretski. 1974. Some physiological and biochemical effect of a Coxiella burnetii lipopolysaccharide preparation on guinea pigs. Infect. Immun. 9:939-945.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0F6416	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'S DIST'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	050
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE (Provide with security Classification Code)

(U) Therapy of disease transmitted by aerosol: Legionnaires' disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
75 02	80 09	DA			C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR 80 (PREVIOUS)	1.0	225
FISCAL YEAR 81 (CURRENT)	0	0

19a. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

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20a. PERFORMING ORGANIZATION

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PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic participation)

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Provide KWOC with security Classification Code) (U) Military medicine (U) BW defense; (U) Respiratory diseases; (U) Antibiotic therapy; (U) Legionnaires' disease bacillus

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

23 (U) Devise more efficient methods for treating and monitoring the effects of treatment of respiratory bacterial infections. This information is essential for the expeditious therapy of troops subsequent to BW attack.

24 (U) Determine feasibility of aerosol therapy. Compare efficacy with conventional techniques; determine pharmacokinetics, toxicity and biochemical sequelae to monitor progress of disease.

25 (U) 79 10 - 80 09 - Either prior influenza virus infections or treatment with immunosuppresant drugs markedly increased fatality in guinea pigs and monkeys exposed to Legionella pneumophila. It has also been possible to produce secondary infection in one of 3 immunosuppressed guinea pigs. Prior infection protects guinea pigs against a normally fatal L. pneumophila rechallenge, but rechallenged animals die very readily after cardiac puncture (blood sampling), suggesting possible immune complex disease. L. pneumophila, suspended in tryptose-saline, is very unstable in aerosols at 30% relative humidity (RH) but relatively stable at 50 and 80% RH. The addition of 6% raffinose and 0.1% dipyridyl greatly enhanced stability; the latter was found to be the more active ingredient. Suspending the organism in spent media in which blue-green algae were grown also stabilized the organism. It is possible that aerosols of L. pneumophila are very sensitive to iron.

Publications: Am. J. Vet. Res. 41:1492-1494, 1980; J. Infect. Dis. 141:186-192, 1980; J. Infect. Dis. 141:689, 1980

Terminated for management efficiency. Continued in W.U. 870 BB 069. (DAOG3814)

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BODY OF REPORT

Project No. 3M162770A870: (3M162776A841) Risk Assessment of Military Disease Hazards (U)

Task No. 3M1662770A870 BB: Assessment of Airborne Microbial Agents of Potential BW Threat

Work Unit No. 870 BB 043: (841 00 050) Therapy of Respiratory Bacterial Infections Transmitted Via Aerosols

Background:

Preliminary experiments on the infectivity of aerosols of Legionella pneumophila were described previously (1). Aerosols of the organism were found to be more lethal for guinea pigs than were IP infected cells. A guinea pig model was developed in thish the major criteria of infection were weight loss, fever and seroconversion. During the past year, the investigation of L. pneumophila has been greatly extended. Studies have included: i.) infectivity for squirrel monkeys, ii.) cross-infection experiments with guinea pigs, iii.) effect of rechallenge of previously infected guinea pigs, iv.) the effect of guinea pig size and sex on susceptibility to infection, v.) sequential respiratory infection with influenza virus and L. pneumophila in AKR/J mice and squirrel monkeys, vi.) survival of L. pneumophila in aerosols, and vii.) effect of immunosuppression on the susceptibility of guinea pigs to Legionnaires' disease agent.

Progress:

Squirrel monkeys were challenged with L. pneumophila by aerosol and by intratracheal (IT) instillation. The most notable reactions of monkeys to IT doses of L. pneumophila are given in Table I.

TABLE I. REACTION OF SQUIRREL MONKEYS (n=2) TO CHALLENGE WITH L. PNEUMOPHILA

DAY AFTER INFECTION	RESPIRATORYS/MIN		WEIGHT CHANGE (%)		DYSPNEA (+/Total)	
	$6x10^5$	$6x10^8$	$6x10^5$	$6x10^8$	$6x10^5$	$6x10^8$
Baseline	100	93	NA	NA	0/2	0/2
1	112	138	-7.3	-5.9	0/2	0/2
2	125	167	-6.3	-6.5	2/2	2/2
3	113	131	-6.5	-6.7	2/2	1/2
4	104	136	-6.7	-5.3	2/2	2/2
5	113	140	-6.6	-6.5	1/2	2/2
6	107	152	-8.1	-6.3	0/2	2/2
7	110	123	-6.7	-6.1	2/2	2/2
8	110	109	-6.7	-3.9	2/2	0/2
9	103	117	-6.2	-5.5	2/2	0/2
10	93	92	-6.5	-5.7	0/2	0/2

All monkeys showed anorexia, lethargy and leukocytosis at some time during the study, but no consistent trends were noted. Also, every monkey seroconverted with a microagglutination (MA) titer (> 1:32 on day 14). The reaction of squirrel monkeys to aerosols of 3×10^6 organisms is given in Table II.

TABLE II. REACTION OF SQUIRREL MONKEYS (n=2) TO AEROSOL DOSES OF 3×10^6 L. PNEUMOPHILA

DAY AFTER INFECTION	LEUKYCYTES/mm^3 ($\times 10^3$)	RESPIRATIONS/MIN	SERUM GEOM. MEAN MA TITER
Baseline	5.2	92	< 8.0
1	9.8	114	
2	13.5	165	
3	6.3	163	
4	7.2	156	
5	6.0	131	
6	5.2	127	
14			32.0
24			45.0

Dyspnea, lethargy, anorexia and weight loss were noted, but as with IT instillation, consistent trends were not seen. The monkeys showed no signs of marked illness after challenge by either route, but there is no doubt that the animals were infected and milkdly ill. In subsequent investigations involving squirrel monkeys, no monkey died of Legionella infection, regardless of dose, it if was otherwise normal.

Secondary infection with L. pneumophila in human patients is very rare. This is a curious situation, since pneumonia is common. To study the transmission of this organism from infected to normal hosts, we have constructed a plastic box. One infected and one normal guinea pig were placed in one pan, and two normal animals were placed in a second pan so situated that all potentially infected air from the infected guinea pig passed over them. The temperature and weight of all guinea pigs was determined daily. The average infecting dose for the single guinea pig was about 10^5 organisms. In 2 of 6 trials, the infected guinea pig died; in the other 4 trials, infection was confirmed by the criteria of weight loss, fever and seroconversion. In one of these trials did the cagemates or "downwind" guinea pigs show any response at all, and none seroconverted. In a separate experiment in which guinea pigs were immunosuppressed by methods that will be discussed, one "downwind" guinea pig showed a significant increase in the 14-day serum MA titer. This experiment will be repeated. This work has been complicated by the fact that L. pneumophila is difficult to isolate from contaminated air samples because of its slow growth rate and the lack of a differential medium. If the immunosuppression experiment can be repeated, then it can be assumed that the organism is shed from infected guinea pigs, but not in sufficient numbers to produce infection in normal hosts. On the other hand, we have recently demonstrated that aerosol doses as low as 129 organisms are sufficient to infect guinea pigs (2).

In the course of a number of experiments, the development of serum MA antibody was measured. Also the presence of cell-mediated immune response as typified by the delayed type hypersensitivity reaction (DTH) at 21 days was determined. Guinea pigs, given 500 L. pneumophila by aerosol, were injected ID 21 days later with 0.05 ml containing 10^6 killed organisms. At 48 h, typical induration and erythema were noted, indicating that DTH occurred.

Since protection against rechallenge must be considered the final criterion of the immune response, several experiments were performed to determine whether previously challenged guinea pigs were immune to rechallenge. Sixteen guinea pigs were infected with aerosols of a relatively small dose of L. pneumophila, ca. 5 x 10^2. Temperatures and body weight were determined daily for 8 days and serum MA titers were measured at 0, 14, 21, and 28 days. The data (Table III) were typical of reactions to the low challenge dose. At 28 days, the guinea pigs were again challenged with higher dose aerosols of L. pneumophila but ca. 10^6 (ca. 6 LD_{50}). At the same time, 8 normal (no prior infection) guinea pigs were exposed as controls. All animals were bled by cardiac puncture at 48 h to determine whether an anamnestic reaction had occurred. Serum MA titers were elevated in rechallenge animals, controls had not titer (Table III).

TABLE III. CLINICAL AND IMMUNOLOGICAL RESPONSE OF GUINEA PIGS INFECTED WITH AEROSOLS OF 5x10^2 L. PNEUMOPHILA AND RECHALLENGED AT 28 DAYS WITH 10^6

DAY	TEMPERATURE, °F	WEIGHT (%)	GEOM. MEAN MA TITER
Baseline	102.0	NA	< 8.0
1	102.0	-0.4	-
4	103.8	-2.2	-
5	103.2	-3.2	-
6	102.7	-2.0	-
7	102.6	-2.8	-
8	107.2	-0.4	-
14	-	-	83.0
21	-	-	30.6
28	-	-	40.3

Rechallenge at 28 Days			
Geom. Mean MA Titer at 48 h		Geom. Mean Time-to-Death (days)	
Convalescent	Control	Convalescent	Control
94.8	< 8.0	2.1*	3.5

*P < 0.01

Fourteen of 16 rechallenged and all control guinea pigs died. The rechallenged died significantly more quickly than did the controls (P < 0.01). This observation suggested that death was due either to an immune reaction (anaphylaxis) or

to the effect of cardiac puncture. These animals were not necropsied, but in later similar experiments it was ascertained that death was not due to hemorrhage into the chest cavity after cardiac puncture.

To examine the phenomenon of apparent lack of protection afforded by recovery from infection two experiments were performed. In the first, 24 guinea pigs were infected with 3×10^3 L. pneumophila and examined daily for fever and weight loss. Both signs were present in all animals. Mean serum MA titer were 1:31 and 1:23 at 14 and 28 days, respectively. At 28 days, the previously infected animals were rechallenged with 10^6 organisms. Sixteen normal guinea pigs were challenged at the same time. Results are shown in Table IV. No blood samples were obtained after rechallenge. None of the rechallenged animals died, whereas 14 of 16 controls succumbed, indicating that the reaction described previously was related to cardiac puncture.

TABLE IV. EFFECT OF CARDIAC PUNCTURE ON SURVIVAL OF GUINEA PIGS AFTER RECHALLENGE WITH L. PNEUMOPHILA

TREATMENT	NO. DEAD/TOTAL	MTD (Days)
Experiment I		
Rechallenged at 28 days[a] - no cardiac puncture	0/24**	NA
Infected controls	14/16	5.8
Experiment II		
Rechallenged at 28 days[b] - cardiac puncture	9/20*	3.4[†]
Infected controls	10/10*	5.2
Cardiac puncture controls	1/20	2.0

[a]Challenge dose = 3×10^3 organisms; rechallenge dose = 1.0×10^6 organisms.

[b]Challenge dose = 3×10^3 organisms; rechallenge dose = 3.0×10^6 organisms.

*$P < 0.025$ vs. cardiac puncture controls, **$P < 0.005$ vs. infected controls (χ^2 test, Yates correction).

[†]$P < 0.005$ vs. infected controls (student's t test).

The second experiment involved exposure of 20 guinea pigs to a dose of 3×10^3 organisms. When clinically evaluated over 10 days, all responded with typical fever and loss of weight. These animals were bled for serum MA titer by cardiac puncture prior to exposure and at 14 and 25 days after exposure. A second group of 20 guinea pigs was bled concurrently with the first, but was not exposed to L. pneumophila. On the 28th day, the previously exposed guinea pigs, as well as 10 additional animals which had not been used previously, were challenged with 3.0×10^6 organisms. All guinea pigs were bled by cardiac puncture at 48 h. Nine of 18 rechallenged and all 10 infected-control guinea pigs died (Table IV). The mean time to death of the rechallenged animals was once again less than that of infected controls. An interesting observation in the case of the rechallenged animals was that those guinea pigs that ultimately died showed a transient rise

in temperature 24 h after exposure, whereas survivors did not. In addition to serology, fever and weight determinations, the number of viable L. pneumophila in the lungs of the rechallenged and infected groups was determined. Two guinea pigs from each group were killed at 1, 28, and 72 h after rechallenge; lungs were homogenized and the number of viable bacteria in each sample determined by routine plating methods. The data show that more than 10^5 L. pneumophila were found in the lungs of immune animals at 72 h (Table V).

TABLE V. EFFECT OF PRIOR INFECTION ON NUMBER OF VIABLE L. PNEUMOPHILA IN LUNGS OF RECHALLENGED GUINEA PIGS

GROUP	NO. L. PNEUMOPHILA IN LUNGS		
	1 h	48 h	72 h[a]
Previous uninfected - controls	2.1×10^5	3.2×10^8	5.4×10^8
Previously infected - rechallenged[b]	4.1×10^5	1.4×10^5	1.1×10^5

[a]Cardiac puncture at 48 h.

[b]Initial dose = 3×10^4, rechallenge dose = 3×10^6 organisms.

Presently, it is not known whether these organisms were intracellular. The serological titers obtained during this experiment are given in Table VI. The slight diminution of titer in the rechallenge guinea pigs 48 h after rechallenge was provably due to complexing. The results suggest that the death of rechallenged guinea pigs was not due to anaphylaxis nor to the trauma of cardiac puncture per se. It may be due to the effect of cardiac puncture (or possibly other stress) on a heart that has been damaged by some form of immune-complex related disease. Another possibility is that of disseminated intravascular coagulation; this will be investigated; blood coagulation studies are planned in collaboration with Dr. Woodruff (Pathology Division).

TABLE VI. MA TITER OF GUINEA PIGS AFTER PRIMARY AND SECONDARY CHALLENGE WITH AEROSOLS OF L. PNEUMOPHILA

GROUP	GEOM. MEAN SERUM MA TITER AT DESIGNATED TIME				
	Baseline	14-day	25-day (3 days prior to rechallenge)	48-h post-rechallenge	14-day post-rechallenge
Experimentals	< 8.0	169	29.9	22.6	53.0
Rechallenge controls[a]	NA	NA	< 8.0	< 8.0	all dead
Bleeding	< 8.0	< 0.80	< 8.0	< 8.0	< 8.0

[a]Initial dose = 3×10^4, rechallenge dose = 3×10^6 organisms.

The protective effect of recovery from infection was also investigated in squirrel monkeys. Three groups of 2 monkeys each were given IT doses ranging from 6.5×10^5 to 6.2×10^8 L. pneumophila. Every monkey became mildly ill after challenge; dosage had no significant effect. Serum MA titers are given in Table VII. At 49 days, all monkeys as well as 2 previously uninfected controls were challenged by IT instillation of 2×10^7 organisms (Table VII).

TABLE VII. MA TITERS OF SQUIRRED MONKEYS (n=2) AFTER INITIAL INFECTION AND RE-CHALLENGE

DAY AFTER INFECTION	GEOM. MEAN SERUM MA TITER AT INDICATED DOSAGE			
	6.2×10^5	6.2×10^7	6.2×10^8	Control (rechallenge only)
Baseline	< 8.0	< 8.0	< 8.0	NA
7	22.6	22.6	256.0	NA
14	64.0	45.3	181.0	NA
21	64.0	45.3	128.0	NA
28	256.0	22.6	453.3	NA
	Rechallenge (all monkeys received 2×10^7 on day 49)			
Baseline	32.0	45.3	181.5	< 8.0
2	22.6	32.0	22.6	< 8.0
9	512.0	128.0	724.0	1024.0
15	256.0	724.1	1024.0	724.1
21	128.0	362.0	1024.0	363.0
29	256.0	362.0	1024.0	256.0

As in guinea pigs, the titer dropped at 48 h. The pattern thereafter suggests that the monkeys that received the highest dose on original challenge developed the highest titers after the second inoculation. The numbers, however, were too small for statistical analysis. Clinical observations are shown in Table VIII.

TABLE VIII. VALUES OF SELECTED PARAMETERS IN SQUIRREL MONKEYS RECHALLENGED 49 DAYS AFTER INITIAL INFECTION WITH 2×10^7 L. PNEUMOPHILA

DAY AFTER RECHALLENGE	RESPIRATORY RATE/MIN		% FOOD CONSUMED		BODY WEIGHT Δ%	
	Previous Infection (n=6)	Control (n=2)	Previous Infection	Control	Previous Infection	Control
Baseline[a]	89	85	100	100	NA	NA
1	142	142	32	13	-2.3	-0.6
2	145	132	77	31	-012	-016
3	155	154	81	81	-0.2	-7.4
4	163	160	71	69	-1.7	-9.0
5	132	161	90	54	-3.5	-9.1
6	114	140	94	94	-0.7	-9.9
7	119	133	98	100	-0.6	-8.4
8	119	129	94	100	-0.5	-6.5
9	123	129	83	100	-0.8	-6.5
10	110	156	92	100	-1.1	-5.1

[a]49 days after initial infection.

The response of the 3 dose groups (i.e., original challenge) did not vary at rechallenge so the data were combined. Unfortunately, the effects were difficult to measure because the disease was very mild in controls. However, weight loss was more pronounced, anorexia was greater, and increased respiratory rates were of longer duration in controls than in the previously-infected monkeys. The squirrel monkey, however, cannot be regarded as a good host for the study of the protective efficacy of recovery from acute legionellosis.

The study of L. pneumophila also included determination of the effect of two risk factors: prior viral infection and immunosuppression. To study the effect of prior virus infection, we selected a mouse-adapted variant of the Aichi/2/68 strain of type A influenza virus (H3N2). The virus was propagated in embryonated eggs and titered $10^{8.2}$ median egg infectious doses (EID_{50}/ml) of allantoic fluid.

Two hosts were chosen for study: the AKR/J mouse, which has been reported to be susceptible to L. pneumophila challenge by Hedlund et al (3), and the squirrel monkey, which demonstrates a mild clinical response to IT instillation with either influenza virus (4) or L. pneumophila (unpublished observation).

For infection with both organisms, mice were lightly anesthetized with halothane and inoculated intranasally (IN) with 0.05 ml of appropriate dilutions. Preliminary titrations with graded doses established the LD_{50} of the influenza virus by the route to be $10^{4.8}$ EID_{50} and that of L. pneumophila to be 1.1×10^8 organisms. Fifteen mice in each of 3 groups were than treated: one group received $10^{4.0}$ EID_{50} of influenza virus in 0.5 ml of heart-infusion broth (HIB); 3 days later, they were given 0.05 ml of tryptose saline diluent. A second group was inoculated with virus and 3 days later were inoculated IN with 10^5 L. pneumophila. The third group

The protective effect of recovery from infection was also investigated in squirrel monkeys. Three groups of 2 monkeys each were given IT doses ranging from 6.5×10^5 to 6.2×10^8 L. pneumophila. Every monkey became mildly ill after challenge; dosage had no significant effect. Serum MA titers are given in Table VII. At 49 days, all monkeys as well as 2 previously uninfected controls were challenged by IT instillation of 2×10^7 organisms (Table VII).

TABLE VII. MA TITERS OF SQUIRRED MONKEYS (n=2) AFTER INITIAL INFECTION AND RE-CHALLENGE

DAY AFTER INFECTION	GEOM. MEAN SERUM MA TITER AT INDICATED DOSAGE			
	6.2×10^5	6.2×10^7	6.2×10^8	Control (rechallenge only)
Baseline	< 8.0	< 8.0	< 8.0	NA
7	22.6	22.6	256.0	NA
14	64.0	45.3	181.0	NA
21	64.0	45.3	128.0	NA
28	256.0	22.6	453.3	NA
	Rechallenge (all monkeys received 2×10^7 on day 49)			
Baseline	32.0	45.3	181.5	< 8.0
2	22.6	32.0	22.6	< 8.0
9	512.0	128.0	724.0	1024.0
15	256.0	724.1	1024.0	724.1
21	128.0	362.0	1024.0	363.0
29	256.0	362.0	1024.0	256.0

As in guinea pigs, the titer dropped at 48 h. The pattern thereafter suggests that the monkeys that received the highest dose on original challenge developed the highest titers after the second inoculation. The numbers, however, were too small for statistical analysis. Clinical observations are shown in Table VIII.

received 0.05 ml of HIB followed 3 days later by 10^6 L. pneumophila. The mortality rate among the mice given the sequence of virus followed by bacteria was significantly higher than that of either of the single organism control groups (Table IX).

TABLE IX. RESPONSE OF AKR/J MICE TO IN INSTILLATION OF $10^{3.0}$ EID_{50} OF INFLUENZA VIRUS FOLLOWED BY 10^5 L. PNEUMOPHILA

TREATMENT	GEOM. MEAN TIME-TO-DEATH (days)	NO. DEAD/TOTAL	P[a]
Influenza virus alone on day 0	8.4	5/14	0.025
Influenza virus on day 0 + L. pneumophila on day 3	7.1	13/15	0.005
L. pneumophila alone on day 3	NA	0/15	

[a] χ^2 test, with Yates correction.

Confirmatory experiments with monkeys were restricted in scope because of ost and availability. Preliminary observations on groups of 4 monkeys each given 10^6 L. pneumophila either by IT instillation or aerosol showed that clinical signs of illness as dyspnea, coughing, sneezing, nasal crusting and lethargy were present at various times, but were inconsistent and could not be considered as reliable indicators of infection. The IT group monkeys showed significant leukocytosis, anorexia, weight loss and increased respiratory rate; responses were somewhat less marked in aerosol-exposed monkeys. Serum MA titers were greater in IT-monkeys than in the aerosol group, but all were infected. No monkeys died or were markedly ill.

To determine the effect of sequential respiratory infection, 8 monkeys were infected IT with 10^7 EID_{50} of influenza virus, and 4 with sterile HIB. Three days later, 4 of 8 influenza-infected monkeys and the 4 HIB-instilled monkeys were exposed to an aerosol dose of 10^7 L. pneumophila. The 4 remaining influenza-infected animals were reserved as controls with no further treatment. Two of the 4 sequentially-infected monkeys died, on each on days 5 and 8 (Table X).

TABLE X. RESPONSE OF SEQUIRREL MONKEYS TO SEQUENTIAL RESPIRATORY INFECTION WITH INFLUENZA VIRUS AND L. PNEUMOPHILA

EXPOSURE AND DAY	DEAD/TOTAL
10^8 EID_{50} influenza virus - IT (day 0)	0/4
10^8 EID_{50} influenza virus (day 0) + $10^{8.0}$ L. pneumophila aerosol (day 3)	2/4
$10^{8.0}$ L. pneumophila aerosol (day 3)	0/4

Lungs of both contained at least 10^7 L. pneumophila. None of the monkeys in this experiment, including those that died, had fever > 103°F.

Although small numbers preclude statistical analysis, it appeared that the sequentially-infected monkeys lost more weight, ate less and had more marked leukocytosis than either of the control groups. The sequence of influenza followed by Legionnaires' disease may be relatively uncommon in nature because of the differing seasonal patterns of the 2 diseases. What these data suggest, however, is that respiratory viruses may enhance host susceptibility to subsequent L. pneumophila infection. The second risk factor investigated during the past year was that of immunosuppression. A number of reports in the literature linking immunosuppression and increased susceptibility to fatal Legionnaires' disease provided the rationale for developing a model of use in therapy studies. The approach adopted for use in the guinea pig was based on that developed by Pennington and Ehrie (5), who used immunosuppression to produce fatal Pseudomonas aeruginosa pneumonia. The immunosuppressant regimen for all of the experiments reported here was 20 mg/day (40 mg/kg) of cyclophosphamide (Cytoxan, Mead Johnson) injected SC and 25 mg/day (50 mg/kg) of hydrocortisone sodium succinate (solu-Cortef, Upjohn) injected IM. Guinea pigs were treated once daily for 4 days. When the effects of infection were studied, the guinea pigs were exposed to aerosols of L. pneumophila on day after the last dose of drugs was administered.

Because frequent glood samples were required to monitor the effects of drug treatment and because cardiac puncture is a hazardous procedure for the host, an experiment was performed to determine whether sufficient blood for leukycyte, erythrocyte, and hematocrit determinations could be obtained by clipping the toe-nails. Three guinea pigs were employed, the nails were cleaned with alcohol, dipped, the first drop of blood wiped off, and a small amount of blood collected in a heparinized capillary tube. A small sample of blood was then collected from the heart, The results of this comparison are given in Table XI.

TABLE XI. EFFECT OF BLOOD SAMPLING PROCEDURE IN GUINEA PIGS

	Mean No./mm^3 ± SD[a]		
ROUTE OF SAMPLING	LEUCOCYTES	ERYTHOCYTES ($x10^6$)	HEMATOCRIT (%)
Cardiac puncture	3790 ± 790	4.14 ± 0.62	34.0 ± 5.3
Toe clip	5753 ± 922	5.16 ± 0.61	42.9 ± 4.9
P (t-test)	< 0.05	< 0.005	< 0.025

[a]Each value is the mean of 3 determinations for 3 days.

The values for blood obtained by the toe clip technique were significantly higher than by cardiac puncture, possibly because the toe clip procedure provides capilary rather than venous or arterial blood. Novertheless, the toe clip procedure has proven to be reliable, reproducible, and has been adopted for routine use.

Table XII presents total and differential leukocyte data obtained on groups of 8-10 guinea pigs treated on days 1-4, either with cyclophosphamide, hydrocortisone, or a combination of the 2 drugs.

TABLE XII. EFFECT OF IMMUNOSUPPRESSION ON TOTAL AND DIFFERENTIAL LEUKOCYTE COUNTS IN GUINEA PIGS

DAY AFTER INFECTION	TOTAL WBC/mm^3			% NEUTROPHILS[a]		
	Cytoxan	Hydrocortisone	Combination	Cytoxan	Hydrocortisone	Combination
Baseline	9,100	9,300	8,200	72.0	72.3	48.0
1	6,400	9,400	6,900	51.5	58.3	38.0
2	6,200	10,200	6,300	56.3	52.3	57.0
3	5,800	9,000	5,000	64.8	53.3	57.0
4	3,200	8,800	2,900	48.8	62.0	38.0
5	1,800	8,500	1,700	11.3	61.5	12.0
6	1,600	7,600	1,800	2.0	55.3	2.7
7	2,200	7,800	1,900	2.0	51.0	0.7
8	2,200	8.400	1,800	5.3	52.8	2.8
11	8,400	9,600	9,200	52.5	49.8	47.0

[a]Lymphocyte percentages are approximately equal to the difference between % neutrophils and 100%

An additional group of 10 guinea pigs were not treated and were bled daily. The latter group showed no deviation from baseline. and data obtained from them is not presented. Cortisone had no discernible effect. Leukopenia, reaching a minimum on days 5-6, was observed in all guinea pigs treated with cyclophosphamide, alone or in combination with hydrocortisone. The leukopenia was primarily a neutropenia, the effect on lymphocytes was less marked.

The response of immunosuppressed guinea pigs to challenge with L. pneumophila was determined in 2 experiments (Table XIII).

TABLE XIII. RESPONSE OF IMMUNOSUPPRESSED GUINEA PIGS TO AEROSOLS OF L. PNEUMOPHILA

TREATMENT	DOSE	ORGANISM DEAD/TOTAL	LD_{50}
Experiment I	1.5×10^5		
Cytoxan		4/5	NA
Hydrocortisone		0/5	
Cytoxan + hydrocortisone		7/7	
None (infected controls)		0/5	
Experiment II			
Cytoxan	7.6×10^2	0/8	$> 10^5$
	7.6×10^3	1/6	
	9.1×10^4	1/6	
Hydrocortisone	7.6×10^2	1/8	$> 10^5$
	7.6×10^3	0/6	
	9.1×10^4	1/6	
Cytoxan + hydrocortisone	7.6×10^2	0/8	4.5×10^4
Cortisone	7.6×10^3	1/6	$(1.1 \times 10^4 - 1.8 \times 10^5)$[a]
	9.1×10^4	4/6	
None (infected control)	7.6×10^2	0/8	$> 10^5$
	7.6×10^3	0/6	
	9.1×10^4	1/6	

[a] 95% confidence limits

In the first, guinea pigs treated according to the regimens given above were exposed to aerosol dosages of 1.5×10^5 L. pneumophila. None of the corticosteroid or control animals died, 80% of the guinea pigs receiving cyclophosphamide alone and 100% of those receiving it in combination succumbed.

Because the first experiment failed to show whether or not the steriod was having any effect, even in combination, a second experiment was performed in which treated guinea pigs were exposed to graded doses of L. pneumophila. These data show that significantly more guinea pigs died after treatment with the combination of drugs than with the cyclophosphamide alone. It was interesting to note that the surviving guinea pigs that received the combination of drugs lost more weight and had more pronounced fevers than any of the control groups (not shown). Another observation of interest was that serum MA titers at 14 days and DTH at 21 days, were not affected by treatment. This study will be expanded in scope to include more measurements of cell and humoral immune factors and to determine the effect of continuous immunosuppressive treatment. Also, when a guinea pig model is established, therapy studies will be initiated and a primate model developed.

The last subject to be reported is that of the survival of L. pneumophila in aerosols. In the first of 4 experiments, the survival of L. pneumophila suspended in tryptose-saline diluent was determined over a 32-min period as a function of RH

(constant temperature of 24°C). Four replications of each condition were carried out. Survival was best at 80% RH, almost the same at 50%, and very poor at 30% (Table XIV).

TABLE XIV. SURVIVAL OF L. PNEUMOPHILA IN AEROSOLS AS A FUNCTION OF RH

RH (%)	% RECOVERY AT INDICATED[a] TIME (min) 4 min	18 min	32 min	DECAY RATE[b] (%/min)	$t_{½}$ (min)
30	8.7 ± 2.3	0.2 ± 0.2	0.03 ± 0.02	21.2	3.3
50	29.9 ± 13.8	10.3 ± 2.1	4.4 ± 1.3	6.7	11.1
80	18.9 ± 6.5	14.1 ± 7.3	8.5 ± 7.2	4.9	15.2

[a]Mean of 5 replications.

[b]Decay rate = 100k from the simple exponential equation $\% Rt_i = \% Rt_o e^{-kt}$, where Rt_i and R_o represent % recoveries at time t_i and t_o, respectively, and t is total elapsed time.

The next experiments were designed to use both a more "natural" suspending fluid (Culler Lake water), and a suspending medium that has been repeatedly shown to be an effective aerosol stabilizer for certain bacteria (5% raffinose + 0.1% dipyridyl). The 50% RH condition was not not included. The data show raffinose and dipyridyl have a marked stabilizing effect and Culler Lake water a slight effect (most marked at 80% RH, Table XV).

TABLE XV. AEROSOL SURVIVAL OF L. PNEUMOPHILA IN TRYPTOSE SALINE, CULLER LAKE WATER, RAFFINOSE-DIPYRIDYL SOLUTION, AND ALGAL SUPERNATANT

SUSPENDING MEDIUM	% RECOVERY AT 4 MIN 30% RH	80% RH	DECAY RATE (%/min) 5-32 min 30% RH	80% RH	$t_{½}$)min) 30% RH	80% RH
Experiment I						
Tryptose-saline	1.8	2.7	15.9	9.1	4.4	7.6
Culler Lake water	3.9	12.3	15.1	5.2	4.6	13.3
5% Raffinose + 0.1% dipyridyl	20.4	24.0	6.4	6.3	10.8	11.0
Experiment II						
5% Raffinose	4.6	ND	16.2	ND	4.3	ND
0.1% Dipyridyl	8.4	ND	4.4	ND	17.4	ND
Combination of above	40.5	ND	6.7	ND	10.4	ND
Experiment III						
Tryptose-saline	0.6	ND	17.5	ND	4.0	ND
Raffinose + dipyridyl	7.0	ND	6.7	ND	10.4	ND
Algal supernatant	4.3	ND	5.7	ND	12.9	ND

The second experiment was performed to determine which of the components of he raffinose-dipyridyl combinations was most active in terms of stabilization. The means of 3 replicate determinations at 30% RH are presented as experiment II Table XV). For this and subsequent experiments, only the most adverse RH, 30% was employed. The data clearly demonstrate that most of the stabilizing effect was due to the dipyridyl, suggesting that the chelation of iron was of importance.

The third experiment (Table XV) shows the effect of blue-green algae (Fischerella species) upon the survival of L. pneumophila. This work is based upon published reports that Legionella are frequently isolated from lakes, ponds and air-conditioning systems in association with these algae. Algae were obtained from Dr. Carl Fliermans, DuPont Co., Aiken, SC, and grown for 5-7 days at 45°C. The algae were centrifuged and the supernatant fluid sterilized by filtration (0.45 μm). The stabilizing effect of this material was compared to that of tryptose-saline (worst case) and raffinose-dipyridyl (best case) in 3 replicate trials. The data clearly show the stabilizing effect of the algal supernatant. Experiments are currently in progress to determine the chemistry of the naturally occurring stabilizer. An interesting sidelight to these experiments is that these algae also chelate iron (Fliermans, personal communication). A preliminary experiment to determine whether algal products also enhance respiratory infectivity was equivocal and will be repeated.

The last topic that has not been presented in any detail is that of the effect of age and sex upon susceptibility to L. pneumophila infection. Data obtained have been equivocal, and thus far neither age (weight) nor sex seems to affect the susceptibility of guinea pigs to L. pneumophila challenge.

Publications:

1. Berendt, R. F., H. W. Young, R. G. Allen, and G. L. Knutsen. 1980. Dose-response of guinea pigs experimentally infected with aerosols of Legionella pneumophila. J. Infect. Dis. 141:187-192.

2. Berendt, R. F. 1980. Survival of Legionella pneumophila in aerosols: effect of relative humidity. J. Infect. Dis. 141:689.

3. Berendt, R. F., R. D. Magruder, and F. R. Frola. 1980. Treatment of Klebsiella pneumoniae respiratory tract infection of squirrel monkeys with aerosol administration of kanamycin. Am. J. Vet. Res. 41:1492-1494.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6428	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'S INSTR'S	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	051
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE: (U) Analysis of subcellular structures in microbial infections of potential BW importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE

	PREVIOUS/CURRENT	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	80	1.0	210
	81	0	0

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20. GENERAL USE
Foreign intelligence considered

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NAME:

POC:DA

21. KEYWORDS: (U) Military medicine; (U) BW defense; (U) Rickettsia; (U) Influenza; (U) Mycoplasma; (U) Ultrastructure; (U) Toxins; (U) Microscopy

22. TECHNICAL OBJECTIVE, 23. APPROACH, 24. PROGRESS (Provide individual paragraphs identified by number. Provide end of each with security Classification Code.)

23 (U) Study infections and toxemia states at the ultrastructural level by scanning and transmission electron microscopy so as to elucidate mechanisms by which infectious microorganisms enter and leave cells and to identify target organelles damaged by microorganisms and toxins. These studies should provide basic information relative to specific therapy and protection against diseases caused by these agents and could lead to early detection of agents of potential BW importance.

24 (U) Infected animals and cell cultures provide experimental material for examination by scanning and transmission electron microscopy. Conventional techniques and more sophisticated approaches, i.e., immunolabeling, freeze fracture, replication by metal casting, and stereology, are used in these studies.

25 (U) 79 10 - 80 09 - KHF virus, strain 76-118 conforms by morphology, intracellular development, and physical characteristics, to criteria for inclusion in the Bunyaviridae family of viruses. The virus is round with unit membrane and fringe and is 100 nm in diameter. Squirrel monkeys infected with the Lee strain of KHF virus developed renal lesions compatible with human disease; virus particles identical to strain 76-118 were seen in cells of the kidneys from these animals. Identity of the Lee and 76-118 strains of KHF virus is further suggested by the fact that antiserum produced against Lee strain reacted specifically with strain 76-118, as shown by negative stains of virus clumping in the presence of antiserum and by immunolabeling of virus with ferritin.

Terminated for management efficiency. Continued in W.U. S10 AP 198. (DAOG1526)

* Available in computer upon originator's request.

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

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BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A841 BC: Prevention of Viral Diseases of Potential BW Importance

Work Unit No. 871-BC-138: Analysis of Subcellular Structures in Microbial
(841 00 051) Infections of Potential BW Importance

Background:

Ultrastructural studies are useful in infectious diseases and microorganisms of potential BW importance. Immunolabeling procedures for use with out scanning beam electron microscope (EM) have been demonstrated with Rickettsia rickettsii, Coxiella burnetii, Mycoplasma pneumoniae, as well as with influenza and Pichinde viruses. Transmission electron microscopy has provided information related to diseases caused by yellow fever, Sandfly fever, Rift Valley fever (RVF), Ebola and VEE viruses; more recently we have examined the virus of Korean hemorrhagic fever (KHF). Preliminary work last year had suggested that KHF was a Bunyamwera-like virus.

Progress:

Although KHF was first recognized in Korea in 1951, it was not until 1978 that Lee and coworkers isolated an etiologic agent from the lungs of a rodent, Apodemus agrarius coreae (1). This agent was a round 50-mm virus that formed crystalline arrays within alveolar epithelial cells (2). More recently, KHF has been propagated in vitro by French et al. (3). They used a cultured cell line designated A-549, which is derived from a carinoma of the lung, to propagate the virus from the 5th passage of strain 76-118 in Apodemus lung.

The present report describes the morphology of KHF virus strain 76-118. It was obtained from H.W. Lee as the 5th passage in Apodemus lung by R. French (Virology Division). He demonstrated that strain 76-118 has absolute serologic identity with the human disease and heterologous strains of KHF virus and that strain 76-118, at the 6th passage level in A-549 cells, did not produce CPE but established a persistent infection (3).

Cultures of infected A-549 cells were prepared for EM with virus from stocks of 76-118 at the 9th passage level in A-549 cells. Monolayers were incubated at 37°C for various lengths of time, usually 2-3 days. There was no evidence of CPE; the final virus concentration, which ranged from 10^5-10^7 infectious units/ml, was directly proportional to the input multiplicity. Monolayers were washed with balanced salt solution and covered with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7. The cells were removed by scraping or by glass beads, centrifuged at 2000 x g; pellets were suspended in fresh fixative. At the end of 2 h, the pellets were rinsed in buffer, osmicated, dehydrated, and embedded in Epon 812. Sections (60-100 nm) were cut and stained with uranyl acetate and lead citrate.

Examination of the cultures in the lower magnification range showed that the morphology of infected cells resembled normal ones. There was no evidence of cytolysis in infected cell cultures. The A-549 cell culture is considered to be a type II alveolar epithelial cell (3). The cell surface usually has an irregular appearance caused by folds and microvillous projections. Numerous myelin inclusions were seen peripherally; mitochondria, smooth and rough endoplasmic reticulum, Golgi apparatus and lysosomes were located in a tight perinuclear concentration. The remaining cytoplasmic area was devoid of organelles and the nucleus was surrounded by bundles of microfilaments.

Cells in which virus particles were seen contained empty cytoplasmic vesicles, imparting a vacuolated and less dense appearance to the cell, particularly in the area of organelle concentration. There was no toxicity or cell injury evidenced by the appearance of the mitochondria or nucleus or damage to membranes. Individual virus particles or clusters were seen in the cytoplasmic area near Golgi lamella. A narrow band of electron-lucent space encircled virus particles; occasionally the clear area was delimited by some fragments of membrane. Virus particles were round and had an average diameter of 95 nm with a fringe 6-9 nm in length. These particles were not seen in normal noninfected A-549 cells.

In an attempt to describe morphogenesis with immunolabel techniques, A-549 cells were infected with a large input of virus per cell. Suspensions of virus and cells, 600 infectious units per cell, were placed at 4°C. At the end of 1 h aliquots were put into compartments of a slide-culture device and incubated at 37°C. At predesignated times, suspensions or monolayers were either directly fixed for dehydration and embedding or were handled for immunolabeling. Suspensions were pelleted; monolayer cultures were processed in situ. The samples used for immunolabeling were fixed 15 min in 0.5% glutaraldehyde, treated with digitonin, washed in buffer and glycine, washed in saline, incubated in immune rabbit serum; they were then washed in saline, incubated in ferritin-tagged goat and anti-rabbit serum, washed in saline, and postfixed in 2% glutaraldehyde before osmication, dehydration and embedding. Controls for immunolabeling were noninfected cells using the same technique with replacement of immune serum with preimmunization samples of rabbit serum. The antigen used for preparing the immune rabbit serum was the heterologous Lee strain of KHF virus at the 3rd passage level in Apodemus lung. The titer of this antiserum was 1:2560 by an indirect fluorescent antibody (IFA) technique (3). Ferritin-tagged reagent was purchased from Miles-Yeda Ltd.

Extracellular virions seen in suspensions held 1 h at 4°C were 100 nm in diameter, round and electron dense. A membrane and fringe were seen. The particles in samples treated with immune serum were coated with ferritin granules and in controls identical particles were not tagged. At the end of 1 h at 37°C, following the holding period at 4°C, virus particles were in intracellular coated vesicles near the cell membrane. Although there was no evidence for a specific receptor site, the presence of virus in coated vesicles seemed to indicate that a specialized area in the plasma membrane is involved in virus uptake by A-549 cells. It was not possible to determine the fate of these vesicles.

Cells in cultures held 20-40 h at 37°C contained numerous virus particles in cytoplasm. The virus and appearance of the cellular localization was

identical to that seen in cells infected at a low dosage rate. None were seen in noninfected control cells. Although digitonin was used to make cellular membranes more permeable to antibody molecules, there was no evidence of intracellular ferritin or specific tagging of structures within the cytoplasm. These observations suggest that KHF virus conforms to the morphologic criteria for inclusion in the Bunyaviridae family.

Examination of crude cell pellets from KHFV-infected A-549 cells and various purified and concentrated preparations of the crude pellets provided additional information to support the identification. Infected A-549 cells were disrupted mechanically and centrifuged at 6000 x g. Negative stains consistently failed to demonstrate virus in samples which contained $\leq 10^6$ infectious units/ml. It was apparent that the major portion of the virus sedimented with the cellular fraction. Negative stains of these samples were difficult to interpret because large amounts of cellular debris obscured all detail and spoiled the negative stain. Occasionally, round 100-nm particles with a fringe were seen. Crude pellets were fixed and embedded in Epon 812 for thin sections. Two types of samples were obtained: those in which larger cell fragments predominated and those which consisted of ribosomes and fragments of the endoplasmic reticulum (ER). The ribosomal and ER fraction contained 95-nm round particles identical to intracellular virus. Virus particles in samples of the large cell fragments were round, 100-nm, dense particles similar to the extracellular virus demonstrated by ferritin tagging. In order to obtain higher concentrations of virus without cellular material, infected A-549 cells were mechanically disrupted, centrifuged at 500 x g and filtered successively through 0.8- and 0.45-μm pore Millipore filters. Samples of the pellet obtained by centrifugation at 50,000 x g were examined by negative contrast stain and in thin sections prepared from Epon. Virus particles in these preparations were round, 100 nm in diameter and possessed a fringe. They were similar to RVF virus in negative stains made in our laboratory, as well as to published micrographs of this virus and other members of the Bunyaviridae.

A suspension of KHF virus mixed with dilutions (1/10-1/100) of the specific rabbit antiserum used in the ferritin procedure showed clumping when examined in negatively stained preparations. No clumps were observed in samples mixed with preimmunization samples of serum or with a rabbit antiserum to RVF virus. The reciprocal cross of antisera and virus suspensions KHF and RVF viruses did not produce clumping.

At the time this work was in progress, a group of squirrel monkeys was injected with the Lee strain of KHF virus. This strain of KHF virus, heterologous to the 76-118 strain, was at the 3rd passage level in Apodemus lung. The monkeys were sacrificed when the level of blood urea nitrogen (BUN) increased to a clearly abnormal level. CPT G. Knutsen provided material for EM from necropsy samples that had been fixed in buffered neutral formalin. Gross and microscopic lesions in these monkeys were compatible with renal lesions seen in humans. Virus particles seen in necrotic tubular epithelial cells and a plasmablast were identical to the intracellular particles of strain 76-118 seen in A-549 cells.

To recapitulate the morphologic observations, the virus particle seen in negatively stained preparations of pellets and suspensions from A-549 cells infected with KHF virus, strain 76-118, is round and 100 nm in diameter. It resembles RVF virus treated similarly in our hands and other viruses in the Bunyaviridae. Examination of thin sections of material used for negative stains

reveals a particle compatible with the morphology of viruses in the Bunyaviridae family and consistent with the appearance of particles in our negative stains. The round, 100-nm virus particle in the infected A-549 cells is identical to the particle seen in pellets. The morphology and cytoplasmic localization of the virus in the Golgi area of the cytoplasm is compatible with cytoplasmic localization and morphogenesis of members in the Bunyaviridae family. Frequency and prevalence of particles in negatively stained specimens and in thin sections of infected cells or pellets correlated consistently with the virus concentration determined by in vitro assay. Particles were never seen in normal cells or pellets prepared from them. KHF virus, strain 76-118, reacts specifically with antiserum prepared with the heterologous Lee strain of KHF virus. This was demonstrated by immunolabeling with ferritin and negative staining of antibody induced clumping. It is further noted that a similar difference in size between extra- and intracellular virus was reported for Crimean hemorrhagic fever virus, a member of the Bunyaviridae (4).

These observations combined with physical evidence developed by French that strain 76-118 of KHF virus is an RNA virus which is extremely acid-sensitive and labile to organic lipid solvents and will not pass through a 100-nm pore Millipore filter, show that this virus fulfills many criteria for classification as a bunyavirus.

These data were summarized in a planning and review meeting on a grant with Dr. H.W. Lee, principal investigator. At that time he indicated that he was isolating a virus from Apodemus lungs and KHF patients that was different from strain 76-118. This virus particle is approximately 80 nm in diameter and resembles certain orbiviruses. It produces large crystalline arrays and a marked CPE in cultures of A-549 cells.

The relationship between this agent and strain 76-118, obtained from Dr. Lee at the 5th passage level in Apodemus lung, is not clear at this time. It is certain that an 80-nm particle is not in the strain 76-118 used in our studies, nor is there an agent which produces a lytic CPE. Strain 76-118 has absolute serologic identity to the human disease agent (3); we have demonstrated a serologic and morphologic identity with the Lee strain of KHF virus which produces a disease in monkeys with clinical signs and renal lesions that are compatible with the human disease.

Publications:

None

LITERATURE CITED

1. Lee, H.W., P.W. Lee, and K.M. Johnson. 1978. Isolation of the etiologic agent of Korean hemorrhagic fever. J. Infect. Dis. 137:298-308.

2. Lee, P.W., and H.W. Lee. 1978. Electron microscopic findings of Korean hemorrhagic fever. J. Korean Med. Assoc. 21:405-418.

3. French, G.R., R.S. Foulke, O.A. Brand, G.A. Eddy, H.W. Lee, and P.W. Lee. 1981. Propagation of the etiologic agent of Korean hemorrhagic fever in a cultured continuous cell line of human origin. Science (in press).

4. Donets, M.A., M.P. Chumakov, M.B. Korolev, and S.G. Rubin. 1977. Physicochemical characteristics, morphology and morphogenesis of virions of the causative agent of Crimean hemorrhagic fever. Intervirology 8:294-308.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0F6423	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. SMSP/N HISTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. BASIC RSCH

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M162776a841	00	052
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Include with Security Classification Code): (U) Therapeutic manipulation of mestabolic-endocrine controls during infections of unique military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS:
003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
75 12	80 09	DA			C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 80	1.0	69
FISCAL YEAR CURRENT 81	0	0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
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ASSOCIATE INVESTIGATORS
NAME: Merrill, G. A.
NAME: POC:DA

21. GENERAL USE
Foreign Intelligence considered

22. KEYWORDS (Provide KEY with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Infections; (U) Hormone therapy; (U) Metabolic defects; (U) Rats; (U) Receptors; (U) Virus

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

23 (U) Determine the role of insulin and glucagon in the metabolic effects and feedback control during infection and infection-induced stress. Characterize the optimum manipulation of the various hormone axes involved in carbohydrate, fat and protein supply and use as a means of eliminating the undersirable metabolic changes associated with disease in either the physiological manifestations of that disease or a prolonged recovery period. In addition to the relation of this product to BW defense the expertise gained through work in this area is immediately applicable to the soldier infected secondary to either combat or noncombat injuries.

24 (U) Using isolated islets of Langerhans, the effects of bacterial and viral infection on insulin production and release willbe measured. The physiological action of insulin in vivo will be assessed by use of glucose tolerance and insulin tolerance testing. In addition, the effect of infectious stress and its associated metabolic changes on binding of insulin to its receptors and thus the physiological action of insulin will be characterized.

25 (U) 79 10 - 80 09 - No inhibition of insulin secreting ability in the susceptible mouse strains was noted in animals infected with VEE-TC83. Histologic alterations of islets of Langerhans were observed in several animals. Methionine enkephalin, an endogenous opiate, has been found to be significantly elevated in plasma 30 min after injection of endotoxin in dogs, preceding the hyperinsulinism and hyperglucagonism associated with the endotoxemia. Naloxone inhibits the hyperinsulinism without altering the magnitude of the elevation of methionine enkephalin. The work unit is terminated due to transfer of the principal investigator.

Publication: Diabetes 29 (Suppl. 2): 97A, 1980

*Available to contractors upon [illegible]'s approval

DD FORM 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

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M162776A841)

Task No. 3M161102BS10 AQ: Enhancement of Host Defense Against Agents of Potential BW Importance

Work Unit No. S10 AQ 174: Therapeutic Manipulation of Metabolic-endocrine Controls
(841 00 052) During Infections of Unique Military Importance

Background:

A causal association between virus infection and juvenile-onset diabetes has been suggested for a number of viral agents, with the strongest evidence to date implicating a role for Coxsackie B viruses and mumps. Animal models of virus-induced diabetes have also been reported; the most extensively studied model being encephalomyocarditis virus-induced diabetes, although similar studies have been reported for VEE-TC-83. A Fort Detrick employee, diagnosed as having insulin-deficiency diabetes approximately 3 months following administration of VEE vaccine, was shown to have serological antibody rise in this time period to only VEE, suggesting further evaluation of the potential diabetogenicity of the vaccine strain of VEE (VEE-TC-83).

An observed segment of the pathophysiology of most infectious diseases of military importance is hyperinsulinism and hyperglucagonism. Although reports are available indicating both beneficial and detrimental effects of these hormonal changes, to date the direct causal factor for these hormonal changes remains unknown. Evidence that endogenous opiates (peptide synthesized by brain and pituitary and perhaps elsewhere having morphine-like activity) are secreted into the peripheral bloodstream suggests a hormonal-type action on some tissue. Based on the hyperglycemic response of morphine, the endocrine pancreas was suggested as a bioactive site and infusion experiments have shown that β-endorphin does initiate both glucagon and insulin secretion dependent on glucose availability. Both of these responses are blocked by use of naloxone, an opiate receptor antagonist. Because the response to β-endorphin resembles the clinical picture of endotoxin shock in dogs, the possibility that endogenous opiates are involved in the pathophysiology of endotoxin shock was hypothesized. The potential therapeutic effect of naloxone in endotoxin shock and other infectious diseases in the prevention of protein depletion by inhibiting hyperinsulinism warranted investigation of this hypothesis.

Progress:

Antibody production. An anti-insulin antibody is normally produced in guinea pig so that insulin concentrations cannot be determined in guinea pigs. Therefore, an attempt was made to produce anti-insulin antibody in an alternative model not generally used in infectious disease research. Five white leghorn roosters were subdermally immunized with porcine insulin alone or made more immunogenic by use of Freund's complete and incomplete adjuvants (FCA and FIA), CP 20,961, or lipid emulsion. No demonstrable antibody was observed following booster injections given on days 3, 10, 17 and 24 at any time point tested. It has since been reported that guinea pigs have 2 types of insulin; a normal used in peripheral tissues and a second

found in plasma, which is not reactive with antibody produced against normal insulin. Future attempts to produce antibody to measure circulating guinea pig insulin levels would require purified guinea pig insulin (second type) for injection into chickens.

Iodination. A modification of the chloramine T iodination of insulin using enzymobeads (BioRad) has yielded an insulin tracer with less damage to the insulin molecule. The procedure is less time critical and appears to be more reproducible than is possible with chloramine T iodinations. This procedure is also used for viral antibody labeling.

Receptor studies. Preliminary characterization of insulin binding to hepatic nuclei performed in conjunction with Mr. Hauer (Physical Sciences Division) has been completed. The use of N-butylphthalate to separate bound from free insulin is inferior to the use of 10% bovine serum albumin. Separation by the latter procedure has a coefficient of variation approximately 20% of that seen when the N-butylphthalate procedure is used. Binding has been shown to be pH-, time- and protein-dependent, and also reversible. Nuclei from Streptococcus pneumoniae-infected rats demonstrate significantly higher binding than those from controls.

Insulin binding to RBC has been successfully accomplished. Utilization of this assay would enable use of only 25 ml of blood to study insulin receptor changes due to various stresses, as opposed to 250 ml as are presently needed to do such studies on peripheral monocytes. Dual assays to demonstrate the similarities of the two assays under conditions of known changes in the monocyte assay are still required before elimination of the monocyte radioreceptor assay as the standard tool.

Binding to monocytes of a lipoatrophic diabetic showed no significant alteration of receptor binding ability by this patient mentioned earlier. Altered binding is frequently observed in lipoatrophic diabetics and such a patient would be a valuable asset as a control in many studies.

Endogenous pyrogen. In cooperation with CPT Critz (Physical Sciences Division), Work Unit S10 AQ 173, a human monocyte cell line has been successfully stimulated with heat-killed Staphylococcus epidermidis to produce EP. This is of substantial benefit because the EP produced is essentially free of fetal calf serum and albumin and eliminates the requirement of several purification steps to eliminate these substances. Presently large-scale production is being investigated to produce large lots of EP suitable for antibody production and ultimately for an EP radioimmunoassay. Appropriate controls for the positive demonstration that the fever-producing agent produced by the cells is EP are being incorporated into the study.

VEE studies. Two strains of mice, SJL (susceptible to other diabetogenic strains of viruses) and C57 Bl (resistant to diabetogenic viruses) were inoculated with TC-83 strain of VEE. Both control and inoculated animals were periodically sacrificed over a 21-day experiment. Fasting glucose and insulin values for the 2 strains have not indicated an impaired insulin secretory ability for either strain of mice. Preliminary histological studies have, however, suggested islet changes in some infected SJL mice. More detailed studies of histologic changes are currently being evaluated by Dr. Marshall Austin of Bethesda Naval Hospital. Dr. Jahrling (Virology Division) has demonstrated a 100% correlation after 5 days postinoculation of the presence of neutralizing antibody (1:80 dilution of plasma) in all inoculated mice and no neutralizing antibody at the same dilution in uninoculated mice. This experiment has been repeated over a 35-day period and in hamsters over a 5-month period. Evaluation of the islets and blood parameters has not been performed as yet.

Endogenous opiates. Studies demonstrating the ability of NaF (5 mg/ml), a potent enzyme inhibitor, to prevent degradation in plasma of both methionine and leucine enkephalin by carboxypeptidases has been completed. Heparanized blood spiked with exogenous enkephalins was both acid-extracted (the recommended enzyme inactivation procedure) and placed in NaF. NaF not only proved superior because of the elimination of the costly and time-consuming acid extraction step but also because in the NaF procedure approximately 95% of the calculated enkephalin was recovered, whereas only 20-25% was recovered by the acid-extraction procedure. Varying NaF concentrations (0-30 mg/ml) do not appear to alter the assay for the enkephalins and standards prepared in NaF are essentially identical to standard curves generated when NaF is not present.

In determining the role of the endogenous opiates in endotoxin shock, dogs were utilized in an anesthetized state. Dogs were maintained at a 200 mg/dl glucose level and given endotoxin (Escherichia coli lipopolysaccharide). Hyperinsulinism occurred (> 1000 μU/ml) by 4 h. Thus far, methionine and leucine enkephalins are the only endogenous opiates measured. The latter's concentrations were unaltered following endotoxin administration. However, methionine enkephalin was markedly elevated by 15 min following endotoxin administration and remained elevated throughout the 6-h experiments. Naloxone therapy (0.2 mg/kg/h) was incapable of diminishing the methionine enkephalin response following endotoxin; however, it markedly diminished the magnitude of the hyperinsulinemic response in the hyperglycemic endotoxin shock dogs. Glucagon and β-endorphin plasma concentrations have yet to be determined and the influence of endotoxin shock and naloxone on these parameters is being investigated. The rise in methionine enkephalin preceding the hyperinsulinism is consistent with the hypothesis suggested by our research proposal that endogenous opiates are associated in a causal relationship with the hyperinsulinism of hyperglycemic endotoxin shock in dogs.

The work unit is terminated due to transfer of the investigator.

Presentation:

Anderson, Jr., J. H. and G. Merrill. Carbohydrate metabolism and insulin receptor response in humans with acute viral infection. Presented, Am. Diabetes Assoc. Mtg., Washington, DC, 15-17 Jun 1980 (Diabetes 29 (Suppl. 2):97A, 1980).

Publications:

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0F6418	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'G RSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	053
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Include Security Classification Code)

(U) Characterization of nonindigenous tick-borne rickettsiae for vaccine development

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
75 03	80 09	DA	C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE	PREVIOUS/CURRENT	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	80	1.0	103
	81	0	0

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NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Provide KEYWORD and Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Vaccines; (U) Tick-borne rickettsioses; (U) Q fever

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

23 (U) Characterize in vitro and in vivo biological and chemical markers of rickettsial cultures to enhance vaccine efficacy and to facilitate identification of strains and species. Assess low virulent organisms of potential military importance for use as live vaccines for protection of troops exposed to a biological warfare environment.
24 (U) Grow rickettsiae in tissue culture, embryonated eggs or animals to obtain sufficient quantities of organisms for chemical or physical fractionation. Vaccinate animals with various fractions and test for protective capacity by challenge with virulent agents.
25 (U) 79 10 - 80 09 - A study is underway to investigate properties of the NDBR-105 phase I Q fever vaccine in animals prior to its use in human volunteers. Experiments to determine the protective potency of the vaccine have been conducted in guinea pigs on all 5 lots of the vaccine. The lots all appear similar, but statistical analyses of the data have not been completed. Serological studies of the vaccine, using the microagglutination test, gave similar indications with graded dose levels in general showing graded antibody response. As little as 0.001 microgram of vaccine will produce detectable antibody response and induce some protection in guinea pigs. Dose levels of 1.7, 8.6 or 262 micrograms per guinea pig at 20 days postvaccination have not shown significant increases in stimulation indices over controls in the lymphocyte transformation test.
Publication: Abstracts, Annu. Mtg. ASM 1980, p. 91.

Terminated for management efficiency. Continued in W.U. 871 BB 149.(DAOG3813)

* (If available in accordance with originator's approval)

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BB: Prevention of Bacterial and Rickettsial Diseases of Potential BW Importance

Work Unit No. 871 BB 129: Characterization of Non-indigenous Tick-borne Rickettsiae for Vaccine Development

Background:

In 1960, a phase II formalin-killed Q fever vaccine suitable for human use was prepared by Berman et al. (1) using the Henzerling strain of Coxiella burnetii. Over the years, this vaccine has been shown to provide good protection against the disease, although its use has caused a significant, but acceptable, number of systemic or local reactions, especially among recovered or previously immunized individuals (2). Supplies of this vaccine have been gradually reduced to the point where a replacement will be needed in the near future.

Some years after this vaccine was prepared, phase I strains of C. burnetii were shown by Ormsbee et al. (3) to be more effective agents for the preparation of vaccines since they were easier to purify and required lower doses for the same degree of protection. In 1970, Spicer et al. (4) published a method for the purification of Q fever organisms from chicken embryo yolk sacs involving extraction with Freon 113 and passage through a brushite column to remove host material. Using this procedure, Spicer and DeSanctis (5) prepared 5 large lots of a phase I antigen suitable for vaccine use as a potential replacement for the vaccine in current use. The present work was initiated to study the properties of the NDBR-105 phase I Q fever vaccine in vitro and in vivo to lay the groundwork for human volunteer testing. The initial objective of this study was to determine whether there were detectable differences in the protective potencies among the lots, and to define a standardized assay system for testing new lots. Other objectives were to measure humoral and cellular immune response in vaccinated guinea pigs, to compare the protective porperties of the new and old vaccines, and to measure selected physical properties of the preparation. Some of these studies have been reported previously on lot 4.

Progress:

Before testing the vaccine, a stock of phase I C. burnetii challenge inoculum was prepared and assayed in guinea pigs to determine its median fever dose (FD_{50}). Phase I and phase II antigens were also prepared by purifying rickettsiae from infected yolk sacs.

An experiment was conducted to determine whether guinea pig weight (age) affected susceptibility to Q fever infection and to find the optimun sized animal for the vaccine studies. Guinea pigs in 3 weight ranges were obtained (200-300, 400-500, and > 800 g each). Animals in each range were divided into 3 groups of 6 each and inoculated IP with 98, 9.8, or 0.98 guinea pig FD_{50} of stock C. burnetii. The temperature of each animal was recorded daily for 10 days following infection Table I summarizes the results.

TABLE I. THE EFFECT OF WEIGHT (AGE) ON THE DEVELOPMENT OF C. BURNETII INFECTION IN GUINEA PIGS (n=6)

DOSE (GP FD_{50})	NO. WITH > DAYS FEVER		
	200-300 g	400-500 g	> 800 g
98	6	6	2
9.8	6	6	1
0.98	4	3	0

These data indicate that larger guinea pigs were least susceptible and the smallest the most susceptible to Q fever infection. However, smaller animals tend to become ill, develop fever and occasionally die from nonspecific causes more frequently then larger animals. At the 400-500-g level, this problem appeared to stabilize and since guinea pigs at this level are about as susceptible to infection as the smaller group, they were used for vaccine evaluation.

The initial approach to comparing properties of these vaccine lots was to measure the median protective dose (PD_{50}) of each lot separately with appropriate controls to evaluate the effects of using different groups of guinea pigs. Vaccine dilutions were prepared containing the following concentrations: 0.02, 0.70, 3.4, 17.2, and 524 µg/ml, representing 2 PD_5, 2 PD_{30}, 2 PD_{50}, 2 PD_{70}, and 2 PD_{95}, respectively, as calculated from previous data on lot 4. Ten guinea pigs were vaccinated SC with 0.5 ml of each dilution and allowed to rest for 4 weeks. They were bled by cardiac puncture to obtain sera for serologic tests and were challenged IP with 10^5 $GPFD_{50}$ of phase I Henzerling strain C. burnetii. Temperatures of all animals were recorded daily for 10 consecutive days following challenge. Groups of 6 normal guinea pigs were inoculated IP with dilutions of the challenge dose varying around the FD_{50} and temperatures were recorded as above. These animals as well as a group of uninfected guinea pigs, served as controls to compare the challenge inoculum and the guinea pig lot for another experiment.

The experiment described above was conducted with vaccine lots 1, 2, and 5, and the guinea pig temperature data obtained was analyzed statistically, using a temperature of $\geq$ 40.0 as a febrile reaction.

The results of these studies were disappointing. The guinea pig temperature data were not consistent with those from lot 4 obtained several years ago. Dose-response curves could not be derived with any reasonable degree of accuracy because significant fevrile responses occurred throughout all dose levels and some groups of guinea pigs seemed to develop greater febrile responses than others. Serologic data from these animals were more consistent. Microagglutination(MA) titers were more closely related to dose in all 3 experiments. Graded doses of antigen showed graded serologic responses.

Because of the variations among groups of animals, it was decided to test all 5 vaccine lots in one large group of guinea pigs and compare the results, again using the febrile reaction for measuring clinical response. Each lot of the vaccine was tested at 4 concentrations (0.35, 1.70, 8.60, and 262 µg). Each

concentration was inoculated SC into 8 guinea pigs. On day 20 postvaccination, animals were bled by cardiac puncture for serologic and cell-mediated immunity studies. The following day all vaccinated animals, along with 24 normal guinea pigs from the same group, were challenged IP with 10^5 $GPFD_{50}$ of phase I Henzerling strain C. burnetii. The temperature of each animal was recorded daily for 10 consecutive days following challenge as a measure of the clinical response to the infection. A second experiment similar to this was also conducted, but some dose levels were reduced (0.001, 0.01, 0.35, and 1.7 µg). Results are being analyzed statistically in an attempt to estimate a PD_{50} for each vaccine lot, or at least compare their potencies.

The first analysis of the data used animals showing temperatures of $\geq 40^{\circ}C$ on > 2 consecutive days as an indication of infection. With this criteron, a dose-response curve could not be developed for any of the vaccine lots since the number of responders did not vary regularly as the dose was increased. The calculation of a PD_{50} could not be made and the vaccines could not be compared on this basis. In a second analysis, the critical fever temperature was dropped to $\geq 39.8^{\circ}C$ on > 2 consecutive days. Here again, the dose-response data were unsuitable for calculating a PD_{50}. In another analysis, a comparison was made among the average heights of fever for each dilution within each vaccine lot. This was expected to show a reciprocal relationship with the vaccine dose, but no correlations were observed. Of 18 comparisons made at the $40^{\circ}C$ cutoff temperature, no significant differences were observed among any of the dilutions. At the $39.8^{\circ}C$ level, only 7 of 30 showed a significant difference from the overall mean, but these were scattered in an apparently random fashion throughout the group. Other parameters being investigated are the use of temperature > $40^{\circ}C$ for determining either the number of fever days or of guinea pigs infected and combinations of temperature height and duration of fever.

Serologic studies were also conducted on the sera of these guinea pigs. The animals were bled 20 days after vaccination and their sera tested for phase I and phase II Q fever antibodies by MA test. The results are shown in Table II.

TABLE II. GEOMETRIC MEAN MA TITERS OF GUINEA PIGS (n=8) VACCINATED 20 DAYS PREVIOUSLY WITH NDBR-105 Q

VACCINE CONC. (µg)	EXP 1 MA TITERS BY LOT NO. 1	2	3	4	5	EXP 2 MA TITERS BY LOT NO. 1	2	3	4	5
Phase I										
0.001	-	-	-	-	-	1	1	1	1	1
0.01	-	-	-	-	-	1	1	1	1	1
0.35	2.6	1.5	1.8	1.1	1.1	1	1	1	1	1
1.70	4.0	3.1	1.4	1.1	2.4	1.2	1.8	1	1.7	1.8
8.60	8.0	6.7	1.1	2.2	3.4	-	-	-	-	-
262.0	34.0	32.0	27.0	34.9	45.3	-	-	-	-	-
Phase II										
0.001	-	-	-	-	-	2.8	1.2	1	1.3	1.2
0.01	-	-	-	-	-	2.0	2.8	2.4	1.5	4.0
0.35	17.3	8.7	19.0	17.4	17.4	4.4	8.7	14.7	5.7	11.3
1.70	17.4	32.0	13.5	8.0	10.8	20.7	29.3	6.7	14.7	35.3
8.60	64.0	45.3	24.7	38.1	41.5	-	-	-	-	-
262.0	156.0	234.8	139.6	256.0	512.0	-	-	-	-	-

In general, MA titers increased with dose for both experiments, although at the lowest doses the changes were hardly observable even for 10-fold differences. Phase II antibody responses were detectable at very low dose levels (0.001 µg), where some protection was also evident. Phase I antibody responses needed dose levels about 100 times greater to be detectable.

For estimating cell-mediated immunity, the whole blood lymphocyte transformation (LT) test was used. Blood was drawn by cardiac puncture from animals receiving the 3 highest vaccine doses on day 20 postvaccination. Lymphocytes were cultured in the presence of phase II antigen for 5 days. Tritiated thymidine was added to the culture and 24 h later lymphocytes were harvested and the degree of stimulation estimated by uptake of the isotope. The dose levels used and the results of these studies are shown in Table III.

TABLE III. LYMPHOCYTE TRANSFORMATION STUDY (PHASE II ANTIGEN) ON GUINEA PIGS 20 DAYS AFTER A SINGLE SC DOSE OF NDBR-105 PHASE I, Q FEVER VACCINE

EXP. NO.	DOSE (µg)	GUINEA PIG NO.	MEAN STIMULATION INDICES BY LOT				
			1	2	3	4	5
2	1.7	1	1.20	0.75	0.76	1.09	1.16
		2	1.21	0.08	1.31	1.03	1.24
		3	1.17	0.96	0.77	1.08	1.19
		4	1.36	0.80	1.10	1.34	1.25
		5	1.50	0.94	0.92	1.25	1.02
		6	1.47	1.31	0.77	0.37	0.80
		7	0.90	1.02	0.93	0.84	1.11
		8	1.00	1.22	1.15	0.78	1.77
1	8.6	6	0.67	1.60	1.40	1.20	0.74
		7	1.30	1.10	0.98	1.10	1.10
		8	0.78	0.97	1.40	0.73	1.86
1	262.0	6	0.92	1.00	1.80	1.20	1.50
		7	1.10	1.20	1.60	1.30	0.32
		8	0.90	1.00	0.89	1.00	3.50

No significant stimulation was observed. This finding is in line with reports by others that a single vaccine dose without adjuvant does not cause significant LT titers to develop in animals.

This report summarizes the progress being made toward the development of a model system for evaluating the protective and serologic properties of the NDBR-105 phase I, Q fever vaccine. While some difficulties have developed with the guinea pig mode, further studies are underway to resolve these problems and provide a method for evaluating and comparing this and other Q fever vaccines.

Presentation:

Johnson, J. W., R. F. Wachter, G. A. Higbee, and C. E. Pedersen, Jr. Physical and immunological comparisons of a phase I and a phase II Q fever vaccine. Presented, 80th Ann. Mtg. ASM, 11-16 May 1980. (Abstracts, 141, p. 91).

Publications: None

LITERATURE CITED

1. Berman, S., R. B. Gichenour, G. Cole, J. P. Lowenthal, and A. S. Benenson. 1961. Method for the production of a purified dry Q fever vaccine. J. Bacteriol. 81-794-799.

2. Vivona, S., J. P. Lowenthal, S. Berman, A. S. Benenson, and J. E. Smadel. 1964. Report of a field study with Q fever vaccine. Am. J. Hyg. 79:143-153.

3. Ormsbee, R. A., E. J. Bell, D. G. Lackman, and G. Tallent. 1964. The influence of phase on the protective potency of Q fever vaccine. J. Immunol. 92:404-412.

4. Spicer, D. S., A. N. DeSanctis, and J. M. Beiler. 1970. Preparation of highly purified concentrates of Coxiella burnetii. Proc. Soc. Exp. Biol. Med. 135:706-708.

5. Spicer, D. S., and A. N. DeSanctis. 1976. Preparation of phase I Q fever antigen suitable for vaccine use. Appl. Environ. Microbiol. 32:85-88.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6422	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. ENTER ACTY	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	054
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE: (U) Characterization and evaluation of selected hemorrhagic fever agents for vaccine development

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
76 10	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. [illegible]
FISCAL YEAR PRECEDING 80	1.0	205
FISCAL YEAR CURRENT 81	0	0

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NAME: POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS: (U) Military medicine; (U) BW defense; (U) Korean hemorrhagic; Hantaan virus; (U) Fluorescent antigen.

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS:

23 (U) Complete characterization of the agent of Korean hemorrhagic fever (KHF) and assessment of the squirrel monkey model for human disease. Assess the potential of ribavirin for chemotherapy. Initiate KHF vaccine development. Study CHF-C virus. 24 (U) KHF virus will be disrupted to isolate and examine viral RNA. Determination of the number of RNA strands and segments will be utilized to complete the classification of the virus. The efficacy of ribavirin as a chemotherapeutic agent will be assessed in the squirrel monkey model of KHF. Vaccine development will be initiated by re-isolation of the virus in vaccine certified cells from known infectious human blood. 25 (U) 79 10 - 80 09 - Infection of squirrel monkeys suggested this system as a possible disease model for KHF. Infection with the Lee strain of Hantaan virus resulted in elevated BUN and creatinine values in some animals. Pathologic lesions were detected in the kidneys of both monkeys sacrificed at the time of elevated enzymes. It appears that elevated enzymes may be an indicator of infection in the primate model. Terminated for management efficiency. Continued in W.U. 871 BC 148. (DAOG1537)

*Available to contractors upon originator's approval.

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

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BODY OF REPORT

Project No.	3M162770A871: (3M162776A841)	Prevention of Military Disease Hazards (U)
Task No.	3M162770A871 BC:	Prevention of Viral Diseases of Potential BW Importance
Work Unit No.	871 BC 139: (841 00 054)	Characterization and Evaluation of Selected Hemorrhagic Fever Agents for Vaccine Development

Background:

The Korean Hemorrhagic Fever agent has recently been named Hantaan virus by Dr. H. W. Lee. Hantaan virus has been successfully isolated in A-549 tissue culture cells (type II alveolar cells derived from a human lung carcinoma). It has the ability to establish persistent noncytopathic infection in these cells; however, the presence of KHF is detectable by immunofluorescence. This has led to the development of a fluorescent foci unit (FFU) assay which has been utilized to semiquantitate KHF samples, in addition to being utilized in a neutralization test. Hantaan virus was shown to be lipid-solvent sensitive, to replicate in the presence of DNA inhibitors, and to be strongly cell-associated. Hantaan virus is serologically distinct from other RNA viruses; however, serological relationships have been demonstrated between KHF antigen and convalescent sera of similar diseases in other countries.

Progress:

In initial experiments, disease was induced in four squirrel monkeys inoculated with Hantaan virus (Lee strain). In a follow-up study, infection of 8 squirrel monkeys with the Lee strain was completed. Three (#16, 27, 538) of the 8 monkeys (Table I) developed elevated BUN and creatinine values on days 16, 21 and 23. Two (16, 27) experienced an early leukocytosis of >14,000 cells that occurred from days 2-7. There was no evident shift to the left in differentials for either of these animals. Three (#569, 570, 571) developed late leukocytosis on days 21-25. There is, at present, no explanation for this event but it suggests the possibility that the incubation period for disease may be quite prolonged in some animals. There is no follow-up serum chemistry on these animals after day 25 due to a relocation of the hot clinical laboratory. Two of these 3 animals were sacrificed (days 22 and 35) in hopes that the histopathologic examination might provide some insight into this question.

Four of 8 monkeys (16, 27, 569, 570) were sacrificed for histopathologic examination during the experiment, one each on days 17, 21, 22, and 35. Monkeys 16 and 27 had elevated BUN and creatinine values prior to or on the day of sacrifice; at necropsy, kidneys were abnormal grossly with a severely congested medulla and clear cortex. The third and fourth monkeys sacrificed were chosen on the basis of normal clinical appearance, without elevated BUN; these monkeys had normal appearing kidneys.

The only significant pathologic findings (# 16 and 27) were in the kidney, and varied in severity, but were morphologically similar. On gross examination,

TABLE I. CLINICAL DATA FROM SQUIRREL MONKEYS INFECTED WITH LEE STRAIN OF HANTAAN VIRUS

MONKEY NO	BUN		CREATININE		TEMPERATURE (°F)		WBC	
	MAX	DAY	MAX	DAY	MAX	DAY	MAX	DAY
16[a]	68	21[a]	2.7	21	1024	18	14,700	4
27[a]	178	16[a]	9.7	16	102.2	7	14,600	4
30	41	0	1.6	14	102.9	21	10,600	21
531	27	0	1.1	0	103.1	21	7,300	21
538	50	23	1.7	23	103.4	2	8,200	21
569[a]	34	0	<1.0	1-23	103.4	9	13,400	21
570[a]	39	16	1.4	7	103.4	0	16,000	23
571	41	0	1.3	2	102.4	0	28,900	21
Controls								
7	40	7	0.9	2	102.2	2	7,500	2
20	34	21	1.5	0	102.6	21	8,400	0
537	25	18	2.0	16	102.4	2	7,100	23
Normal								
Mean Range (Range)	25 (10.0 - 40)		0.8 (0.1 - 1.5)		101.9 (100.3 - 103.3)		6,700 (400 - 13,000)	

[a] Sacrificed.

the kidney medulla was much darker in color and easily delineated from the cortical area. Upon microscopic examination, it was evident that the congested and hemorrhagic areas were severely compressed and the tubular pattern disrupted. Necrosis, whether patchy or segmental, occurred simultaneously with regeneration as evidenced by high mitotic index, and flattening and crowding of lining cells; indicative of a active reparative process. The severe medullary intertubular vascular congestion with lesser amounts of hemorrhage and the usual pattern of necrosis were characteristic of the lesions seen in human KHF.

There were no significant pathologic changes in monkeys 569 and 470. On gross examination, kidneys appeared normal. This was confirmed by histologic examination which indicated no scarring or any indication of having undergone a reparative process. This substantiates the use of BUN and/or creatinine assays as the primary indicators of pathological changes in squirrel monkeys and provides a means for determination of when monkeys are infected.

The clinical description of KHF in humans includes fever, weakness, thirst, anorexia, malaise, emesis, nausea, petechiae and flush, in addition to other signs and symptoms. Severe disease involved shock and renal failure leading to fluid and electrolyte imbalance. BUN was used as an indicator of the renal phase of the disease and first became elevated when the febrile phase was ending and remained elevated until the convalescent phase.

In humans, characteristic pathologic findings include retroperitoneal edema, diffuse hemorrhage in the right atrium of the heart, severe congestion, hemorrhage and infarct-like necrosis in the renal medulla, and hemorrhage and necrosis in the anterior lobe of the pituitary gland. The most consistent and prominent pathologic findings were in the kidney and consisted of a congested dark red medulla with areas of necrosis separating the medullary tubules. Compression of tubules was prominant and the epithelium was flattened, distorted, and hypocellular. Some exhibited epithelial regeneration but this was not evident in all instances.

The Lee strain produces pathologic changes in the kidneys in some squirrel monkeys; however, it does not appear to produce consistent clinical signs in infected animals. All monkeys were infected as evidenced by seroconversion (Table II). With the exception of #521, monkeys with the higher titers were the ones that had the more prominent clinical signs.

The squirrel monkey appears to be a promising model for human disease. Kidney suspensions from monkeys #16 and 27 were used to infect additional squirrel monkeys in hopes that by passage the Lee strain might increase in pathogenicity. That experiment is currently in process and preliminary results indicate that a higher percentage of monkeys (2 of 3) are developing pathologic changes in the kidney.

Persistent infection of A-549 cells with Hantaan virus, the etiologic agent OF KHF. Due to low titers of Hantaan virus, persistent infection of A-549 cells was evaluated. In acute-phase infection, yield is proportional to virus input. In fact, under the best high-yield circumstances of primary infection one has to put in virus at an input equivalent to the maximum yield, i. e., the multiplicity of input of infection equals or exceeds the yield of infection.

TABLE II. IMMUNOFLUORESCENT ASSAY OF PERIODIC BLEEDINGS FROM SQUIRREL MONKEYS INFECTED WITH LEE STRAIN HANTA/N VIRUS

MONKEY NO.	RECIPROCAL TITER BY DAY			
	0	14	21	35
531	<10	20	160	640
571	<10	20	160	160
569[a]	<10	20	40	160
570[a]	<10	40	320	160
27[a]	<10	320	160[+]	
16[a]	<10	20	320	
538	<10	<10	40	80
30	<10	10	80	80
20	20	<10	10	
537	<10	10	40	
7	160	80[+]	160	

[a]Sac - sacrificed

Hence, the reference to the negative economics of large-scale virus production via primary acute infection. For example, in a recent in-depth study of primary infection of A-549 cells, we infected cells in suspension with an input of 9 infectious particles/cell. After 160 min of adsorption at 36 C, 60% had been removed from the cell suspension medium. Thus, a maximum input of infection of slightly more than 5 infectious particles/cell was achieved. Newly produced extracellular virus first appeared at 15 h postinfection, and logarithmic cellular replication and viral production continued for 8-10 h more. Peak production was seen at 39 h, at which time a maximum yield of 1.3×10^7 FFU/ml was detected. This yield, although it is nearly the best seen with this virus, converts to 7.5 FFU/cell, i.e., less than that required to achieve the infection conditions to attain maximal yield. Three-fold higher yields have been achieved, but they require inputs of several hundred infectious particles/cell. In comparison, inputs of one infectious particle/cell yield 4 infectious particles/cell, while inputs of one infectious

particle/50 - 100 cells have peak yields of one infectious particle/30 cells. Clearly then, there is no way to achieve the desired result, that of large volumes of infectious cell culture fluids that exceed 10^7 FFU/ml, via the harvest of spent media from primary infection at peak yield. Although peak yield can be increased 2-3 fold, i.e. to 15-20 infectious particles/cell, by harvesting the cell contents as well as the extracellular virus, the resulting product is unsatisfactory for biochemical analysis because of the resulting massive contamination with host proteins and nucleic acids.

Persistent infection was, therefore, examined as a possible solution to this problem. Yields were expected to be considerably lower than at peak production during primary infection. Multiple harvests from the same infected cells, combined with methods to concentrate culture fluids to the desired infectivity titers, might achieve the goal that appeared to be impractical with the single harvest methods of primary infection. Persistent infection was studied in the same infected cells described above (Table III). Two T-150 flasks of 2×10^7 cells/flask were infected at high multiplicity (input was 9/cell and MOI was 5/cell) and sampled for virus infectivity at 24-h intervals. Spent cell culture fluids were harvested daily and replaced with fresh medium. Cells were passaged at 4-7 day intervals and carried for 8 passages for a total of 47 days.

In 850-CM^2 roller bottles containing 2×10^8 cells each, an 80-fold increase of viral production was detected. Infectivity titrations indicated that the cells were yielding an average 1-2 infectious particles/cell every 24 h. Although this represents a daily yield/unit volume only 20% of that at peak yield during primary infection, the yield volume is 80 times that of the original infection, doubles every 5 days, and can be continued at 24-h harvest intervals, apparently, indefinitely.

Growth of Hantaan virus in persistently infected A-549 cells gave a 58% increase in titer when the volume of media was decreased from 150 to 50 ml.

TABLE III. HANTAAN VIRUS TITER FOR SEQUENTIAL PASSAGES OF PERSISTENTLY INFECTED A-549 CELLS

PASSAGE	MAXIMUM TITER	DAY*
1	2.1×10^7	1.5
2	1.3×10^7	1.5
3	1.9×10^6	3
4	3.4×10^6	4
5	5.5×10^7	4
6	1.3×10^7	4
7	3.1×10^6	5
8	1.5×10^6	4
9	6.9×10^5	3

*Day Maximum titer occurred.

Treatment of persistently infected cells with 0.1 mg of actinomycin D increased the yield for the first 24 h, varying from 37 to 183% in different experiments.

Membrane filtration of Hantaan virus established the size range at >100 nm. It is not known why some infectious particles would not have come through the membrane.

Hantaan virus infectivity and replication were evaluated at various fetal calf serum concentrations (0, 1, 2, 3, 6 and 10%). It appeared that concentrations of 1-2% allowed more fluorescent foci to form by the standard assay procedure than did identical samples containing 3-10% fetal calf serum. Culture of Hantaan virus with the same serum concentrations noted above indicated that the highest titers during the 14-day incubation period were produced when 2-6% fetal calf serum was used. Preliminary studies at various temperatures (37, 35, 32 and 26 C) indicated highest titers were produced by 72-96 h at 37 and 35 C.

Work on concentration, and purification of Hantaan virus and methods to develop procedures required to achieve virus concentrations adequate for biochemical analysis are continuing simultaneously with the work described above. Some 70-90% of the virus sediments at relatively low centrifugal forces ($\geq$ 3000 XG). An adequate explanation of this phenomenom is still not available; however, it is assumed that at least part of the explanation rests in the very close association of the virus with cell fragments. Regardless of the reason, the event is extremely useful in that 20-100-fold concentrations of virus can be achieved with insignificant virus loss. The main disadvantage to the procedure is the inevitable simultaneous concentration of cell debris with virus. A partial solution to this problem has been found recently with the observation that virus in spent cell culture fluids passes an 0.8-μ filter with subsequent removal of 50-75% of the cell debris, but with immeasurable losses in virus titer. Thus, virus can be concentrated 2-3 fold by very simple means without increasing the relative concentration of cell debris.

Publications:

1. Rice, R. M., B. J. Erlick, R. R. Rosato, G. A. Eddy, and S. B. Mohanty. 1980. Biochemical Characterization of Rift Valley Fever Virus. Virology 105:256-260.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6417	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. GRP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	056
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Provide with Security Classification Code)

(U) Effects of experimental respiratory infection on pulmonary function

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
77 06	80 09	DA			C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: NA d. AMOUNT:

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

18. RESOURCES ESTIMATE

	PRECEDING/CURRENT	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	80	1.0	172
	81	0	0

19a. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Provide KEYWORDS with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Immunology; (U) Respiratory diseases; (U) Lung physiology; (U) Pulmonary Function

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

23 (U) Determine effects of respiratory infection on mechanical and immunologic functions of the lung. A better understanding of these effects will aid in improvement of prophylaxis and treatment of diseases of military importance. This is especially important in defending against possible airborne BW pathogens.

24 (U) Develop and utilize capability to study pulmonary function in small laboratory animals during experimental respiratory infection.

25 (U) 79 10 - 80 09 - Pulmonary function variables of Fischer-344 rats were measured by plethysmography under halothane anesthesia. Measurements included spontaneous breathing patterns, lung volumes, dynamic and quasistatic pressure-volume relationships, forced expiratory flow-volume and volume-time relationships. These measurements were made possible by the development of numerous techniques and fabrication of specialized equipment. It is expected that the techniques described in this report will yield useful information on pulmonary function before, during and after experimental respiratory infection, immunization or therapy.

Publication: Infect. Immun. 30:51-57, 1980.

Terminated due to transfer of the principal investigator to another Division at USAMRIID.

27a. relate to or comment upon originator's report.

DD, FORM 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

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BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BB: Prevention of Bacterial and Rickettsial Diseases of Potential BW Importance

Work Unit No. 871-BB-130: Effects of Experimental Respiratory Infection
(841 00 056) on Pulmonary Function

Background:

Infections in U. S. Military and Civilian populations resulting from operational use of biological weapons by enemy forces are likely to be induced by inhalation of infectious agent aerosols. Infections of the respiratory tract, therefore, may represent the initial mode of entry of a pathogen into the susceptible host.

The effects of such infections on pulmonary function are essentially unknown and may be present before clinical signs of infection become apparent. Pulmonary function measurements are expected to provide useful information on the pathogenesis of respiratory infections and the effects of aerosol immunization or therapy. The present investigations were designed to develop the capability to study pulmonary function in small laboratory animals.

Progress:

Pulmonary function measurements are expected to provide useful information in studies of lung disorders of small laboratory animals caused by inhalation of aerosols of infectious agents. As only limited measurements can be performed on unsedated animals, techniques for determining lung volumes and pressure-volume relationships of anesthetized rats are being developed. Measurements of dynamic lung mechanics in rats have been demonstrated by Diamond and O'Donnel (1) and adapted for serial use by Mauderly (2).

A 1.5-L combination pressure and volume plethysmograph was used. Flow rates in the volume-mode were determined by measuring pressure differences across 6 layers of 400-mesh wire cloth covering a 6.4-mm hole, using a differential pressure transducer. Volumes in the pressure-mode were determined by the same transducer with the plethysmograph sealed. Transpulmonary and airway pressures were measured using water-filled catheters placed in the esophagus and airway. Positive and negative pressure reservoirs, made from size E gas cylinders, were kept charged at ± 40 cm H_2O by pumps and pressure limit switches. A system of switches and solenoid valves permitted connection of the tracheal catheter to the pressure reservoirs; needle valves permitted control of inspiratory and expiratory flow rates.

Male Fischer rats, weighing 250-300 g, were studied. Each rat was anesthetized with halothane in air and a tracheal catheter, made from a 14-gauge IV catheter, was introduced via the mouth. An esophageal catheter (#5 French infant feeding tube) was inserted into the esophagus and adjusted for maximal pressure deflections

during spontaneous breathing. The rat was placed in sternal recumbency in the plethysmograph which was then closed; halothane concentration was adjusted to give a respiratory frequency of 60 breaths/min.

Spontaneous respiratory patterns were measured with the plethysmograph in the volume mode. Pressure changes proportional to lung volume were measured using a differential pressure transducer. Variables measured during spontaneous breathing included respiratory frequency, tital volume, minute volume and dynamic lung compliance. Minute volume, the product of respiratory frequency and tidal volume is a measure of ventilation; dynamic lung compliance is the relationship of transpulmonary pressure (P_{tp}) to tidal volume.

Each of the subsequent measurements were made during forced ventilatory maneuvers. Prior to each measurement, the rat was hyperventilated to induce temporary apnea and to establish a uniform lung volume history. Inspiratory capacity (IC) was measured by inducing a slow inspiration from the apneic lung volume (functional residual capacity, FRC) to + 30 cm H_2O P_{tp} (total lung capacity, TLC) using an inspiratory flow rate of 5 ml/sec. The expiratory reserve volume (ERV) was then measured by inducing a low expiratory flow rate from FRC to -30 cm H_2O P_{tp} residual volume. A slow vital capacity (SVC) maneuver was performed by inflating and deflating the lungs to + and -30 cm H_2O P_{tp} using an expiratory flow rate of 3 ml/sec. A forced vital capacity (FVC) maneuver was conducted using the same procedure as for SVC but without limiting flow rate. The plethysmograph was then switched to the pressure mode and functional residual capacity was measured using Boyle's law, as the rat attempted to inspire against a blocked airway. All maneuvers were repeated 3 times; the animal's spontaneous respiration was allowed to stabilize between tests. Each rat was weighed and rectal temperature recorded prior to recovery from anesthesia.

Quasistatic lung compliance was calculated using pressure-volume signals from the slow vital capacity maneuver. Forced expired volume and peak expiratory flow rate were determined graphically from forced vital capacity maneuvers. Total lung capacity was determined by adding IC and FRC.

Results of spontaneous and forced ventilatory maneuvers are summarized in Table I.

TABLE I. RESPIRATORY FUNCTION OF ANESTHETIZED FISCHER RATS (n=8)

VARIABLE	SYMBOL	UNITS	MEAN ± SD
Body weight		g	279 ± 27
Respiratory frequency		breaths/min	62 ± 10
Tidal volume	TV	ml	1.4 ± 0.21
Minute volume	MV	ml/min	88 ± 21
Dynamic	C_1dyn	ml/cm H_2O	0.36 ± 0.05
C_1dyn x 100/TLC		ml/cm H_2O/ml	2.9 ± 0.3
Inspiratory capacity	IC	ml	9.8 ± 0.7
Expiratory reserve volume	ERV	ml	3.7 ± 1.3
Two-stage vital capacity		ml	13.7 ± 1.2
Slow vital capacity	SVC	ml	12.9 ± 1.2
Quasistatic C_1 at 10 cm H_2O	$C_{qs}10$	ml/cm H_2O	0.61 ± 0.19
Forced vital capacity	FVC	ml	12.6 ± 1.3
Forced expired volume at 0.2 sec.	$FEV_{0.2}$	ml	11.0 ± 1.3
Peak expiratory flow rate	PEFR	ml/sec	83.2 ± 13.9
% VC at PEFR		% VC	68.8 ± 7.5
Expiratory time		sec	0.52 ± 0.14
Functional residual capacity	FRC	ml	2.8 ± 1.3
Total lung capacity	TLC	ml	12.4 ± 1.5
TLC/kg		ml/kg	44.0 ± 2.8
VC/TLC		ml/ml	1.04 ± 0.11
FRC/TLC			0.34 ± 0.34

These results compare favorably with those reported by Diamond and O'Donnell (1) and Mauderly (2), using similar techniques. Techniques reported here are specifically suited to repeated use in the same animal because tracheal intubation was not invasive and the forced ventilatory maneuvers did not require pharmacological respiratory paralysis. The positive and negative pressure system provided reproducible forced ventilatory maneuvers. These maneuvers permitted measurement of TLC, VC, flow rates during maximal expiration, expiratory time, and % VC in 0.2 sec, all of which have been suggested as being useful for detecting pollutant-induced lung disorders in man. It is expected that the techniques reported here will yield useful information on pulmonary function before, during and after experimental respiratory infection, immunization or therapy.

Presentations:

1. Kastello, M. D. Pulmonary alveolar macrophages and infection. Presented, Inhalation Toxicology Research Institute, Albuquerque, NM, 26 Jul 1979.

2. Emmert, A. D, L. A. Brown, and M. D. Kastello. Lung lavage technique for reqovery of alveolar macrophages from monkeys. Presented, National Capital Area Branch, Am. Assoc. Lab. Anim. Sci., Cockeysville, MD, 25 Oct 1979.

3. Kastello, M. D. A comparative approach to regulation of body fluids and electrolytes. Presented, Uniformed Services University of Health Sciences, Bethesda, MD, 1 Apr 1980.

Publications:

1. Reynolds, J. A., M. D. Kastello, D. G. Harrington, C. L. Crabbs, C. J. Peters, J. V. Jemski, and G. H. Scott. 1980. Glucan-induced enhancement of host resistance to selected infectious diseases. Infect. Immun. 30:51-57.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6418	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	057
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE: (U) Metabolic alterations in fatty acid metabolism during infections of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 012300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 07	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 80	1.0	115
	CURRENT 81	0	0

19. RESPONSIBLE DOD ORGANIZATION
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ASSOCIATE INVESTIGATORS
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NAME: Neufeld, H. A. POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Provide EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Inflammatory stress; (U) Tularemia infection; (U) Viral infection; (U) Lipid metabolism; (U) Therapy

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

23 (U) Identify mechanisms responsible for the altered fatty acid metabolism and decreased ketogenesis observed during bacterial or viral infections. Study the muscle-liver-adipose tissue interrelationships during starvation and militarily important infections. Determine if lipids or enzyme replacement are potentially useful in supportive therapies during severe infections. When completed this work will provide the basis for more rational and more effective therapy for the soldier suffering from an infectious disease of natural or BW origin.

24 (U) Using the established rat Streptococcus pneumoniae and Francisella tularensis models, exam e mechanistic changes in lipid metabolism by in vivo and in vitro methods. Extend work to study viral encephalitis.

25 (U) 79 10 - 80 09 - Livers from S. pneumoniae- and F. tularensis-infected rats have a decreased ketogenic capacity associated with an accumulation of carnitine and a decrease in CoA fatty acyl esters. The subcellular distribution of carnitine and CoA indicates a decreased mitochondrial acetyl-CoA and an increased concentration of cytosolic acetyl-CoA and acetylcarnitine. Carnitine palmityltransferase activity decreased and alpha-glycerophosphate acyltransferase and acetyl-CoA carboxylase increased during infection. These results suggest a shuttling of long-chain acyl-CoA into triglyceride synthesis and acetyl units into the cytosol away from the enzymes of ketogenesis. Thus, necessary energy-yielding fuel, in the form of fatty acid, is directed away from oxidative pathways toward pathways designed for synthesis and storage, establishing what might be considered a fatty acid-futile cycle in the liver of the infected host.

Publications: Fed. Proc. 39:1123, 1124, 1727, 1980.

Terminated for management efficiency. Continued in W.U. S10 AQ 197.(DAOG1529)

* Available to contractors upon authorization 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.

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BODY OF REPORT

Project No. 3M162776A871: Prevention of Military Disease Hazards (U).
(3M162776A841)

Task No. 3M162770A871 BB: Prevention of Bacterial and Rickettsial Diseases of Potential BW Importance

Work Unit No. 871 BB 131: Metabolic Alterations in Fatty Acid Metabolism during
(841 00 057) Infections of Military Importance

Background:

A complex variety of metabolic alterations that occur during infectious illness are manifested principally by the wasting of body proteins (1). The ability of the liver to synthesize ketone bodies which can serve as metabolic fuel and thereby reduce gluconeogenesis and protein wastage is impaired during infection (2, 3). To study the effect of bacterial infection on hepatic fatty acid (FA) metabolism, a series of experiments were performed in the rat infected with Streptococcus pneumoniae or Francisella tularensis. These studies have shown livers from fasted-infected rats have (a) a decreased ketogenic capacity (3); (b) an accumulation of carnitine, especially acetylcarnitine; and (c) a > 50% decrease in coenzyme A (CoA) and its derivatives. (d) Isolated perfused livers from infected rats direct more long-chain FA into triglyceride synthesis and less into oxidative pathways and ketogenesis (3). Since the cofactors and enzymes involved in FA metabolism are compartmentalized (4), studies directed at determining changes in subcellular concentrations and enzyme activities are essential to the understanding of the control of FA metabolism during infectious illness.

Progress:

Rats infected with either S. pneumoniae or F. tularensis were used to study the effect of bacterial infection on hepatic FA metabolism. The studies included determination of the ketogenic capacity of livers from 48-h fasted-infected rats; hepatic concentrations and subcellular distribution of carnitine and CoA; and activities of enzymes that catalyze the activation, esterification and oxidation of FA.

The isolated liver perfusion model was used to evaluate the ketogenic capacity of livers from infected rats. These studies showed a decreased utilization of long-chain FA for ketogenesis during both these infections, while medium-chain length FA was oxidized to ketone bodies at rates equal to fasted-controls. The decreased ketogenic capacity was associated with an accumulation of hepatic carnitine and a decreased concentration of hepatic CoA fatty-acyl esters.

Since carnitine and CoA are compartmentalized within the liver cell, the decreased ketone production could have been related to alterations in the hepatic mitochondrial and cytosolic pools of these cofactors. Therefore, livers from fasted and fasted-S. pneumoniae infected rats were fractionated by differential and isopycnic sucrose density gradient centrifugation; each fraction was assayed for marker enzymes, carnitine and CoA. In both fasted-control and fasted-infected

rats 6% of the total carnitine and 30% of the total CoA was in the mitochondria-rich fraction. Acid-soluble cytoplasmic carnitine, especially the acetyl derivative, increased while the long-chain acyl derivative decreased in the infected rats compared to fasted controls. No changes were seen in the mitochondrial pool size of carnitine but there was a > 50% decrease in mitochondrial acetyl-CoA. The carnitine acylation ratio reflected a decreased rate of FA oxidation during the infection.

Liver homogenates and samples from the fractionation study were assayed for the enzymes that catalyze activation, esterification and oxidation of FA. The overall activation of long-chain FA was not affected by infection. The acylation of glycerophosphate increased in fasted-infected rats and carnitine palmityltransferase activity (forward reaction) decreased compared to fasted-controls. While carnitine acetyltransferase per mg of mitochondrial protein increased during infection, the peroxisomal acetyltransferase activity decreased by 50%. This decrease could be related to the decrease observed in catalase, the marker enzyme for peroxisomes. Mitochondrial enzymes of β-oxidation, the Krebs cycle and acetoacetate synthesis were functional during the infection. However, in the infected rat less acetyl-CoA was directed toward ketone production and more was transferred into the cytosol via citrate or transferred to carnitine.

A careful examination of the 3 studies suggests that the infection-related decrease in ketone body production from long-chain FA results from an increased production of triglyceride, a decreased utilization of acyl groups for oxidative processes and an increased shuttling of acetyl groups to carnitine. Alterations observed in enzymic activities during infection confirm this altered flow of precursors for ketone synthesis. This investigation supports the concept that alterations in hepatic FA metabolism and ketogenesis during bacterial infections may promote the continued use of amino acids for gluconeogenesis and contribute to the protein-wasting state that accompanies the illness. Thus, necessary energy-yielding fuel in the form of FA is directed away from oxidative pathways toward pathways designed for synthesis and storage of fat, setting up what might be considered a FA futile cycle.

Presentations:

1. Pace, J. G. Carnitine and coenzyme A distribution in liver of meal-fed, fasted and Streptococcus pneumoniae-infected rats. Presented, George Washington University Biochemistry Department, Washington, Nov 1979.

2. Pace, J. G. The effect of Spreptococcus pneumoniae infection on hepatic carnitine, coenzyme A and carnitine acyltransferases. Presented, symposium sponsored by Cutter Laboratories, "The role of carnitine in fat metabolism in infection and the efficacy of intralipid during infection", Chicago, IL, Feb 1980.

3. Pace, J. G., H. A. Neufeld, and R. W. Wannemacher, Jr. Intracellular distribution of hepatic carnitine (CAR), coenzyme A (CoA) and their acyl derivatives in fasted and fasted-infected rats. Presented, Annu. Mtg. FASEB, Anaheim, CA, 11-18 Apr 1980 (Fed. Proc. 39:1123, 1980).

4. Beall, F. A., and J. G. Pace. Ketogenesis in isolated perfused livers from rats with bacterial infections. Presented, Annu. Mtg. FASEB, Anaheim, CA, 11-18 Apr 1980 (Fed Proc. 39:1124, 1980).

5. Pace, J. G., and R. W. Wannemacher, Jr. Carnitine palmityltransferase (CPT) and acetyltransferase (CAT) in rats infected with Streptococcus pneumoniae. Presented, Annu. Mtg., Am Soc. Biol. Chemists, New Orleans, LA, 1-5 Jun 1980 (Fed. Proc. 39:1727, 1980).

Publications:

None

LITERATURE CITED

1. Wannemacher, Jr., R. W., and W. R. Beisel. 1977. Metabolic Response of the host to infectious disease, pp. 135-139. In Nutritional Aspects of Care in the Critically Ill. (J. R. Richards and J. M. Kinney, eds). Churchill Livingstone, Edinburgh.

2. Neufeld, H. A., J. A. Pace, and F. E. White. 1976. The effect of bacterial infections on ketone concentrations in rat liver and blood and on free fatty acid concentrations in rat blood. Metabolism 25:877-884.

3. Wannemacher, Jr., R. W., J. G. Pace, F. A. Beall, R. E. Dinterman, V. J. Petrella, and H. A. Neufeld. 1979. Role of the liver in regulation of ketone body production during sepsis. J. Clin. Invest. 64:1565-1572.

4. Aas, M. 1971. Organ and subcellular distribution of fatty acid activating enzymes in the rat. Biochim. Biophys. Acta 231:32-47.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. Agency Accession	2. Date of Summary	Report Control Symbol
DA OH6425	80 10 01	DD-DR&E(AR)636

3. Date Prev Sum'ry	4. Kind of Summary	5. Summary Scty	6. Work Security	7. Regrading	8. Dis'b'n Instr'n	9a. Specific Data-Contractor Access	9b. Level of Sum
79 10 01	H. TERMINATION	U	U	NA	NL	☒ Yes ☐ No	A. Work Unit

10. No./Codes	Program Element	Project Number	Task Area Number	Work Unit Number
a. Primary	62772A	3M162776A841	00	059
b. Contributing				
c. Contributing	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Pathogenesis of anthrax

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. Start Date	14. Estimated Completion Date	15. Funding Agency	16. Performance Method
77 10	80 09	DA	C. In-house

17. CONTRACT/GRANT

a. Dates/Effective: Expiration:

b. Number:

c. Type: NA d. Amount:

e. Kind of Award: f. Cum. Amt.

18. Resources Estimate	Preceding / Current	a. Professional Man Yrs	b. Funds (In thousands)
Fiscal Year	80	1.0	121
	81	0	0

19. RESPONSIBLE DOD ORGANIZATION

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Responsible Individual

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20. PERFORMING ORGANIZATION

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Principal Investigator

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Social Security Account Number:

Associate Investigators

Name: Middlebrook, J. L.

Name: Spero, L.

POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Anthrax; (U) Toxin; (U) Toxoid; (U) Vaccine; (U) Laboratory animals

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS

23 (U) To elucidate the mechanisms of intoxication of anthrax toxin and to define its relationship to the overall course of anthrax infections. Our ultimate goal is the development of effective countermeasures including, but not limited to, the production of a toxin-derived vaccine suitable for human use, which will protect military personnel from anthrax used as a BW agent.

24 (U) Produce toxin in desired quantity. Fractionate and purify anthrax toxin producted in large quantities (either by fermentation or alternate culture conditions) Study the pathophysiology of toxin in laboratory animals. Prepare and evaluate vaccines and/or toxoids.

25 (U) 79 10 - 80 09 - Satisfactory conditions were established for in vitro production of anthrax toxin and protective antigen. The Sterne strain of B. anthracis produces EF, PA and LF. Under the same growth conditions, strain V770 produces only PA. A serological assay is used for PA; bioassays for LF and EF require the presence of PA, since neither is active alone.

Partial purification of PA has been achieved using ion-exchange resins (DEAE-cellulose) and molecular sieving. Preliminary data indicate that PA is proteinaceous, somewhat thermolabile, and has a molecular weight greater than 100,000 daltons.

Antisera are being prepared to several antigens. Protection studies are being evaluated. Human sera are being screened for antibody levels and duration of titers. Publications: Abstr., Annu. Mtg. ASM - 1980, p.29; Proc. 4th Int. Symp. Staph. and Staph. Infections, in press, 1980; Infect. Immun., in press, 1980.

Terminated for management efficiency. Continued in W.U. 871 BA 150.(DAOG3810)

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162770A371: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BA: Prevention of BW Diseases Caused by Microbial Toxins

Work Unit No. 871 BA 122: Pathogenesis of Anthrax
(841 00 059)

Background:

Although primarily a disease of livestock, anthrax is potentially a high hazard to man. Interest in its use as a biological agent has recently been renewed due to the Soviet accident at Sverdlovsk. Septicemic and pulmonary forms of anthrax are highly lethal (1) and represent a serious potential threat to our military forces.

Much of the early work on anthrax involved pathogenesis. It was not until 1954 that the role of anthrax toxin was suggested (2). There are 3 components of the toxin: lethal factor (LF), protective antigen (PA), and edema factor (EF). Some strains of Bacillus anthracis produce all 3 factors, while others do not. The vaccine strain (V770) produces only the protective antigen factor of the toxin complex. LF and EF have no biological action when isolated. Each must be combined in a specific ratio with PA to elicit a response (3). The PA factor alone is a potent immunogen and is presumably present in high concentration in the current vaccine.

The exact role of the toxin complex and each of the individual components in the pathogenesis of anthrax has not been explained. In order to evaluate these factors, each must be prepared in sufficient quantity and must be highly purified. Only when these materials are isolated can definitive work be done to determine the molecular structure of the toxin and to define the exact role of each component in the pathogenesis of anthrax. Once the evaluations have been made a better vaccine material should be available.

Progress:

Two strains of B. anthracis have been studied fairly extensively for production of anthrax toxin. Vaccine strain V779 produces only PA component, while the Sterne strain produces all 3 components. Each strain has been studied in a 10-L fermentor system. Synthetic medium is used in order to facilitate purification and to define exact metabolic requirements. Glucose is supplied as the sole carbohydrate source. Multiple fermentation runs were done with each strain to determine maximal growth conditions and to study elaboration of toxin or antigen. Samples were removed hourly for these determinations. For both cultures, maximal growth was achieved after 10-12 h, but antigen was not detected in the culture supernatant until 16-18 h postinoculation.

Once satisfactory production was obtained, a number of preliminary purification steps were tried. At this point, the lability of the toxin became evident,

necessitating a rapid removal of the antigenic complex from the culture fluid. A batch adsorption onto DEAE-cellulose has been successful. Following centrifugation to remove bacteria, the resin is stirred into the culture supernatant for 1 h. The toxin (or antigen, depending on strain in use) is bound to the resin and non-attached materials can be removed by washing. The resin is then transferred to a chromatographic column, and the antigen-containing protein peak is eluted with 1 M NaCl. Although the recovery is rather low at this stage, it is necessary to sacrifice yield for improved stability of the antigen. SDS-gel electrophoresis analysis of this partially purified concentrated material indicates the presence of 5-10 proteins. This material is immunologically active and contains biological activity, indicating the presence of all components of the toxin.

Further purification studies have included more selective binding to ion-exchange resins; DEAE-Sephacel and DEAE-Sephadex have been used. In both cases the antigen binds to the resin at buffer strength up to 0.1 M. Selective elution has been accomplished by stepwise increments in NaCl addition. Gradient elution has been attempted. Thus far we have not obtained consistent results with these techniques, in that antigen-containing fractions are eluted at varying buffer concentrations. Standardization of the procedure is currently in progress. Analysis of several preparations has revealed that the antigen at this stage is more highly purified.

Alternate methods of purification have included molecular sieving on Sephadex G-200. Results from these experiments indicate that the MW of the antigen is 110,000-130,000 daltons.

Several different antisera have been prepared in rabbits. The standard human vaccine (adsorbed on alum), standard antigen before alum absorption, and partially purified antigens from our production runs have been used as immunogens. Evaluation of the efficacy of each of these preparations is currently in progress. In addition, human sera are being screened to determine levels of antibody production, as well as the duration of levels of protective antibody.

Presentations:

1. Johnson, A.D., and L. Spero. Toxin components produced by two strains of Bacillus anthracis. Presented, Annu. Mtg., ASM, Miami Beach, FL, 11-16 May 1980 (Abstracts - 1980, B76, p. 29).

2. Johnson, A.D. Production of biochemically different types of exfoliatin from two strains of Staphylococcus aureus. Presented, IVth Int. Symp. Staphylococci and Staphylococcal Infections, Warsaw, Poland, 15-19 Oct 1979 (in press, 1980).

Publications:

Morlock, B.A., L. Spero, and A.D. Johnson. 1980. Mitogenic activity of staphylococcal exfoliative toxin. Infect. Immun. 30: in press.

LITERATURE CITED

1. Wright, G.G. 1965. The anthrax bacillus, pp. 530-544. In Bacterial and Mycotic Infections of Man, 4th ed. (R.J. Dubos, and J.G. Hirsch, eds), J.B. Lippincott, Philadelphia.

2. Smith, H., and J. Keppie. 1954. Observations on experimental anthrax: demonstration of a specific lethal factor produced in vivo by Bacillus anthracis. Nature (London) 173:869-870.

3. Mahlandt, B.G., F. Klein, R.E. Lincoln, B.W. Haines, W.I. Jones, Jr., and R.H. Friedman. 1966. Immunologic studies of anthrax. IV. Evaluation of the immunogenicity of three components of anthrax toxin. J. Immunol. 96:727-733.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6426	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. ORGN INSTR'N	8B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☑ YES ☐ NO	A. WORK UNIT

10A. PROJ./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A041	00	060
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Include with Security Classification Code): (U) Identification of bacterial BW agents using a chemiluminescent immunoreaction procedure

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 012300 Biochemistry; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGCY			16. PERFORMANCE METHOD
77 11	80 09	DA			C. In-house

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

18. RESOURCES ESTIMATE

	FY	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING 80	1.0	121
	CURRENT 81	0	0

19A. RESPONSIBLE DOD ORGANIZATION
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RESPONSIBLE INDIVIDUAL
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TELEPHONE: 301 663-2833

20. GENERAL USE
Foreign intelligence considered

19B. PERFORMING ORGANIZATION
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PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic participation)
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TELEPHONE: 301 663-7181
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Miller, Jr., R. J.
NAME:

POC:DA

21. KEYWORDS (Provide KEYWORDS with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Bacterial diseases; (U) Chemiluminescence; (U) Rapid detection; (U) Viral diseases

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

23 (U) Develop reproducible and reliable techniques for the rapid detection of small numbers of bacterial or viral particles in biological and environmental samples using a chemiluminescent procedure. Following detection of a biological attack, rapid identification of the causative agent is essential in order to begin the proper and most efficacious therapy.

24 (U) Antigen-specific antibodies are allowed to capture antigen. The resulting complex may be analyzed directly if the primary antibody is labeled. If not, the complex is treated with an appropriately labeled second antibody. The labeled antibodies or their enzymatic products are then determined using specific luminescent techniques.

25 (U) 79 10 - 80 09 - Studies in our laboratory as well as in other laboratories have demonstrated the capability to detect small numbers of microorganisms by luminescent immunochemical procedures. Assays developed for Francisella tularensis, live vaccine strain, have suffered from nonreproducibility due to antibody problems. A very sensitive assay for Venezuelan equine encephalomyelitis has been developed but problems have been encountered with only 60-90 day storage possible. Manipulation of the assay procedures to include antigen immobilization and immune complex isolation are in progress in an attempt to overcome these difficulties.

Publications: Fed. Proc. 39:919, 1980; Army Sci. Conf. Proc. I:169-179, 1980; Proc. 2nd Int. Symp. on Bioluminescence and Chemiluminiscence, in press, 1980.

Terminated for management efficiency. Continued in W.U. 870 BC 068. (DAOG3811)

(Available to contractors upon originator's approval)

DD FORM 1498 MAR 88 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 88 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3M162770A870: Risk Assessment of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A890 BC: Prevention of Viral Diseases of Potential BW Importance

Work Unit No. 870 BC 045: Identification of Bacterial BW agents using a
(841 00 060) Chemiluminescent Immunoreaction Procedure

Background:

Previous work on this unit concerned itself with instrumentation, antibody production horseradish peroxidase detection levels and reproducibility of literature reports (1, 2). Studies have also shown that peroxidase-coupled antibodies maintain the immunogenicity required for an immunoassay. Work has continued on the immobilized antibody LVS and VEE assays previously reported.

Progress:

The LVS assay using passively immobilized primary (capture) antibody has proven to lack the reproducibility for an effective assay. In our assays as well as other immunoassays utilizing immobilized antibodies, several formidable problems present themselves, namely: the specificity and concentration per surface area of the antibody used to capture the antigen, and the shelf-life of immobilized antibodies. In the bacterial assay, the first problem has been identified as the cause of nonreproducibility. Although a VEE assay has been developed (a highly specific antibody is available), the second consideration has surfaced, i.e., immobilized antibody being nonfunctional after 60-90 days.

Therefore, a shift in emphasis from antibody immobilization to antigen immobilization has been recognized and is being investigated. Such schemes could utilize lower specificity material and also different classes of immunoglobulins.

Investigation of density gradient separation of immune complexes has suggested that such an approach may be suitable for early identification. Also under investigation are systems using ATP generating enzymes conjugated to antibodies as another possible luminescent system. Some work has also been done on the possibility of filtering antigen or antigen/antibody complexes as a very rapid identification scheme.

Presentations:

1. Miller, Jr., R. J., and D. W. Reichard. Chemiluminescent immunoreactive assay (CIRA) for rapid detection of Venezuelan equine encephalomyelitis virus. Presented, FASEB, Anaheim, CA, 13-17 Apr 1980 (Fed. Proc. 39:919, 1980).

2. Reichard, D. W. Chemiluminescence immunoreaction and its optimization for rapid identification. Presented, Army Research Office workshop, Raleigh, NC, 10-12 Jun 1980.

3. Miller, Jr., R. J., and D. W. Reichard. Chemiluminescent immunoassay for

the detection of virus/antibody aggregates. Presented, 2nd Int. Symp. Bioluminescence and Chemiluminescence, LaJolla, CA, 26-28 Aug 1980.

4. Reichard, D. W., and R. J. Miller, Jr. Bioluminescent immunoassay: a new enzyme-linked analytical method for the quantitation of antigens. Presented, 2nd Int. Symp. Bioluminescence and Chemiluminescence, LaJolla, CA, 26-28 Aug 1980.

Publication:

Reichard, D. W., and R. J. Miller, Jr. 1980. Chemiluminescence immunoreactive assay (CLIA): a rapid method for the detection of bacterial and viral agents - Francisella tularensis, live vaccine strain (LVS) and Venezuelan equine encephalomyelitis vaccine strain (VEE TC-83). Army Sci. Conf. Proc. III:169-179.

LITERATURE CITED

1. Halmann, M., B. Velan, and T. Sery. 1977. Rapid identification and quantitation of small numbers of microorganisms by a chemiluminescent immunoreaction. Appl. Environ. Microbiol. 34:473-477.

2. Velan, B., and M. Halmann. 1978. Chemiluminescent immunoassay; a new sensitive method for determination of antigens. Immunochemistry 15:331-333.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6410	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	063
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Provide with Security Classification Code)

(U) Rapid diagnosis of viral diseases of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 0100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 06	80 09	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: NA d. AMOUNT:

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR — PRECEDING		
80	1.0	277
FISCAL YEAR — CURRENT		
81	0	0

19. RESPONSIBLE DOD ORGANIZATION

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RESPONSIBLE INDIVIDUAL

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Luscri, B. J.

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Provide EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Arboviruses; (U) Arenaviruses; (U) Virus diagnosis (FA, RIA, ELISA)

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

23 (U) Develop and standardize immunofluorescent or alternate techniques for isolation, detection and identification of viruses of military importance and high-hazard viruses. Provide diagnostic reagents and protocols to implement the objectives.

24 (U) Produce specific virus antigens and antisera for use as reagents in serodiagnosis and virus isolation and identification; evaluate clinical and laboratory-generated specimens for diagnosis of virus infections; develop specific SOP's and systematize laboratory procedures; develop protocols for and train personnel of the clinical laboratory in their use.

25 (U) 79 10 - 80 09 - The specificity of indirect FA reagents was determined for all alpha- flavi, and arenaviruses of interest. The stability of antigen containing spot-slides held at -20 and 4C was determined; spotslides are stable for 123 days, the last time period tested. Inactivation studies of virus contained on spotslides was reviewed; a cobalt-60 irradiation inactivation study was initiated. Plaque reduction neutralization, IFA using monolayer cultures and spotslides, and RIA tests were examined to assess the sensitivity of each to detect antibodies to Rift Valley fever in human sera. The PRNT and IFA monolayers were accurate, RIA gave one false positive and IFA spotslides 4 false negatives for the 15 sera tested. Studies adapting the amplification SOP for the isolation and identification of viruses from clinical specimens, to samples of fluids received from the XM-2 collector were initiated. ELISA for RVF and VEE viruses are approaching late stages of development.

Terminated for management efficiency. Continued in W.U. 870 BC 068. (DAOG3811)

* Available to contractors upon originator's approval

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

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BODY OF REPORT

Project No. 3M162770A870: Risk Assessment of Military Disease Hazards (U)
(3M176776A841)

Task No. 3M162770A970 BC: Prevention of Viral Diseases of Potential BW Importance

Work Unit No. A870 BC 046: Rapid Diagnosis of Viral Diseases of Military Importance
(841 00 063)

Background:

This work unit "Rapid Diagnosis of Viral Diseases of Military Importance" was approved 18 September 1978. Development was based on the assumption that immunofluorescent techniques would be the first generation of studies so as to put a system on-line as soon as possible. Alternate methods for serodiagnosis or isolation and identification of viruses of interest would come later. Initial objectives were to develop and standardize immunofluorescent techniques for the detection and identification of viruses of military importance, and to develop and standardize rapid immunofluorescent techniques for the serodiagnosis of high hazard viruses in man. Viruses of interest are listed in Table I. It is apparent that not all viruses are of critical importance and that others do not infect humans. Some such as Langat (LGT) have even been used as potential human vaccines but it is of interest and included as a simulant for the Russian spring-summer complex which requires P-4 containment facilities. Others are included due to cross-reactions with viruses of interest, they are found in the endemic area or present clinically as a virus of interest. A few, such as Saint Louis encephalitis (SLE) and LaCrosse (LAC) viruses are included so as to have a diagnostic capability for viruses of emerging concern in the US.

TABLE 1 VIRUSES OF INTEREST TO THE RAPID DIAGNOSIS PROGRAM

Alphaviruses:	EEE, WEE, VEE, Mayaro (MAY), Chikungunya (CHIK), O'Nyong- Nyong (ONN).
Flaviviruses:	Japanese Encephalitis (JE), Langat (LGT), Dengue (DEN)-1, 2, 3, 4; Yellow Fever (YF) West Nile (WN), SLE.
Arenaviruses:	Lymphocytic choriomeningitis (LCM), Junin (JUN), Machupo (MAC), Lassa (LAS), Tacaribe (TCR), Pichinde (PIC).
Bunyaviruses:	Sandfly fever (Naples, Sicilian), Oropouche (ORO), Rift Valley fever (RVF), Congo-Crimean hemorrhagic (C-CHF), Hazara (HAZ), LaCrosse (LAC).
Ungrouped	Korean hemorrhagic fever (KHF), Ebola (EBO), Marburg (MBG).

Progress:

Specificity of fluorescent reagents for IFA. The assessment of fluorescent reagents requires in part, the determinations of homologous and heterologous serological reactions with other serogroup viruses. The ultimate reagent is high-titered, monospecific, and generally unattainable with the current state of the art. The new field of hybridoma monoclonal antibody production will hopefully breach this impass.

The homologous and heterologous indirect fluorescent antibody (IFA) titers of known positive alphavirus sera tested on mono- and polyvalent virus antigen containing spotslides are shown in Table II.

As generally expected, homologous reactions are strongest and subgroup cross-reactions evident, i.e., relatedness of CHIK, MAY and ONN. The polyvalent slide containing EEE, WEE, VEE and CHIK antigens reacted adequately with each specific antiserum. Titers generally support antigenic relationship previously reported by other serological tests.

TABLE II. IFA SPECIFICITY OF ALPHAVIRUS REAGENTS[a]

	ANTIBODY					
ANTIGEN	MAY	EEE	WEE	CHIK	VEE	ONN
MAY	512	64	NEG	16	32	NEG
EEE	NEG	256	32	NEG	16	NEG
WEE	64	64	≥ 4096	16	128	8
CHIK	128	16	32	512	64	128
VEE	8	16	8	NEG	1024	NEG
ONN	256	64	16	128	64	256
Polyvalent	256	512	2048	512	≥ 4096	256

[a]Reciprocal homologous titers

MAY - guinea pig, 512
EEE - rabbit, 256
WEE - rabbit, ≥ 4096
CHIK - monkey, 512
VEE - mouse, 1024
ONN - mouse, 256

Table III shows similar data for arenavirus reagents.

Results agree somewhat with published data (1) although direct comparisons should not be made due to the known fact that cross-reactions observed are dependent on the homologous titers of the sera used, and the manner in which

sera were produced, i.e., compare the results of LCM hyperimmune and 2 injection sera. The latter are more specific.

TABLE III IFA SPECIFICITY OF ARENAVIRUS REAGENTS[a]

	ANTIBODY						
ANTIGEN	MAC	PIC	JUN	LAS	LCM[1]	LCM[2]	TCR
MAC	≥ 1024	64	≥ 1024	8	64	NEG	≥1024
PIC	512	>1024	256	32	NEG	NEG	256
JUN	≥ 1024	128	≥ 1024	32	128	NEG	≥1024
LAS	± 8	NEG	NEG	≥1024	64	16	NEG
LCM	64	± 0	NEG	256	≥1024	256	32
TCR	≥1024	32	512	16	16	NEG	≥1024

Reciprocal homologous titers

MAC - monkey, 4096

PIC - hamster, 4096

JUN - guinea pig, 2048

LAS - guinea pig, 2048

LCM[1] - mouse hyperimmune sera (NIH),2048

LCM[2] - 2 guinea pig-injection sera, 256

TCR - mouse hyperimmune sera (NIH), 1024

Homologous antibody reactions are strongest for PIC, LAS and LCM and each is separable from other arenavirus antibodies. The large degree of cross-reaction between MAC, JUN and TCR is apparent and end-points were not reached. If one eliminates the presence of TCR antibody from consideration (TCR is not a naturally acquired human disease) the presumptive differentiation of MAC and JUN antibodies from those of other arenavirus is possible. The pursuit of MAC and JUN end-point titers may be of little value since Wulff et al. (1) reported that neither FA nor CF tests could achieve separation; neutralization tests are required. Fortunately the presumptive separation of LAS from LCM, and MAC from JUN can be supported by the fact that neither is normally present in the others endemic area.

Flavivirus antibodies are historically difficult to identify due to the large degree of cross-reactivity within the group. HA, HI and CF tests are useful but finite identification, especially among DEN serotypes, usually require neutralization tests (2). Flavivirus reagents produced for this program (Table IV) show this reported cross-reactivity. It may not be possible to differentiate the DEN serotypes using these reagents; which is not totally unexpected. DEN antibodies can be differentiated from those of other flaviviruses which in turn are separable from each other. The specificity of the SLE (Hubbard) antigen spotslide 1:8 cross-reaction with JE antibody is surprising in light of the cross-reactivity of SLE antibody and bears repeating, as do

TABLE IV. IFA SPECIFICITY OF FLAVIVIRUS REAGENTS[a]

	ANTIBODY								
ANTIGEN	JE	LGT	DEN-1	DEN-2	DEN-3	DEN-4	YF	WN	SLE
JE	2048	32	128	64	256	256	NEG	64	64
LGT	64	512	NEG	64	128	32	16	16	16
DEN-1	512	128	512	1024	1024	NEG	NEG	64	256
DEN-2	1024	64	64	1024	1024	512	NEG	64	256
DEN-3	256	32	64	1024	1024	1024	NEG	16	64
DEN-4	256	64	64	1024	1024	2048	1024	64	64
YF	128	32	16	128	256	128	32	NEG	16
WN	512	64	32	256	512	128	NEG	1024	64
SLE	8	NEG	NEG	NEG	NEG	NEG		NEG	512

[a]Reciprocal homologous titers
JE - Guinea pig, 2048
LGT - Monkey, 512
YF - Guinea pig, 1024
WN - Guinea pig, 1024
SLE - Guinea pig, 512
DEN-1 - Monkey, 512
DEN-2 - Mouse ascitic fluid (NIH), 1024
DEN-3 - Mouse ascitic fluid (NIH), 1024
DEN-4 - Mouse ascitic fluid (NIH), 2048

spot checks of various crosses with sera from a different species other than those shown. Coded, known positive sera have been requested from Dr. Casals, YARU, and they with other available alpha- flavi- and arenavirus sera from various species will be tested as time, personnel and reagents permit.

As previously indicated, fluorescent techniques were developed as a first-generation system for on-line capability whenever possible. The inability to identify specifically DEN clearly points out the need for development of alternate techniques. We are confident that knowledge of the specific cross-reactions evident in Tables II-IV will allow a serodiagnosis to be obtained for serum samples submitted for testing.

Reagents - general. The production or acquisition of working lots (~200) of spotslides of agents listed in Table I is essentially complete except for MBG virus, which is not being used within USAMRIID. Those considered high-hazard were, or are, produced and maintained by those investigators directly involved with the agent [i.e., LAS (Dr. Jahrling), KHF (LTC French), EBO (LTC Lupton) and MAC (COL Eddy)]. In many instances specific antisera for use as controls and for the production of direct conjugates were also provided. Positive control sera and direct conjugates for all viruses are available except for EBO and MBG. An IFA developed by MAJ Moe exists for EBO. As reported in last years annual, the homologous and heterologous specificities of arenavirus direct conjugates were determined. We do not plan to extend such studies to alpha- and flavivirus conjugates until a study comparing the ability (sensitivity) of direct conjugates to detect specific viral antigens to those used in indirect fluorescent procedures is done. Sensitivity may well vary, depending on the type of specimen being tested, i.e., frozen vs. fixed.

Stability study. It was determined by LTC French that KHF spotslides held in sealed cans from which air had been excluded by the addition of liquid N_2 were stable as to FA intensity at various temperatures (-70, -20, 4 and 28°C for various periods of time. After 6 months at -70, -20, or 4°C, intensities were still 3-4 +; at 25°C intensity was variable to negative. A similar study was initiated to test FA stability of TCR, PIC, EEE, DEN-2, WN, and RVF spot slides at -20 and 4°C. Viruses were selected to represent various serological classifications. No drastic changes in FA intensities were observed during the 123-d period (Table V).

TABLE V. STABILITY OF FLUORESCENCE AT VARIOUS TEMPERATURES

	FLUORESCENCE (1+ to 4+ BY DAYS							
	4C				-20C			
VIRUS	0	30	61	123	0	30	61	123
TCR	4+	3+	4+	3+	1+	3+	4+	3+
PIC	4+	4+	4+	4+	4+	4+	4+	4+
EEE	3+	2+	4+	3+	3+	2+	4+	4+
DEN-2	2+	1+	4+[a]	4+	2+	1+	4+[a]	4+
WN	3+	4+	2+	3+	3+	3+	2+	3+
RVF	4+	4+	4+	3+	4+	4+	4+	4+

[a]Conjugate changed.

Sufficient samples are available for testing at 240 days and another as yet selected time period. This study is of critical importance because we are currently holding 7000-8000 spotslides at -70°C. The decision to move all or part of the sldies from -70 to -20°C could be made now, but the transition should perhaps await the results of the inactivation study to be described.

Inactivation study. Initially, it was deemed desirable to produce non-viable antigen-containing spotslides for serodiagnosis in laboratories without containment facilities. Numerous preliminary studies both in-house and under sections of contracts (YARU, DAMD 17-77-C-7035, DAMD 17-77-C-70480) failed to find a suitable virus-inactivating substance that allowed adequate retention of antigenicity as determined by FA. Short-wave (265 nm) and long-wave (320-380 nm) irradiation, chlorpromazine and 4-aminomethyl-4, 5, 8,-trioxysalen (AMT) as adjuncts to irradiation, alcide class substances, and β-propiolactone each exhibited some problem in FA antigen retention. AMT + long-wave irradiation followed by ^{60}Co irradiation seems most promising (Dr. Jahrling, personal communication). We have started the following preliminary experiment by ^{60}Co irradiation of previously prepared spotslides received from YARU. Four sets, each containing a slide of EEE, CHIK, DEN-2, WN, TCR, PIC, RVF and ORO virus were prepared and stored in air tight tins as described for the stability study. One will serve as an unirradiated control. The remaining 3 sets were treated by ^{60}Co irradiation in which one received 300,000 R, another 150,000 R and the last one 75,000 R. Slides are currently held at -20°C (stable for ~123 days) and will be assayed for FA intensity, and residual live virus in cell and animal systems.

Sensitivity of serological tests:

The ability of various serological tests to detect RVF antibodies was studied using current sera obtained from individuals vaccinated a number of years ago, true negatives, and individuals on a sandfly fever project. The possibility of a RVF and a sandfly fever cross might exist. Results show that the PRN test and IFA, using monolayers of infected cells, accurately identified all known positive and negative sera (Table VI). The RIA (CPT Urbanski) was slightly less accurate with one false positive (#13 1:40) and the IFA using spotslides was least accurate in that 4 false negatives were recorded. Titers by PRN and RIA correspond reasonably; no such correspondence of titer exists between FA and any other test. This decreased lack of sensitivity of spotslides vs. monolayer cultures has been noticed with YF antigens (unpublished data) and is a generally known limitation of the system. Consequently we have produced a test lot of YF virus monolayer spotslides in which virus-infected cells are prepared (as per the SOP for normal spotslides), added aseptically to the slides, allowed to attach and spread out over the spot for 24 h, fixed and processed. This procedure alleviates the necessity of using Leighton tubes and coverslips and allows the testing of 8 samples on a single slide. Whether this is a solution or should be done in any but only special cases remains to be determined.

TABLE VI. ABILITY OF SEROLOGICAL TESTS TO DETECT RVF ANTIBODIES

SAMPLE	SALK PRNT	USAMRIID PRNT	IFA Spot	IFA Monolayer	RIA
1	18	160	Neg	64	160
2	Neg	<10	Neg	Neg	<10
3	264	160	Neg	16	160
4	17	320	Neg	32	113
5	Neg	<10	Neg	Neg	<10
6	289	>320	64	128	462
7	59	160	32	64	89
8	12	80	Neg	16	89
9	Neg	<10	Neg	Neg	<10
10	ND	320	16	64	150
11	ND	>320	512	512	>1280
12	ND	ND	Neg	Neg	<10
13	ND	ND	Neg	Neg	40
14	ND	ND	Neg	Neg	<10
15	ND	>320	18	64	100

Microdot study. We started preliminary studies to determine the feasibility of preparing antigen containing "microdots" for seroepidemiological and clinical specimen screening studies. Microdots, initially developed to screen for trachoma strains, are 1-2 mm dots produced by placing virus-infected cells onto a slide using a C-6 Speedball pen. Virus-infected cells are prepared as (per SOP) for producing spot slides, the pen is dipped into the cell suspension and 5-9 separate microdots are produced within a single 6 mm spot of a conventional spotslide. Such a single spot contains from 90-100 individual cells. Microdots have been produced using YF-infected LLC-MK 2 cells. Such a microdot spot containing a single virus does not offer any advantage over a standard spot slide. Consider though, the advantage in time, and reagents if, in fact, each of the 5-9 individual microdots within a single spot contained a different viral antigen. One could, for instance, screen multiple sera on a single microdot 10 place spotslide for African hemorrhagic fevers (LAS, MAR, EBO, RVF, C-CHF, YF), South American viruses (JUN, MAC, YF, ORO, SLE, DEN) North American viruses (VEE, WEE, EEE, SLE, LAC), Argentine viruses (JUN, LCM, ROC, WEE, VEE, EEE), or any preselected geographic area. Although easy in theory it will require extensive study to infect cells with the viruses selected for a given geographic area so that each contains maximum

TABLE VI. RECOVERY OF RVF VIRUS FROM REPLICATE XM-2 COLLECTION FLUIDS[a] (INPUT:10^4 $SMICLD_{50}$/0.1 ml)

CELL	SAMPLE	OBSERVATION					
		6 h		12 h		18 h	
		FA (1+ to 4+), No. positive cells and foci		FA (1+ to 4+), No. positive cells and foci		FA (1+ to 4+), No. positive cells and foci	
BHK-21	Undil	Neg		3-4	10	3-4	∿100 2F
	1+5	Neg		3-4	20	3-4	∿ 50 5F
	Undil	Neg		3-4	10	3-4	∿ 10 3F
	1+5	Neg		3-4	25	3-4	< 50 5F
	Control[b]	Neg		3-4	25	3-4	<100 5F
Vero	Undil	2+	5	3-4	∿ 50	3-4	∿ 30 F
	1+5	Neg		3-4	>100	3-4	> 50 F
	Undil	2+	5	3-4	∿ 50	3-4	∿ 30 F
	1+5	Neg		3-4	∿100	3-4	> 50 F
	Control	2+	10	3-4	>100	3-4	> 50 F
$LLC-MK_2$	Undil	Neg		Neg		3-4	< 5
	1+5	Neg		2	5	3-4	< 10
	Undil	Neg		Neg		3-4	< 5
	1+5	Neg		Neg		3-4	< 10
	Control	Neg		2-3	∿5	3-4	< 30

[a]FBS + 0.05 % Tween-80, pH 7.2.

[b]Input as stated, but in E199, 2% FCS Pen-Strep-Nystatin.

antigen at the time of slide preparation. Alternatively, perhaps each could be added individually with the slides being held in some, yet to be determined, "stable state." Microdots are an interesting technique that will be studied as time and personnel permit.

Biodetection systems. Attendance at the quadripartite meeting (5-6 May 1980 at USAMRIID) indicated that the amplifying systems developed for the isolation of viruses from clinical specimens were directly applicable to fluids from the XM-2 collector. A quick series of short experiments determined that: 0.05%-Tween-80 in the collection fluid is not toxic either undiluted or at a 1+5 (or 1 in 6) dilution to BHK-21, LLC-MK_2, or Vero monolayers when the SOP for cell infection is used; the 45-min period for air sample collection at ambient temperature did not decrease the 10^4 $SMICLD_{50}$/0.1 ml of RVF virus as compared to an identical sample held at 4°C; bacteria, molds, etc., drawn into the collection fluid by 45 min of sampling in as dirty an environment as naturally occurs could be controlled by the addition of penicillin, streptomycin, and nystatin for the 72-h duration of the experiment, and positive FA results could be obtained within 24 h using RVF mock-infected collection fluids. Table VI shows the results of a more detailed study. It is possible within 6 h to detect 10^4 $SMICLD_{50}$/0.1 ml inoculum from undiluted collection fluids used to infect Vero monolayers. By 12 h the infections have increased and FA intensities are: in Vero > BHK-21 > LLC-MK_2. Previous inquiries to the "hardware" people indicated that specifications for the XM-2 were that, it delivers 10^4 assay units and was understood to mean 10^4 units/ml. Subsequent discussions resulted in clarification that the specification is 10^4 units/total volume or ca. 2.5×10^2 units/ml. This is at the level at which RVF virus can be detected 72 h after Vero cell infection (unpublished data).

An ELISA test for RVF virus was our first choice due to its major role in divisional studies, the probability of recurrent epidemics, and the fact that an ELISA test is desirable to complete our comparative study of the sensitivity of various tests to detect RVF virus antibodies in vaccinated humans (see section on test sensitivity). Problems were encountered in obtaining sufficiently pure antigen due to the retention of endogenous cellular alkaline phosphatase and the number of false-positives seen with known negative sera. New antigen was prepared and tested by block-titration against positive and negative sera (working dilution 1:25) this dilution is quite low and will necessitate frequent antigen preparation and standardization. This antigen was tested against a number of coded sera and shown to be usable. Nine negative sera were tested; one reacted at 1:10 and another at 1:20. Of the nine positive sera all but one reacted at titers of 1:20 to 1:80. It was also determined that lipid-rich sera give false-positive reactions. We have been unable to draw a sufficiently low, negative serum baseline. A similar problem was encountered in Dr. Macasaet's VEE ELISA and resolved by increasing the percentage of BSA from 0.5 to 4.0% in the washing fluid and by increasing the standard 3-wash cycle to 10. RVF antibody titers are low to moderate by ELISA, requiring a true negative baseline. Both modifications of the VEE ELISA will be tried as well as the horseradish peroxidase, 2,2 -Azino-di 3-ethyl Benzthfazoline-6-sulfonate, (ATBS) and ortho-Phenylene diamine (OPD) ELISA systems.

In the coming year, emphasis will be placed on antigen detection in both

cell and animal systems. Preliminary studies indicate that formalin-fixed, paraffin-embedded tissue is usuable for testing by fluorescent methods. Losses of FA intensity occur during rehydration but will be resolved by manipulation of the time of rehydration and composition of reagents. Such a system will allow manipulation of tissues containing high-hazard viruses without the requirement for P-4 containment. More emphasis will be placed on cell amplification systems for priority I and II viruses; other viruses will be deemphasized. Alternate methods for serodiagnosis and identification of isolates will be a primary concern. Ancillary studies concerning biodetection, i.e., XM-2 systems, are most interesting and will be pursued as time permits.

Presentations:

1. Rosato, R. R. Immunofluorescent Studies with Viral Antigens and Antibodies, Presented, Workshop on Rapid Identification of BW Agents sponsored by the Technical Cooperation Program Subgroup E, Technical Panel 4, 5-7 May 1980, USAMRIID, Fort Detrick, MD.

2. Rosato, R. R. Rapid Detection of Virus Antigen and Antibody by Immunofluorescence, Presented, Workshop on Detection of Biological Materials in Field Environments sponsored by U.S. Army Research Office, 10-12 June 1980 Raleigh, NC.

Publications:

1. Luscri, B. J., O. M. Brand, and G. A. Eddy, 1980. Sensitivity of selected arenaviruses to a human interferon. Submitted Infect. Immun. March 1980.

2. Foulke, R. S., R. R. Rosato, and G. R. French, 1981. Structural polypeptides of hazara virus. J. Gen. Virol. accepted 6 Oct 80.

3. Rice, R. M., B. J. Erlick, R. R. Rosato, G. A. Eddy and S. B. Mohanty. (1980). Biochemical Characterization of Rift Valley Fever Virus. Virology, 105:256-260.

Literature Cited:

1. Wulff, H., J. V. Lange, and P. A. Webb. 1978. Interrelationships among arenaviruses measured by direct immunofluorescence. Intervirology 9:344-350.

2. Bancroft, W. H., J. M. McCown, P. M. Lago, W. E. Brandt, and P. K. Russell. 1979. Identification of dengue viruses from the Caribbean by plaque-reduction neutralization test; pp. 173-178. In Dengue in the Caribbean, 1977. (Sci. Publ. No. 375) Pan American Health Organization, Washington, DC.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6416	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. GIDEP DATA?	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	065
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Include with Security Classification Code)

(U) Mechanism of action of antimicrobial agents

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
78 09	80 09	DA			C. In-house

17. CONTRACT/GRANT

a. DATE(S) EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
PRECEDING FISCAL YEAR 80	0.5	62
CURRENT FISCAL YEAR 81	0	0

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Jahrling, P.B.
NAME: Stephen, E. L. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Provide KEY with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Ribavirin; (U) Antiviral drugs; (U) Arenaviruses; (U) Togaviruses

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

23 (U) Determine the mechanism of action of ribavirin (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide). This drug shows considerable promise as an antiviral agent. How it exerts its effect will be useful in treatment of viral diseases of military importance.

24 (U) Test in vitro effect in tissue culture of ribavirin on m-RNA structure of arena- and togaviruses. Various biochemical and radiolabeling techniques will be used.

25 (U) 7' 10 - 80 09 - It was found that the antiviral activity of ribavirin resides in its ability to interfere with translation of RNA. Its effect on translation is non-specific and leads to inhibition of both cellular and viral protein synthesis. Ribavirin was shown to alter utilization of nucleic acids by BHK-21 cells at lower concentrations than its in vitro antiviral activity. These data show that expression of ribavirin's antiviral activity requires an alteration in nucleic acid metabolism. Studies on the uptake and incorporation of precursors for RNA metabolism by 2 cell lines, BHK-21 and Vero cells, demonstrate that ribavirin accumulates to a greater extent in BHK-21 cells and brings about a different pattern of RNA synthesis. The differences may reflect the variable efficacy of the drug when tested in different cells. The intracellular distribution of ribavirin following uptake by BHK-21 cells was determined by isopycnic centrifugation of cell homogenates; 90% of the drug has a cytoplasmic localization while the remainder is found in lysosomes. In vivo, ribavirin was found to accumulate in high concentrations in erythrocytes. Chromatography of ribavirin taken up by human RBC indicate that the drug is converted to the triphosphate form. It is concluded that uptake of ribavirin by erythrocytes results in trapping of the drug by phosphorylation. Term. for mngment efficiency. Cont. W.U. 871BE146 (DA0G3815). Publications: Prog. Abstracts No. 977, 19th ICAC Meeting, 1979; Army Sci. Conf. Proc. v. 302-319, 1980; in Current Chemotherapy & Infectious Disease, pp. 1370-1372, 1980.

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 85 AND 1498-1, 1 MAR 88 (FOR ARMY USE) ARE OBSOLETE

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BODY OF REPORT

Project No. 3M152770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BE: Exploratory Antiviral Drug Development

Work Unit No. 871 BE 147: Mechanism of Action of Antimicrobial Agents
(871 00 065)

Background:

Viral infections are a major military health problem in the United States and throughout the world. Effective treatment and prevention of viral diseases to conserve the fighting strength more effectively requires development and evaluation of new antiviral agents.

Use of antiviral drugs in military personnel, however, requires a comprehensive understanding of their pharmacology to ensure selection of the correct drug which combines low toxicity and high efficacy for treating specific virus infections. Civilian pharmaceutical companies in general have not been willing to invest heavily in development of drugs which do not have a national or international market. Thus, elucidation of the pharmacology of potentially useful antiviral drugs for which we have studied the mechanism of action is ribavirin (1). Ribavirin is a nucleoside analogue with demonstrable antiviral activity against the etiological agents of such militarily important diseases as Lassa fever, Bolivian and Argentine hemorrhagic fevers, Rift Valley fever, and Venezuelan equine encephalomyelitis (VEE).

Previous reports form this laboratory have dealt with the mode and specificity of action of ribavirin on the replication of VEE grown in vitro in BHK-21 cells (2). These studies demonstrated that ribavirin's antiviral activity resides in its ability to interfer with translation of RNA. Its effect on translation is nonspecific and leads to inhibition of both cellular and viral protein synthesis (3).

Progress:

Work continued on the effects of ribavirin on metabolism of cultivated cells. The uptake of radiolabeled nucleic acid precursors into tricloractic acid-soluble (uptake) and -insoluble (incorporation) material during a 30-min pulse was examined in control and VEE-infected cells pretreated for 5 h with low doses of ribavirin (32 x 0.5 µg/ml). At the lowest concentration tested ribavirin inhibited both the uptake and incorporation of uridine by control and infected cells. Guanosine uptake was enhanced at the lowest doses but declined at higher concentrations of ribavirin to about 80% of the uptake of untreated control and infected cultures. Incorporation of label into the acid-insoluble material followed a similar pattern. Inosine utilization was strongly inhibited at 0.5 µg/ml of ribavirin.

Ribavirin's effects on utilization of nucleic acids by BHK-21 cells occurs at lower concentrations than its in vitro antiviral activity. One interpretation which may be derived from these data is that expression of ribavirin's antiviral activity requires an alteration in nucleic acid metabolism.

Another characteristic property of ribavirin is that its efficacy against viruses in tissue culture systems varies depending on the host cells. Ribavirin is highly effective against viruses grown in BHK-21 cells, but 10 times more drug is required to obtain approximately the same level of efficacy in vero cells.

A number of studies were conducted, therefore, to determine the cause for the difference between BHK-21 and Vero cells in their response to ribavirin. In one series of experiments, the uptake and incorporation of precursors for RNA metabolism were measured in both cell lines in the presence of ribavirin at concentrations extending from 0 - 800 µg/ml. Uridine incorporation in BHK cells followed a biphasic response. At low concentration of drug (0.5 - 25 µg/ml), uridine incorporation was inhibited. This effect, however, was reversed at higher doses. In Vero cells, on the other hand, incorporation of uridine was nearly 1/5 that of BHK-21 and was inhibited by ribavirin in a dose-dependent manner. Inosine incorporation was inversely proportional to drug concntration. There was no difference between the 2 cell lines in their response to inosine except that uptake in Vero cells was 1/6 that of BHK-21 cells. Guanosine uptake by BHK cells was only slightly altered by ribavirin, but in Vero cells, incorporation decreased markedly at the higher doses of drug. The uptake of ribavirin by both cells lines was determined using ^{14}C-labeled drug. Uptake was linear with respect to time but a plateau was not reached even after 24 h of incubation with 50 µg/ml of ribavirin. The uptake by Vero cells was 1/6 as large as that found for BHK-21 cells. These data demonstrate that ribavirin interacts differentially among tissue culture cell lines. The differences may reflect the variable efficacy of the drug when tested in different cells.

The intracellular distribution of ribavirin following uptake by BHK-21 cells was determined by isopycnic centrifugation of cells homogenates. Cells were incubated with [^{14}C] ribavirin (50 µg/ml) for 1 and 24 h, washed, homogenized and centrifuged on linear 30-60% sucrose gradients. Results show that 90% of the drug has a cytoplasmic localization while the remainder is found in lysosomes. The sequestration of drug in lysosomes may represent trapping of ribavirin by protonzation of the free base. Additional experiments are planned before the impact of this finding can be evaluated.

Experiments were also conducted to determine ribavirin's effects on the synthesis of viral mRNA species. Using the VEE-infected BHK-21 cell model, cultures were treated with ribavirin at a dose of 100 µg/ml. After3-1/2 h, Actinomycin D was added to roller bottles to a final concentration of 1 µg/ml in order to inhibit selectively cellular ribosomal RNA synthesis. At 4 h, [H^3] uridine was added and the cells were incubated for an additional 2 h to label newly formed mRNA. Ribavirin was omitted from control cultres. mRNA was isolated for control and ribavirin treated cultures and anlyzed by electrophoresis. The resulting pattern resolved 2 major peaks corresponding to the 42 and 26S RNA species of the VEE virus. The relative quantity of the RNA peaks was not materially affected by ribavirin. These experiments confirm that ribavirin does not exert its antiviral effects by inhibiting synthesis of viral RNA.

In vivo, ribavirin has been found to accumulate to high concetrations in erythrocytes. Furthermore, the rate of release from red cells is markedly different between rats, monkeys and man. This prompted in vitro studies to characterized the kinetics of the cellular uptake and release of the drug in erytrocytes of rat, monkey, and human using ^{14}C-labeled ribavirin.

At 37° RBC's from all 3 species were found to take up ribavirin rapidly and reach equilibrium with the extracellular pool of drug in less than 3 min. Thereafter, the intracellular concentration of drug increased at an apparently constant rate for at least 2-1/2 h. This uptake was not saturable even at drug concentrations as high as 1000 µg/ml. When ribavirin-loaded erythrocytes were incubated in ribavirin-free media, the rat cells rapidly released about 2/3 of their ribavirin contents, while monkey and human cells released consistently smaller amounts. Little if any drug was released upon continued incubation for up to 2 h.

Chromatography of ribavirin taken up by RBC indicate that a portion of the drug in rat cells is converted to charged moieties which correspond to the di- and triphosphate derivatives of the drug. In human cells, however, the drug is almost totally converted to the triphospate form. It is concluded, that uptake of ribavirin by erythrocytes results in trapping of the drug by phosporylation. However, in contrast to human erythrocytes, rat cells do not appear to convert ribavirin to the triphosphate derivative as efficiently and as a result can release the drug from the cells, probably due to the action of more active phoshpatase.

Presentations:

1. Canonico, P. G., J. S. Little, P. B. Jahrling and E. L. Stephen. Molecular aspects of the antiviral activity of ribavirin on Venezuelan equine encephalomyelitis virus (VEE). Presented, 11th Int. Cong. Chemother. and 19th Int. Conf. Antimicrob. Agents Chemother., Boston, MA, 1-5 Oct 79 (Program and Abstracts, Abstract No. 977).

2. Canonico, P. G., J. S. Little, P. B. Jahrling, and E. L. Stephen. Mechanism of action of ribavirin: an antiviral drug of military importance. Presented, Army Science Conference, Jun 1980, West Point, NY (Proc. I:309-319, 1980).

Publication:

Canonico, P. G., J. S. Little, P. B. Jahrling, and E. L. Stephen. 1980. Molecular aspects of the antiviral activity of ribavirin on Venezuelan equine encephalomyelitis virus, pp. 1370-1372. In Current Chemotherapy and Infectious Disease (J. D. Nelson and C. Grassi, eds). American Society for Microbiology, Washington, DC.

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1. Sidwell, R. W., R. K. Robins, and I. W. Hillyard. 1979. Ribavirin: an antiviral agent. Pharmacol. Therapeut. 6:123-146.

2. Canonico, P. G., J. S. Little, P. B. Jahrling, and E. L. Stephen. 1980. Mechanism of action of ribavirin: an antiviral drug of military importance. Proc., Army Sci. Conf. I:309-319.

3. Browne, M. J. 1979. Mechanism and specificity of action of ribavirin. Antimicrob. Agents Chemother. 15:747-753.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6418	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. GIDEP INTEREST	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	066
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE: (U) Characteristics of aerosol-induced Rift Valley fever infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 12	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING 80	1.0	89
CURRENT 81	0	0

19a. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Provide KEY with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Aerosols; (U) Rift Valley fever; (U) Laboratory animals; (U) Vaccines

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

23 (U) Evaluate potential for aerosol transmission of Rift Valley fever (RVF) isolates. Investigate pathogenesis, therapy and prophylaxis in relation to aerosol challenge. RVF is a disease of military importance in Africa where it is a serious problem for man.

24 (U) Initially, infect mice with dynamic aerosols; follow with other appropriate animal species. Determine effectiveness of the currently available vaccine against Egyptian strains of virus.

25 (U) 79 10 - 80 09 - Pathogenesis studies of RVFV infection induced via the respiratory route suggest that initial virus replication occurs in the lung. Subsequently, viremia develops and the foci of infection shift to the liver. The liver infection is fulminating and usually fatal. Aerosol infectivity trials with the ZH-501 strain were performed in rats using Wistar-Furth (WF), Fischer-344 (F-344) and Maxx strains. Fatalities and neurological disease were noted for each strain. The median lethal dose for WF was 1.01 log PFU. An increasing order of susceptibility was established as follows: Maxx, F-344, and WF. Baseline information was obtained on the relationship between respiratory infectious dose and PRN antibody titer. Aerosol infectivity of SA-51, SA-75, and Entebbe strains for WF rats was examined. Median lethal doses for SA-51, SA-75, and Entebbe were 2.08, 4.3, and 4.0 log PFU, respectively. Terminated for management efficiency. Continued in W.U. 870 BB 069. (DAOG3814).

* Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BC: Prevention of Viral Diseases of Military Medical Importance

Work Unit No. 871 BC 140: Characteristics of Aerosol-Induced Rift Valley
(841 CO 066) Fever Infections

Background:

Rift Valley fever (RVF) is an acute arthropod-borne disease first described by researchers in Kenya in 1931. The causative agent is an arbovirus classified in the family Bunyaviridae. The complete enzootic cycle of RVFV is unknown; however, a maintenance cycle involving mosquitoes and vetebrates is hypothesized. It infects man and several mammalian species, primarily sheep, cattle, and goats.

Historically, RVF has been confined to central and southern Africa and regarded as a disease problem of livestock. Although humans were recognized as being highly susceptible, RVF in humans was described as either a "dengue-like" fever or an "influenza-like" illness noted on occasion in livestock workers. However, in 1977, a RVF outbreak occurred in Egypt which produced widespread and severe human infections.

Evidence supports a conclusion that RVFV is an infectious agent which military forces operating in Africa might encounter. Also, there are scattered reports that RVF has spread beyond the African contenent into the Middle East.

Potency testing of a currently available inactivated vaccine was performed by IC and IP challenge of mice. Further testing was accomplished using cynomolgus monkeys. Efficacy of the vaccine against an aerosol challenge is undetermined.

Progress:

Pathogenesis of RVF infection induced via the respiratory route was investigated using ICR mice. Male mice, 6-8 weeks old, were infected with 2.8 $\log_{10}$ PFU of the ZH-501 strain; sites of viral replication were monitored as a function of time. Beginning at time 0, at 6-, or 12-h intervals through 96 h, 2 mice were killed and tissue or fluid was harvested as follows: brain, upper respiratory tract (URT), lung, heart, kidney, spleen, liver and blood. Tissue was homogenized separately and frozen for subsequent assay. Two additional mice were killed per interval and tissues were fixed for histological examination.

For undetermined reasons, virus assay was not accomplished for homogenates at dilutions less than 1:1,000. Perhaps homogenates at lower dilutions were toxic, or the virus was either not present or was present but masked. Assay was accomplished on tissues collected 72-96 h postexposure; results are summarized in Table I.

TABLE I. VIRUS CONCENTRATION FOLLOWING AEROSOL EXPOSURE OF ICR MICE (n=2/group) TO 2.78 LOG_{10} PFU, ZH-501 STRAIN

TISSUE OR FLUID	MEAN LOG_{10}/g OR ml			
	72 h	78 h	84 h	96 h
Blood	5.5	7.5	7.3	6.7
Brain	3.8	6.4	6.4	5.6
URT	3.8	6.9	6.7	6.8
Lung	4.2	6.9	6.7	6.6
Heart	4.7	7.2	7.0	6.5
Kidneys	4.6	7.4	6.8	6.5
Spleen	4.9	7.7	7.8	7.3
Liver	5.4	7.8	8.1	8.2

Virus levels in the liver increased and were consistently higher than in other tissues. At 96 h virus in the liver exceeded that of blood and other tissues except spleen, by > 1 log_{10} PFU. Gross lesions were evident in both the liver and lungs by 82 h. The liver had a white discoloration with small foci of necrosis; lung lesions ranged from isolated areas of congestion to a widespread dark, diffuse consolidation. Lesions in both organs varied between the 2 mice. MAJ Morrissey (Pathology Division) examined tissues histologically and reported that most lesions were evident at 72 and 84 h and included hepatic, splenic, thymic and lymphoid necrosis.

In evaluating these results, it must be considered that the exposure dose was lower than planned, thus infection rate was not well synchronized, and the small number of mice (n=2) examined at each time interval. One could speculate that the lung lesions observed grossly indicate early replication of virus in the lung, However, this speculation was not supported by assay or histopathologic examination.

With an objective of further evaluating the pathogenesis of respiratory RVF and identifying sites of earliest virus replication, a second study was initiated. Male ICR mice, 6-7 weeks, were exposed via the respiratory route to 3.1 log_{10} PFU of ZH-501. A dose in the range of 3-4 log_{10} PFU was selected as representative of a probable dose under natural conditions. Presumably only a few susceptible cells would be infected and the disease would progress as virus replicated and infected additional cells in a series of succeeding cycles. Beginning at time 0 through 96 h, 4 mice were selected twice daily for harvesting of tissues and subsequent virus assay. Assay results are shown in Table II.

TABLE II. VIRUS CONCENTRATION IN MALE ICR MICE (4/group) FOLLOWING RESPIRATORY EXPOSURE TO $10^{3.1}$ PFU RVFV, ZH-501 STRAIN

TISSUE OR FLUID	GEOM. MEAN LOG_{10} PFU/g OR ml BY HOURS								
	0	6	24	30	48	54	72	74	96
Blood	0	0	0.27	0	2.07	4.29	5.80	6.49	6.40
Brain	0	0	0.63	0	0	1.04	3.83	3.95	4.53
Heart	0	0.78	0.93	0.39	1.90	2.60	4.55	5.16	5.12
Kidneys	0	0	0	0	0	1.21	4.07	4.64	5.56
Liver	0	0	0	0	1.10	3.96	5.63	6.25	6.85
Lung	1.32	0	0.74	2.42	2.85	3.08	4.98	5.44	5.49
Spleen	0	0.55	1.23	0	2.00	2.89	5.55	5.99	6.62
URT	0.28	0	0.28	0.37	1.54	3.01	4.18	5.25	5.74

Immediately after exposure at 0 time, virus was demonstrated in the lungs of 3 mice and the URT of one. During the first 48 h, virus levels were low and most consistently demonstrated in lungs. At 30 h, lungs were the only tissue with a significant viremia. Beginning at 54 h, a high virus titer was demonstrated in the liver. Virus levels in both blood and liver gradually increased between 54 and 56 h. Virus did not appear in the brain until after development of viremia.

The pathogenesis studies performed to date are compatible with the hypothesis that after respiratory exposure, the sequence of events develops as follows: virus deposited in lungs; replication in lug cells or cells associated with the lungs; virus released from lungs resulting in viremia and invasion of hepatic cells as well as cells in other tissues throughout the body; between 58 and 54 h, hepatic cells as well as cells in other tissues throughout the body; between 48 and 54 h, hepatic cells extensively infected so that the liver is the primary site of virus replication; and after 54 h, massive viremia resulting in high concentrations of virus in tissues throughout the body.

Infectivity of the ZH-501 strain for rats was investigated by exposing Wistar-Furth (WF), Fischer-344 (F-344), and Maxx inbred strains to graded aerosol doses. All were males, 12-13 weeks old, purchased from Microbiological Associates. Only the WF were exposed to a complete dose range, from partially infective at 0.8 log_{10} PFU, to lethal at 4.5 log_{10} PFU. Results are shown in Table III.

TABLE III. RESPONSE OF RATS TO INFECTIOUS AEROSOL OF RVFV ZH-501 STRAIN

DOSE (log_{10} PFU)	WF (n=6) Dead	WF (n=6) GMTD[a] (range)	F-344 (n=6) Dead	F-344 (n=6) GMTD (range)	MAXX Dead/ Total	MAXX GMTD (range)
0.8	2	9.9 (7-14)				
1.4	6	10.6 (5-15)				
2.3	5	6.0 (4-11)				
3.4	6	5.7 (4-10)	5	9.7 (5-24)	6/6	15.8 (13-20)
5.4			6	5.4 (5-8)	5/5	8.0 (8)

LD_{50} = 1.01 PFU

[a]Geometric mean time-to-death (days)

Calculated by the Reed and Muench method, the LD_{50} for WF was 1.01 log_{10} PFU. Fatal infections were observed in each rat strain. Six rats survived the initial exposure (5, WF and 1, F-344). They were rechallenged at 21 days to a second aerosol dose of 4.5 log_{10} PFU. Only one rat survived this challenge, the WF which had been exposed to 2.3 log_{10} PFU on Day 0. The geometric mean time-to-death ranged from 5.3 to 15.8 days. Results indicate that the GMTD varied between rat strains and was directly related to exposure dose. Based on lethal infection and GMTD, the rat strains varied in susceptibility. Listed in order of increasing susceptibility they were Maxx, F-344, and WF.

Signs of neurological disease were observed in each of the 3 strains (Table IV).

TABLE IV. NEUROLOGICAL DISEASE OBSERVED IN RATS EXPOSED TO SMALL PARTICLE AEROSOL OF RVFV, ZH-501 STRAIN

AEROSOL DOSE (log_{10} PFU)	WF Pos./Dead	WF GMTD (range)	F-344 Pos./Dead	F-344 GMTD (range)	MAXX Pos./Dead	MAXX GMTD (range)
0.8	1/2	14				
1.4	3/6	16 (16-17)				
2.3	0/5					
3.4	0/6		2/5	20.2 (17-24)	4/6	15.2 (13-20)
4.5	0/6					
5.4			0/6		0/5	

In each instance the rats which developed neurological signs were in groups exposed to lower viral doses. Obstensibly, at the lower infectious doses, some rats survive the acute febrile disease only to succumb to a second infection which ensues following invasion of the CNS by virus during the acute period of viremia. Rats which developed neurological signs died without exception. The GMTD was extended to $\geq$ 14 days. Signs were usually as unilateral or bilateral paraplegia with ascending paralysis, or in several instances, only the brain appeared infected as evidenced by torticollis and circling.

Gross lesions were recorded in rats in which postmorten changes were minor. Organs most consistently involved were lungs, thymus and liver (Table V). Although an effort was made to be objective, recognition of liver and lung lesions was difficult and, at best subjective.

TABLE V. ORGANS SHOWING CROSS LESIONS AFTER EXPOSURE OF WF, F-344, AND MAXX RATS TO INFECTIOUS AEROSOL OF RVFV, ZH-501 STRAIN

RAT STRAIN	NO. WITH LESIONS		
	Lung only	Lung + thymus	Lung, thymus + liver
WF	9	7	6
F-344	4		1
Maxx	2	3	2
Total	15	13	9

In an additional study, male WF rats (n=30), purchased from GIBCO, were infected via the respiratory route with graded doses of ZH-501 strain. The results shown in Table VI indicated the LD_{50} was 1.01 $\log_{10}$ PFU, a value similar to that obtained using WF procured from Microbiological Associates. Rats which survived were bled on day 18; the serum antibodies were assayed by the PRN technique.

TABLE VI. RESPONSE OF WF RATS, MALE, 13 WEEKS, TO INFECTIOUS AEROSOL OF RVFV, ZH-501 STRAIN (SOURCE: GIBCO) AND DAY 18 ANTIBODY TITERS OF SURVIVORS

VIRAL DOSE ($\log_{10}$ PFU)	DEAD/TOTAL	GMTD (range)	RECIPORAL	
			PRN_{50}	PRN_{80}
0.2	1/6	10.0	< 10-20	< 10
1.0	4/6	7.5 (6-11)	< 10	< 10
1.9	4/6	10.4 (7-13)	20	10
3.2	5/6	6.3 (5-10)	80	40
4.2	5/6	10.4 (4-16)	320	80

On postexposure days 1-4, rats exposed to 4.2 $\log_{10}$ PFU were bled for viremia studies. The blood virus level for each of the days was 5 PFU/ml. Although the number of animals is small, the PRN results provide baseline information concerning the minimum infectious dose and the relationships between the PRN_{50} and PRN_{80} values. Apparently, at a dose level of 4.2 $\log_{10}$ PFU, viremia does not develop until after day 4.

A small-scale study was performed to ascertain the aerosol infectivity of SA-51, SA-75, and Entebbe (ENT) strains for WF rats. Male rats, 13-14 weeks old, purchased from GIBCO, were exposed to graded doses of one of the virus strains. The death pattern, GMTD, and LD_{50} are shown in Table VII.

TABLE VII. RESPONSES OF WF RATS (SOURCE: GIBCO) TO INFECTIOUS AEROSOLS OF SA-51, SA-75, AND ENT STRAINS OF RVFV AND RECHALLENGE OF SELECTED SURVIVORS ON DAY 21 WITH $10^{3.4}$ PFU ZH-501 STRAIN

LOG_{10} DOSE	Dead	GMTD	RECHALLENGE OF SURVIVORS ON DAY 21 Dead	GMTD
Strain SA-51 (n=5)				
1.03	0	-		
2.04	1	16.0	3	3.3
3.33	5	10.2		
4.37	3/4	9.3	1	20.0
5.36	4	6.0		
LD_{50} = 2.08				
Strain SA-75 (n-5)				
1.02	0	-		
2.14	0	-		
3.24	1	16.0	1	14.0
4.29	2	9.5	0	-
5.42	5	10.0		
LD_{50} = 4.3				
Strain ENT (n=5)				
1.39	0	-		
2.45	0	-		
3.51	0	-	2	12.0
4.47	5	13.0		
5.39	5	6.6		
LD_{50} = 4.0				

This single experiment involving a relatively small number of animals requires cautious interpretation. It is clear that all 3 strains produced fatal infections. Less clear is the apparent greater virulence of the SA-51 strain with an LD_{50} of 2.8 PFU vs. an LD_{50} of $\sim$ 4.0 $\log_{10}$ PFU for SA-75 and Entebbe. The death pattern of SA-51 infected rats was skewed; additional testing should be performed to verify this observation.

Surviving rats from the preceeding study were rechallenged by the respiratory

route on day 21 with 3.4 $\log_{10}$ PFU, ZH-501 strain. Antibody levels prior to challenge were not determined. Mortality and GMTD results are also shown in Table VII and provide preliminary evidence of cross-protection, at least with SA-75 and ENT strains, against the AH-501 strain. The challenge dose of 3.4 $\log_{10}$ PFU represented approximately 150 rat respiratory LD_{50}, but killed only 25% of these previously challenged animals.

Results of these studies indicate that the WF rat is a suitable animal model to examine more critically the relationship of immune mechanisms to vaccine and therapeutic regimes in respiratory RVF. Studies are planned to esamine the immunogenicity of RVF vaccine. WF rats will be vaccinated with decreasing quantities of vaccine and challenged to determine an ED_{50} ag-inst an airborne challenge of 5,000 LD_{50} RVFV. Relationships between protection and antibody titer will also be examined. More definitive studies will follow with the objective of evaluating protection against a range of airborne exposure doses.

Publications: None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 0J6419	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	067
b.				
c.	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code) (U) Effect of Infection on Energy Metabolism, Muscle Enxymes and Host Immune Response in Relation to Physical Performance and Training

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical medicine; 004900 Defense; 012300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
79 01	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 80	1.5	81
	CURRENT 81	0	0

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21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Infectious disease; (U) Exercise; (U) Stress; (U) Physical performance; (U) Laboratory animals

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS

23 (U) Determine influence of: (a) infectious diseases on exercise capacity and biochemical variables related to performance; (b) exhaustive exercise prior to disease exposure; and (c) strenous exercise on the course of infection and on host cellular and humoral defense mechanisms. This research is essential for ascertaining a soldier's ability to perform physical work, assessing impact of physical conditioning and developing approaches for successful immunoprophylaxis during potential BW agent assaults.

24 (U) Infect rats with various organisms or toxins. Measure a variety of enzymes in thigh muscles, substrates and byproducts of energy metabolism and cellular and humoral immune responses in exercise- and disease-stressed rats.

25 (U) 79 10 - 80 09 - A rodent wheel-running apparatus was established as an excellent means of stressing animals by physical work intensities. A running speed of 16 m/min for 60 min (666 m total) was established as a prolonged submaximal exercise test. Infection-induced reduction in performance capacity and exercise-induced increases in disease-related mortalities was attributed to S. pneumoniae, F. tularensis, S. typhimurium and its endotoxin, and a sterile turpentine abscess. F. tularensis and the exercise model of wheel-running were utilized for studies on exercise/infection-induced alteration in energy metabolism and host immune responses. Increased energy substrate utilization during strenuous exercise with no discernible change in infection-related alterations in metabolic pathways occurred, with one exception, starvation-induced ketosis. Strenuous exercise also exhibited an immunosuppressive effect on both cellular and humoral responses. Challenge with SCHU S4 of LVS-immune animals postpones onset and time of death, and a 10% increase in mortality in the immune-conditioned rats.
Publications: Fed. Proc 39:344, 1980; Clin. Res. 28:643A (2X), 1980.
Terminated for management efficiency. Continued in W.U. S10 AQ 197. (DAOG1529)

*Available to contractors upon originator's approval

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

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BB: Prevention of Bacterial and Rickettsial Diseases of Potential BW Importance

Work Unit No. 871 BB 132: Effect of Infection on Energy Metabolism, Muscle Enzymes
(841 00 067) and Host Immune Response in Relation to Physical Performance and Training

Background:

The military has long recognized the value of physical fitness, and as a consequence has especially designed physical fitness programs to condition troops. Animal models capable of simulating "conditioning" are relatively simple to use since animals will run voluntarily or can be "trained" to run great distances for long periods of time. Some levels of physical exercise and conditioning have been observed to have beneficial effects (1). However, we recognize that the effects of physical conditioning on military personnel is only one aspect of military operations. The most important aspect is that related to physical performance capacity during "forced" exercise conditions either by design or emergency situations. Therefore, an animal model must be developed to simulate a "forced exercise" regime.

The detrimental effects of strenuous exercise during the course of acute febrile infections have been reported (2-4). Reduction in physical performance capacity, exercise-induced increases in infection-related mortalities, myalgia, decreased static muscle strength (isometric) and endurance are common observations. Our major research emphasis has been to examine the relationship between physical performance decrements and metabolic fuel, hormonal, and immune response alterations caused by exercising during the different stages of infectious illness cuased by Streptococcus pneumoniae, Salmonella typhimurium, Francisella tularensis, and VEE.

Progress:

Comparative studies were conducted this year to ascertain the effect of a 24-h fast and turpentine inflammation on physical performance capacity and biochemical variables using the 3 available exercise models (swimming, wheel-running, and treadmill running). The treadmill study was performed in collaboration with Dr. J. Bone and P. Deuster of the Department of Nutrition and Health, University of Maryland, College Park, MD, using the Quinton Model 42-15 rodent treadmill. All exercising rats were physically stressed to exhaustion. Swimming stress (3-h swim) and wheel-treadmill (timed running periods) results demonstrate a clear differentiation of response between swimming and running models; the most striking difference was in work intensity. Swimming is a mild submaximal exercise, wheel-running, moderately submaximal, and treadmill running (20 m/min speed with 15° incline), strenuous maximal. The metabolic responses were more drastically altered in the wheel-treadmill running groups, and therefore represent a more strenuous means of stressing animals using physical activity.

The next objective of this project was to construct a wheel-running apparatus. A direct-drive wheel-running apparatus was designed, modified and operationally tested in which 85-200-g Sprague-Dawley rats can be forced to run at varying speeds (0-27 m/min). The apparatus consists of 8 specially modified standard Wahmann activity cages to prevent injuries and provides a useful means of altering metabolic parameters related to performance capacity.

In order to establish a clearly defined running exercise stress test, Sprague-Dawley rats (200 g) were exercised in wheel-running apparatus at various speeds and times to establish varying work intensities (maximal, submaximal, and minimal tests). The standardized test we established for further infectious illness studies is a forced running exercise in a motor-driven wheel apparatus at a speed of 16 m/min for 60 min. The influence of 10^9 F. tularensis (LVS), 10^3 S. pneumoniae, 10^9 S. typhimurium and 2.5 mg S. typhimurium endotoxin on running performance and lethality of the various inflammatory stresses were studied. All three bacterial infections and administration of endotoxin cause: (a) progressive reduction in performance times during intermediate and late stages of the illnesses and (b) increased mortalities to all inflammatory stresses due to moderately severe exercise. In both variables tested, the differences between control and infected groups were greatest during the exhaustive bout of exercise on day 3.

Studies were also completed and established biochemical and functional responses (which are presumably highly correlated with performance capability) of the exercising-infected rats and their controls. During the 60-min submaximal exercise period, tissue glycogens progressively decreased, plasma glucose and free fatty acids increased to the same extent in both control and fasted-infected exercised rats. Blood lactate and pyruvate increased proportionately with time of exercise. Although infection caused decrease in plasma ketone concentrations, it was evident that there was a progressive increase in circulating ketone bodies in the infected-exercised rats.

A fall in plasma insulin and rise in plasma glucagon was observed in all control exercised rats, while the infected-exercised rats showed a progressive increase in both insulin and glucagon up to 30 min. The hormones exhibited a drastic drop at 40 min with a gradual recovery over the next 20 min. This fluctuation at 30 min is probably due to O_2 debt, lactate accumulation, and depleted tissue carbohydrate reserves. It is believed that the rat may respond by converting its metabolic systems to triglyceride and ketone body utilization to overcome these alterations.

Various studies in the literature have suggested that exercise had detrimental effects on the host defense mechanisms by suppressing cellular and humoral responses (5). Attempts to demonstrate exercise-induced and conditioning-complimented alterations in cell-mediated and humoral immunity have been initiated. Immune and non-immune, conditioned-exercised and nonconditioned-exercised rats were utilized.

A preliminary study indicates that basal counts of circulating white blood cells and especially lymphocytes are rapidly increased by physical activity. The reactivity of lymphocytes as measured by [^{14}C]thymidine incorporation using peripheral blood collected after exercised (60-min run at 20 m/min) from conditioned rats in response to concanavalin A (ConA) and phytohemagglutinin (PHA) were both impaired in the exercised groups. Lymphocyte stimulation to Foshay type tularemia antigen was also impaired in the immune-exercised group.

A challenge study with virulent 10^9 F. tularensis (SCHU S4) by Dr. Jemski (Aerobiology Division) showed that 87.5% of the nonimmune sedentary controls were dead at 48 h. The remaining 12.5% did not succumb to the disease during the 14-day study. Conditioning seemed to delay onset of illness, but afforded little or no protection. Mortalities in this nonimmune-conditioned group were 50% at 48 h, 75% at 72 h and 87.5% at 84 h, with 12.5% surviving challenge. Although immunity provided protection in the immune-sedentary group, one rat in the immune-conditioned group died on day 7.

The acute-phase protein, α_2-macrofetoprotein, showed a pronounced elevation in exercised-immune rats, while MA titers to F. tularensis were lower in the exercised-immune group compared to their controls.

A collaborative study was conducted by CPT McCarthy (Physical Sciences Division) to determine the effects of exercise on PMN chemiluminescence. Nonimmune and immune rats were inoculated with a dose of 10^7 LVS F. tularensis/100 g body weight. Luminol-assisted endogenous PMN CL was measured at 24 h after inoculation in all groups, plus a naive control. While previous studies using immune rats challenged with LVS exhibited resistance to infection (absences of fever response and high antibody titers), in this study both immune-conditioned exercised and nonexercised groups showed enhanced PMN CL to LVS compared to nonimmune rats. The immune-conditioned exercise group showed the greatest response of all groups (probably due to chronic exercise-induced suppression of antibodies and altered cell-mediated immunity). The nonimmune conditioned exercised animals showed drastic suppression of CL response to LVS compared to their sedentary controls.

Exercise seems to be immunosuppressive, but for only a finite time, followed by recovery. Conditioning, on the other hand, does not seem to have any discernible or beneficial effects on host immune response. Further studies are planned to clarify all aspects of these immune response studies.

Presentation:

Crawford, D. J., and P. Z. Sobocinski. Skeletal muscle and liver glycogen metabolism during bacterial infections and endotoxemia in rats. Presented, Annu. Mtg. FASEB, Anaheim, CA, 11-18 Apr 1980 (Fed. Proc. 39:344, 1980).

Publications:

1. Friman, G., and N. G. Ilbäck. 1980. Effects of physical exercise on course and complications of F. tularensis infection in the rat. Clin. Res. 28:643A.

2. Friman, G., and N. G. Ilbäck. 1980. Effects of bacterial infections on oxidative and glycolytic enzyme activity in red and white skeletal muscle in the rat. Clin. Res. 28:643A.

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2. Friman, G. 1976. Effects of acute infectious disease on human physical fitness and skeletal muscle. Uppsala: Acta Universitatis Upsaliensis No. 245.

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4. Alluisi, E. A., W. R. Beisel, B. B. Morgan, Jr., and L. S. Caldwell. 1980. Effects of sandfly fever on isometric muscular strength, endurance, and recovery. J. Motor. Behavior 12:1-11.

5. Reyes, M. P., and A. M. Lerner. 1976. Interferon and neutralizing antibody in sera of exercised mice with coxsackie B-3 virus myocarditis. Proc. Soc. Exp. Biol. Med. 151:333-338.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6420	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GROUP	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	068
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE: (U) Ebola virus infection: characterization of virologic, immunologic, and host-parasite relationships

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
79 02	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 80	1.0	191
FISCAL YEAR CURRENT 81	0	0

19. RESPONSIBLE DOD ORGANIZATION
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ASSOCIATE INVESTIGATORS
NAME: Moe, J. B.
NAME: Cole, Jr., F. E. POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS: (U) Military medicine; (U) BW defense; (U) Vaccines; (U) Laboratory animals; (U) Ebola virus

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS

23 (U) Develop a vaccine that is effective against Ebola virus (EBOV) infection. Characterize Ebola virus in terms of cell culture, animal infectivity and pathogenesis. Develop therapeutic measures for treatment of this highly infectious virus which produces high mortality in man, and could be a major problem for the military in Africa.
24 (U) Optimize cell culture technique for production of EBOV in certified cells. Evaluate inactivated vaccine for safety and efficacy. Attenuate virus for possible live virus vaccine. Establish animal models for determination of dose-response, pathogenesis, pathologic changes, therapeutic prevention or treatment, and immune response. Develop serologic assay system for diagnosis.
25 (U) 79 10 - 80 09 - Significant advances were achieved in EBOV research after about one year devoted to establishment of P-4 containment and preliminary investigations. Trials established that inactivated vaccines were immunogenic and protective in guinea pigs. This was particularly encouraging because this is the first vaccine against any virus of this type. Vaccine development is important because no known therapeutic regimen is available for EBOV infection. Convalescent serum from a Zaire-infected patient failed to protect cynomolgus monkeys. Monkeys treated with ribavirin lived 3.5 days longer than control monkeys. Lethality of virus infection was related to route of inoculation; SC inoculation may enable development of a less lethal and more realistic model for therapy studies. A plaque assay technique was developed in Vero cells. Preliminary data implied feasibility of developing a plaque-reduction neutralization test. Some serologic test is a prerequisite to vaccine control of EBO disease.
Publication: Milit. Med. 145; in press, 1980.
Terminated for management efficiency. Continued in W.U. 871 BC 148. (DAOG1537)

* Available to contractors upon originator's approval.

DD, FORM 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.

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BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BC: Prevention of Viral Diseases of Potential BW Importance

Work Unit No. 871 BC 141: Ebola Virus Infection: Characterization of Virologic,
(841 00 068) Immunologic, and Host Parasite Relationships.

Background:

In 1976, outbreaks of Ebola hemorrhagic fever (EBO) occurred in Southern Sudan and Northern Zaire. A second outbreak of EBO occurred in Sudan during 1979. The illness was characterized by prostration, fever, headache, myalgia, arthralgia, abdominal pain and high mortality. The high mortality rate of EHF has been associated, at least in part, with the quality of medical care in these underdeveloped countries, where relatively primitive medical care has contributed, in part, to patient-to-patient transmission of disease. Furthermore, lack of proven prophylactic or therapeutic measures for EBO has limited medical care to supportive therapy combined with isolation of patients and barrier nursing techniques. Successful treatment of a British scientist, who suffered a laboratory infection with EBOV included treatment with immune plasma and interferon. Studies at USAMRIID have been directed toward development of an effective vaccine. Concomitant studies have been directed toward establishment of laboratory animal models, development of *in vitro* diagnostic tests, evaluation of treatment regimens and determination of effectiveness of disinfectants.

Progress:

Inactivated Vaccine Trial: A preliminary vaccine trial was conducted in guinea pigs using vaccines produced by heat- and formalin-inactivated Zaire strain EBOV (titer 1.7×10^5 PFU/ml). Groups of 7 guinea pigs each were inoculated IM as follows: (a) one dose of formalin-inactivated vaccine (FIV), (b) one dose FIV + lipid emulsion (LE) adjuvant, (c) 2 doses FIV, (d) 2 doses FIV + LE, (e) one dose heat-inactivated vaccine (HIV), (f) one dose HIV plus LE, (g) 2 doses of HIV, (h) 2 doses of HIV + LE, (i) 2 doses heat-inactivated cell controls (HICC), (j) 2 doses HICC + LE, (k) 2 doses formalin-inactivated cell controls (FICC), and (l) 2 doses FICC + LE. Guinea pigs that received 2 doses of vaccine or cell control were inoculated on day -21; all guinea pigs were inoculated/reinoculated on day 0 and challenged with 4.0 $\log_{10}$ PFU of Zaire strain EBOV by IP injection on day +21. Each vaccine dose consisted of 1 ml of inactivated virus; vaccine was combined with an equal volume of adjuvant as indicated. Results are detailed in Table I. The protection demonstrated in guinea pigs is very encouraging and indicates that an inactivated vaccine has potential efficacy. Improved ability to culture EBOV in tissue culture (titers of 8 - 10 $\log_{10}$ PFU/ml) further indicate the feasibility of developing an inactivated vaccine.

In vivo Effect of Antiviral Substances Against EBO. Experimental therapy of EBO infection in cynomolgus monkeys was investigated using convalescent human serum (from a patient that survived infection with Zaire strain) and ribavirin. Experimental design and results are detailed in Table II. Results confirmed reports by British workers that passively transferred antibodies did not alter the course of disease. Available EBO antiserum had a low specific antibody titer (1:20 by IFA test) which

may have accounted for the lack of effect. Ribavirin therapy, consisting of a loading dose of 50 mg/kg and then 20 mg/kg of ribavirin twice daily, increased survival of monkeys by 3.5 days. However, the monkey that survived the longest (11 days) received virus by SC inoculation. Detectable specific antibodies were present in monkeys at 7 days.

TABLE I. RESULTS OF TRAIL USING FORMALIN- AND HEAT-INACTIVATED EBO VACCINE IN GUINEA PIGS

GROUP	VACCINE & DOSE	NO. FEBRILE/7	MEAN TIME TO FEVER ± SD	NO. DEATHS/7	MEAN DAYS TO DEATH ± SD
Formalin-Inactivated Vaccine					
a	FIV	1	7.0	0	-
b	FIV + LE	0	-	0	-
c	FIV-2	0	-	0	-
d	FIV + LE-2	1	11.0	1	14.0
	Total	2/28*	9.0	1/28**	14.0
Heat-Inactivated Vaccine					
e	HIV	3	5.7 ± 0.5	1	-
f	HIV + LE	3	6.0 ± 0.8	3	10.7 ± 0.5
g	HIV-2	1	6.0	0	-
h	HIV + LE-2	1	8.0	2	11.0
	Total	8/28*	6.1	5/28	10.8
Cell Control					
i	HICC-2	6	5.2 ± 0.4	1	10.0
j	HICC + LE-2	6	5.2 ± 1.1	3	10.3 ± 1.2
k	FICC-2	7	5.4 ± 0.5	3	11.7 ± 0.9
l	FICC + LE-2	7	6.0 ± 1.1	3	11.0 ± 1.6
	Total	26/28	6.5 ± 1.0	10	10.9 ± 1.4

*$P < 0.00001$ and **$P = 0.003$, compared to controls (Fisher's exact test).

Effect of Route of Inoculation on EBO in Guinea Pigs. The observation that the route of inoculation affected the time course of EBO in monkeys was evaluated in guinea pigs. Groups of 4 guinea pigs each were inoculated IP or SC with 4.0 $\log_{10}$ PFU of Sudan or Zaire strains. Results (Table III) indicated that EBO was lethal when administered IP and nonlethal SC. This phenomenon will be investigated further because of the significance of being able to establish an infection in monkeys that is not overwhelming and invariably fatal. Infection of monkeys with Zaire strain has resulted invariably in death regardless of the dose administered. This high lethality may have prevented investigators from prolonging life with therapeutic measures. Monkeys, in this respect, are not ideal models for the study of the human disease, in which survival is reportedly as high as 50%.

TABLE II. THERAPY OF EBO IN CYNOMOLGUS MONKEYS (n = 2/GROUP) CHALLENGED WITH 60 PFU ZAIRE STRAIN ($718V_5$)

GROUP	ROUTE	TREATMENT[a]	DAYS TO DEATH
1	IP	20 ml serum (Zaire) IV	5, 6
2	IP	Ribavirin - 50 mg/kg leading + 20 mg/kg, BID, IM	8.5, 9.5[b]
3	IP	None	5, 6
4	SC	None	5, 11

[a]Given 4 hours postinoculation.
[b]Pulmonary hemorrhage and other evidence of disseminated intravascular coagulation.

TABLE III. EFFECT OF ROUTE OF INOCULATION ON EBOV OF GUINEA PIGS

INOCULUM	ROUTE	NO. FEBRILE/ TOTAL	MEAN DAYS TO FEVER	NO. DEATHS/ TOTAL	MEAN DAY TO DEATH
Zaire	IP	3/4	4.7	3/4	8.7
	SC	4/4	5.7	0/4	-
Sudan	IP	4/4	5.5	3/4	10.7
	SC	3/3	5.3	0/3	-

Development of Plaque Assay Technique. Numerous cell lines were evaluated as suitable substrates for plaque assay of EBOV. Vero cells were the only cells examined in which plaques were formed by both Sudan and Zaire strains. Plaques were produced using a double-overlay of agarose and neutral red dye or by using a single-overlay of agarose and staining cells with crystal violet dye. It was necessary to incorporate 4 or 8% fetal bovine sera in the agarose overlay to maintain cell viability during the required 9-day incubation period. Pinpoint plaques appeared after 5 days incubation and plaques increased in size to 2- to 5-mm on days 6 and 7. Plaques produced by Zaire and Sudan strains of EBO were consistently different in appearance. Plaques produced by Zaire strain were "punched out," whereas, Sudan strain plaques consistently had cells remaining within a plaque. The potential application of the PRN procedure was demonstrated in preliminary tests. Further development of this procedure and comparison with the IFA technique is required before the full potential of the test can be stated.

Inactivation of EBOV. The radiation inactivation rates for Sudan and Zaire strains were determined to be linear with 1.0 $\log_{10}$ PFU/ml reduction in titer after 8 min exposure to 2.8×10^4 R/min. In a separate trial, 5.0 $\log_{10}$ PFU/ml of virus was reduced below detectable levels (0.7 $\log_{10}$ PFU/ml) after 30 min exposure to 3.0×10^3 R/min. The inactivation of EBOV-infected specimens with ^{60}Co irradiation has numerous potential applications. Irradiated spot-slides for IFA examination were subjectively evaluated to be superior to spot-slides inactivated by UV irradiation, with an elimination of false positive tests.

TABLE IV. IN VITRO ANTIVIRAL EFFECT OF RIBAVIRIN AGAINST EBOV

CELL LINE	CONCENTRATION OF RIBAVIRIN (μg/ml)	VIRAL TITER (LOG_{10} PUF/ML) ON DAY AFTER INOCULATION[a]									
		0	1	2	3	4	5	6	7	8	9
Vero	0	<0.7	<0.7	1.7	3.3	4.8	4.9	8.0	8.5	8.3	7.7
	10	<0.7	<0.7	2.1	3.9	4.9	4.9	8.1	8.4	8.0	8.0
	100	<0.7	<0.7	1.2	2.7	3.8	4.2	5.8	6.7	7.8	8.1
	500	<0.7	<0.7	1.7	3.2	4.9	5.7	6.8	6.8	6.8	6.6
MRC-5	0	<0.7	<0.7	<0.7	2.5	3.8	4.9	5.7	5.9	8.6	8.0
	10[b]	<0.7	<0.7	<0.7	2.4	3.9	4.7	5.8	6.8	8.1	8.1
	100[b]	<0.7	<0.7	1.2	2.1	2.5	–[c]	–	–	–	–
	500[b]	<0.7	<0.7	<0.7	<0.7	1.5	2.0	2.2	2.5	2.7	2.2
FRhL	0[b]	<0.7	<0.7	<0.7	2.7	3.5	4.9	5.8	6.8	7.8	6.7
	10[b]	<0.7	<0.7	1.9	3.0	3.6	4.6	4.9	–	–	–
	100[b]	<0.7	<0.7	<0.7	1.7	<0.7	–	–	–	–	–
	500[b]	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7

[a] Inoculated with 3.0 log_{10} PFU/flask

[b] Cellular toxicity

[c] Cell monolayer detached

Inactivation of EBOV with a variety of chemical disinfectants produced equivocal results. However, it was determined that working concentrations and 1:10 dilutions of working concentrations of Lysol and Roccal were consistently effective. In contrast, quaternary ammonium compounds were unsatisfactory.

In Vitro Effect of Antiviral Substances Against EBOV. The in vitro effect of ribavirin was determined by PR in Vero cells and by viral yield-reduction in Vero, MRC-5, and FRhL cells. A nonspecific enhancement of plaque formation occurred when concentrations of 10 - 500 ug/ml of ribavirin were added to the overlay medium. Samples treated with ribavirin had a 2-fold increase in plaque numbers compared to untreated controls. The mechanism of this action is unknown. This apparent increase in plaque efficiency made the plaque reduction assay uninterpretable.

Similarly, the effect of ribavirin on yield-reduction of EBOV in vitro was difficult to interpret because of cytotoxicity observed in MRC-5 and FRhL cells (Table IV). Replication to maximal titers was delayed in Vero cells with 100 ug/ml of ribavirin, and 500 ug/ml of ribavirin reduced peak titers by $\sim 2.0 \log_{10}$ PFU/ml. Ribavirin was toxic to FRhL cells in concentrations of 10, 100 and 500 ug/ml. The toxic effect was characterized by rounding of cells, increased cellular granularity, and loss of cellular attachment. FRhL and MRC-5 cells treated with 500 ug/ml of ribavirin exhibited an initial toxic effect (rounding of cells), then recovered and remained viable throughout the experiment; these cultures yielded very low titers. The toxic effect was not evident until days 5-7 at concentrations of 10 and 100ug/ml of ribavirin, at which time the cells became detached. Additional study will have to be conducted to determine if ribavirin is effective in vitro against EBOV.

Presentations:

1. Lupton, H. W. and D. E. Reed. Evaluation of experimental subunit vaccines for infectious bovine rhinotracheitis. Presented, Annu. Meet. Conference of Research Workers in Animal Diseases. 26 Nov 1979, Chicago, IL.

2. Moe, J. B. Fetal-maternal interactions during intrauterine infection with SV-20. Presented, Annu. Meet. Am. Coll. Vet. Pathol. Dec 1979, Denver, CO.

Publications:

Moe, J. B., and C. E. Pedersen, Jr. 1980. The impact of rickettsial diseases on military operations. Milit. Med. 45: in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G2229	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADE	8a. GROUP/SUBJ	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 12 07	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	069
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Procedure and Security Classification Code)

(U) Growth hormone and infection

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. PERFORMING AGENCY	16. PERFORMANCE METHOD
79 12	80 09	DA	C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR 80	1.0	86
FISCAL YEAR 81	0	0

19a. RESPONSIBLE DOD ORGANIZATION

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20. GENERAL USE

Foreign intelligence considered

19b. PERFORMING ORGANIZATION

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NAME: Hauer, E. C. POC:DA

21. KEYWORDS (Provide KEY WORD and Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Metabolism; (U) Growth hormone; (U) Infectious diseases

22. TECHNICAL OBJECTIVE, 23. APPROACH, 24. PROGRESS (Provide brief individual paragraphs identified by number. Precede each with appropriate Security Classification Code.)

23 (U) Measure and correlate growth hormone (GH) release with other metabolic parameters in laboratory animals and man during infections and mild hyperthermia. GH may play a major role in maintaining defense barriers, improving immune responses and aiding in healing and recovery from infection of military importance.

24 (U) Develop rat and monkey models to measure continuously GH and other parameters during various infections and hyperthermia. Conduct studies in man using sandfly fever, malaria and mild hyperthermia.

25 (U) 79 12 - 80 09 - A rat GH assay was established; rats have been shown to have clear-cut lowering of GH after fasting, infection with or without fasting, and after exercise even without infection. A tentative trial of human GH in rats seemed to improve survival with a probability value of 0.06; a repeat study did not verify this using rat GH.

Considerable progress has been made in the use of the jacketed monkey model and at this time an attempt is being made to use a catheter placed in the right atrium with no arterial catheter as a number of problems with thrombotic disease resulted from the arterial catheter. Data are now being collected in the basal monkey as well as after infection and endotoxin. GH is released in approximately 3-h cycles with little difference between day and night except after feeding in the morning. This is in distinction from man who has substantial nighttime release with much less release during the daytime hours. No striking differences have actually been noticed in the GH levels in monkeys in spite of prior reports. Local approval of 2 human research protocols with sandfly fever and artificial hyperthermia was completed in Mar 1980. Final review is scheduled for August; after approval, studies should be initiated promptly. Terminated for management efficiency. Continued in W.U. S10 AQ 197.(DAOG1529)

* Available to contractors upon originator's approval

DD FORM 1498 (1 MAR 80) PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE AND 1498-1, 1 MAR 80 (FOR ARMY USE) ARE OBSOLETE

NOV 80

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BB: Prevention of Bacterial and Rickettsial Diseases of Potential BW Importance

Work Unit No. 871 BB 133: Growth Hormone and Infection
(841 00 069)

Background:

The main thrust has been directed toward evaluation of the role of growth hormone (GH) in infection utilizing rat and monkey models in addition to clinical research studies. The latter are to use subjects infected with sandfly fever and subjects whose body temperatures are artificially elevated. The importance of assessing growth hormone's role stemmed primarily from the fact that it is the single most important anabolic hormone of potential therapeutic importance. Only testosterone compares in the magnitude of the anabolic effect. Combining this with the fact that nitrogen loss and muscle wasting are major contributors not only to disability but to death in a number of infections, and secondly, that changes in GH have been described in infection in man as well as certain animal models all combined to reinforce the importance of assessing the role of GH. GH has also been shown to have an impact on the immune system in specific situations.

Progress:

A monkey and human GH assay was established early in 1980. It is solid-phase and made commercially (PRIST).

Chaired monkeys were studied; major problems in inability to allow self-feeding and continued agitation led us to change over to the jacketed model being used by Dr. Wannemacher and Mr. Dinterman. Over 20 jacketed monkeys have been studied in the basal state, after endogenous pyrogen, endotoxin, and Streptococcus pneumoniae. Problems included bleeding postoperatively and strokes almost certainly related to use of carotid artery catheters. Changing to right atrial catheters seems to have eliminated strokes; use of heparin infusion rather than bolus has probably stopped the problem of postoperative bleeding.

Twenty-four-hour basal studies were done, sampling blood hourly as well as checking temperatures. Glucose, 8-hydroxybutyrate, and free fatty acids (FFA) were also measured. The 24-h results show that sleep-released GH is not prominent in the monkey, but that 3-4 h cyclic release is. Temperatures seem to cycle directly with light (higher) and dark (lower) cycles. Ketone bodies begin to rise about 10 h after feeding stops. Interestingly, glucose levels are not higher during the feeding period.

After fasting from 1600 the prior day, 8-h studies were done from 0800 to 1600 with hourly temperatures and blood samples. Eight-hour fasting studies showed similar cyclic patterns as in the 24-h study except for loss of a

mid-morning peak in GH. After S. pneumoniae infection was established, 8-h studies showed mild increase in body temperature, no change in GH, glucose and a mild decrease in ketogenesis. After endotoxin, GH, glucose, ketone formation and insulin increased.

Jugular vein-right atrial catheterization was performed successfully in a small group of rats with silastic and polyethylene tubing. Sampling in some could be done for 3-4 days. Jackets and protective cabling were not helpful and use of a stylus in the catheter between periods of sampling seemed to maintain patency. However, longer periods for surgical recovery will be required.

GH values were determined in fed and fasted rats with and without S. pneumoniae infection and showed that either fasting or infection markedly lowered the GH. Two survival studies were done giving GH to normal rats infected with S. pneumoniae; results were equivocal and must be repeated.

Coliaborative Studies: (Work Unit S10 AQ 176): Hypophysectomized rats were given thyroxine and cortisone acetate with or without GH. Hypophysectomized rats did not abolish ketones with infection but all other groups did. Further studies are needed. (Work Unit 871 BB 132): Exercised fed rats showed a marked decline in GH within 10 min. This needs to be repeated.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G2700	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. MSTR INSTR'N	8A. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
80 01 09	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	070
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE: (U) Primary evaluation of drugs against viruses of military significance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
80 01	81 09	DA			C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR 80 (DIRECT)	1.0	103
FISCAL YEAR 81 (CURRENT)	0	0

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ASSOCIATE INVESTIGATORS
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NAME: Wannarka, G.L.

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Provide KWOC with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Chemotherapy; (U) Antivirals; (U) Tissue culture; (U) Laboratory rodents.

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

23 (U) Evaluate and screen new antiviral compounds in rodents and tissue culture for prophylaxis and treatment effects in virus infections caused by viruses of military and strategic importance, prior to testing successful compounds in primate models.
24 (U) In model systems screen drugs: (a) with known in vitro antiviral action; (b) close analogs of drugs shown to be active; and (c) new compounds synthesized specifically as antiviral drugs.
25 (U) 80 01 - 80 09 - 36 ribavirin analogs and other contract antiviral drugs were screened and evaluated: in vitro tests were done using RVF, VEE, PIC, SFS, and YF viruses. Evaluations were extended in mice with RVF and VEE viruses in vivo in those instances where sufficient quantities of the compounds were available. In the in vitro tests, while none of the analog compounds were more effective than ribavirin, most had some detectable antiviral activity against one or more of the viruses. Two compounds, BJ-22483 and RA-83, were nearly as effective as ribavirin in vivo. These compounds and BJ-45520, BJ-45529 and BJ-51813 analogs warrant further evaluation since the latter appeared to extend the time to death in RVF virus-challenged mice. Tunicamycin (gift from SKI) was effective against all of the test viruses in vitro at levels from 0.1 to 1.0 microgram/ml. A bonnet macaque study to determine if SFS virus would form a cross-reacting antibody for RVF virus in these monkeys did not indicate the development of this type of antibody. Preliminary collaborative studies to explain the in vitro uptake and metabolism of ribavirin by rat and human red blood cells have been initiated
Terminated for management efficiency. Continued in W.U. 871 BE 1 46. (DAOG3815)

* Available to contractors upon government 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

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BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BE: Exploratory Antiviral Drug Development

Work Unit No. 871 BE 148: Potential Antiviral Drugs for Treatment of Virus
(841 00 070) Infection of Possible BW Significance

Background:

Vaccine prophlaxis is not effective for the prevention of all virus-induced disease. Vaccines, also, are usually virus-specific and ineffective after the onset of infection. Chemotherapeutic agents are critically needed for the prevention or treatment of virus-induced diseases.

The in vitro antiviral activity of ribavirin has been defined against Machupo, VEE, YF, RVF, Pichinde, (PIC) and Sandfly fever (Sicilian) (SFS) viruses. The in vivo antiviral activity of this drug has also been shown against RVF and VEE viruses.

A program was established in January 1980 for the primary evaluation of ribavirin analogs and other promising antiviral drugs. This program evaluates drugs in vitro and in those instances where sufficient quantites of the drugs are available, in vivo. Ribavirin is used as the control drug in all evaluations.

Drugs which show activity comparable to ribavirin will be evaluated in depth in more elaborate studies.

Progress:

Ribavirin analogs and other antiviral drugs were screened and evaluated in vitro using RVF, VEE, PIC, SFS, and YF viruses. In vivo studies were done in mice using RVF and VEE viruses. Results of the evaluations are shown in Table I.

The following compounds were found to be inactive against all of the test viruses: BJ-29928, BJ-29937, BJ-33879, BJ-33860, and BJ-42332.

While none of the analogs appeared to be as effective or more effective in vitro than ribavirin, most of the derivatives had detectable antiviral activity. Three of the compounds, BJ-51813, BJ-22483, and RA-84, were nearly as effective as ribavirin in vitro and in vivo; however, with BJ-22483 and RA-84, skin lesions developed at the site of injection (100 mg/kg) after day 3 of treatment. These lesions were probably due to the low pH of the drug solutions. Tunicamycin (SRI) was very effective against all of the test viruses in vitro, but very toxic to tissue cultures above 5 ug/ml. Several analogs, particularly BJ-45539 and BJ-45548, while not as effective as ribavirin in vivo appeared to extend time-to-death. Further evaluation studies will be performed with those compounds, perhaps with a multiple treatment schedule, when additional drug is procured. Most of the drugs have not been evaluated in vitro against SFS virus because of the non-availability of the SW-13 cell line. However, the virus has recently been adapted to plaque on Vero cells. The drugs will be screened using this virus stock.

TABLE I. IN VITRO EVALUATION OF RIBAVIRIN ANALOGS AND OTHER POTENTIAL ANTIVIRAL DRUGS AGAINST VARIOUS VIRUSES.

COMPOUND	CONTRACT NUMBER	RESPONSE OF VIRUS				
		PIC	RVF	SFS	YF	VEE
BJ22483*	(RA83)	+[a]	+	++	++	+
BJ29893*	(RA98)	-	-	++	+	-
BJ29900*	(RB122)	-	-	+	-	-
BJ29919	(RB124)	-	-	+	-	-
	(RB125)	+	-	+	+	-
BJ33888	(RK15)	+	-	-	+	-
BJ29946	(RH49)	-	-	+	-	-
BJ42341*	(RI5iA)	++	+++	+++	+++	-
	(RA84)	+++	++	+++	+++	++
BJ22492	(RA90)	++	+	++	++	-
BJ3385i	(RA99)	+	++		+	-
BJ42350	(RA105)	-	++	-	-	-
BJ45520*	(RA116)	++	+	+++	+	+
BJ45539*	(RA119)	+++	++	+++	++	+
BJ45548*	(RA121)	+++	+	++	+	+
BJ45557*	(RA125)	-	+	+	+	+
BJ45511*	(RA114)	+++	++	++	+	+
BJ45502*	(RP14)	+[b]	+[b]	-	+[b]	+
BJ46107	(DSC-IV-76)	++	+	-	+	+
BJ46090	(DSC-IV-75B)	+[b]	-	-	-	-
BJ46081	(DSC-IV-51)	++	++	+	+	-
BJ46072	(DSC-IV-46)	++	++	+	-	-
BJ51662*	(KW-II-17)	+	-	-	-	-
BJ51153*	(KW-I-300)	-	-	+	+	+
BJ51813*	(RA111)	+++[c]	++[c]	+++	+++[c]	++[c]
BJ51822*	(RP52)	-	+	+	-	-
BJ51831*	(RV8)	+	++	-	+	+
Sinefungin [d]		++	+	+	-	++
Tunicamycin	(NSC-i77382)	++++	+	+++	++	++
LY122722		++[b]	++[b]	-[b]	-[b]	+[b]
AR30613		++	++	++	+	++
Ribavirin		+++	++	+++	+++	+

a + 10-30% reduction, highest drug concentration (250-500 µg/ml)
++ 31-60% reduction, middle drug concetration (100-250 µg/ml)
+++ 80-90% reduction, lowest drug concentration (10-25 µg/ml)

b Toxic at 500 µg/ml

c Toxic at 100 µg/ml

d + 10-30% reduction (1.0 µg/ml)
++ 31-60% reduction (0.5-1.0 µg/ml)
+++ 61-80% reduction (0.25-0.5 µg/ml)
++++ 80-90% reduction (0.1-0.25 µg/ml)

* Indicates compounds in vivo.

Yield reduction tests were performed to determine if RVF and VEE viruses developed a resistance to ribavirin if they were repeatedly grown in the presence of the drug. Initial test results showed that RVF virus did not develop any decrease in sensitivity to the drug after 6 passages in the presence of ribavirin, but VEE virus may have. Subsequent tests, however, revealed that no significant resistance of the VEE virus had occurred.

Previous studies to evaluate the in vitro activity of ribavirin using a continuous line of macrophage (BWJM) cells showed that the drug was effective against RVF and VEE viruses. A yield reduction test done with VSV in this cell line indicated that 100 µg/ml of ribavirin significantly affected the growth of the virus. Drug concentration in the 25-µg/ml range also showed some effect on the growth rate and total yield of this virus.

In an earlier bonnet macaque study, in which the monkeys were challenged with SFS and RVF virus, no viremia was detected, suggesting the formation of a cross-reacting antibody, since those monkeys normally develop viremias when challenged with RVF virus.

An experiment was designed to check for the formation of cross-reacting antibody, between RVF and SFS. Four bonnet monkeys were inoculated with SFS virus (same virus inoculum used in the human volunteer test in June 1979). These 4 and 4 additional monkeys were subsequently challenged with RVF virus after 42 days. The results of this study did not indicate the presence of a cross-reacting antibody in the serum of the original animals. Both monkey groups showed comparable RVF viremias during the 25-day period following the RVF challenge. Neutralizing antibody tests showed weak SFS antibody titers (1:10-1:40) in the original group inoculated with this virus. All monkeys developed high RVF virus antibody titers at 14 days with this virus, which persisted through the second 42-day holding period. SFS viremias were not determined, because the SW-13 tissue culture cell line was unavailable. This is the only cell line which will plaque SFS virus directly from human or monkey serum.

Preliminary collaborative studies with Dr. Canonico to explore the in vitro uptake and metabolism of ribavirin by rat and human RBC, showed that rat RBC reach a steady concentration of ribavirin within 24 h when exposed to 500 µg/ml of the drug.

The rate of metabolism after equilibration does not appear to be affected by the concentration of ribavirin within the physiologically achievable range. Human RBC metabolize ribavirin at a slower rate than rat cells. In addition, rat cells apparently metabolize ribavirin to mono- and diphosphorylated ribavirin and not to the triphosphate, whereas, human red cells metabolize ribavirin rapidly to the triphospnate, so that mono- and diphosphate derivatives are undetectable.

Approximately one month of laboratory work was lost during this report period because tissue culture trays were not available for the Vero and SW-13 cell lines. Alternate cell lines were of no value for most of the viruses used in the in vitro screening tests.

Publications:

None

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G2596	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. ENTER HISTRY	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
80 02 26	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	071
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE: (U) Development of effective countermeasures against poisoning with microbial toxins of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
80 02	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
80 PREVIOUS	1.0	49
81 CURRENT	0	0

19a. RESPONSIBLE DOD ORGANIZATION
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RESPONSIBLE INDIVIDUAL
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20. GENERAL USE
Foreign intelligence considered

19b. PERFORMING ORGANIZATION
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TELEPHONE: 301 663-7211
SOCIAL SECURITY ACCOUNT NUMBER:

OTHER INVESTIGATORS
NAME: Siegel, L. S. POC:DA

21. KEYWORDS (Provide KEY with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Microbial toxins; (U) Toxoids; (U) Therapy; (U) Prophylaxis; (U) Botulism

22. TECHNICAL OBJECTIVE, 23. APPROACH, 24. PROGRESS (Provide brief individual paragraphs identified by number. Precede text in each with Security Classification Code.)

23 (U) Develop and evaluate biologics suitable against microbial toxicities. Develop materials and methods for detection and assay of microbial toxins of military significance. For several toxins of military importance there are only cumbersome and slow animal test systems for identification and assay. To identify botulinum (BOT) toxin, for example, a mouse protection test is the most sensitive system available, but it requires 2-3 days to perform, and is much too slow to allow optimal management of a military casualty from this BW threat agent.

24 (U) Initial work will be with BOT toxin (types A-G) and suitable toxoids. ELISA techniques will be employed to improve detection and quantitation of the toxin. Collection and testing of botulism immune sera will continue.

25 (U) 80 02 - 80 09 - Immunogenicity and reactogenicity of 2 new BOT toxoids were evaluated. A rapid technique for detection of BOT was refined to identify type A toxin in various fluids. Human BOT antitoxins were evaluated and the efficiency of a chemotherapeutic agent for the treatment of botulism was determined. The immune response elicited in volunteers by 2 new pentavalent botulinum toxoids to type B botulinum toxin was significantly greater than was the response in volunteers to the currently used pentavalent toxoid. Nanogram amounts (20 MIPLD-50) of type A BOT toxin were detected in tap water, milk and urine by a rapid ELISA technique. Half-lives for the neutralizing activity of infused Botulism Immune Plasma (Human) were determined from 17-49 days in volunteers. Pilot lots of human botulism immune globulin prepared by ion exchange chromatography and silicon dioxide methodology contained less extraneous proteins than a similar ethanol fractionated product. 3,4- Diaminopyridine prolonged survival of mice poisoned with a lethal dose of type A BOT toxin.

Publications: In Natural Toxins, in press, 1980; Proc., ICAC Mtg.

Terminated for management efficiency. Continued in W.U. 871 BA 150. (DAOG3810)

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

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BA: Prevention of BW Diseases Caused by Microbial Toxins

Work Unit No. 871 BA 123: Development of Effective Countermeasures Against
(871 00 071) Poisoning with Microbial Toxins of Military Importance

Background:

The lethality, stability, and availability of the crude botulinum toxins qualify them as outstanding threats for use against us as overt, covert, terrorist, and/or biological warfare agents. The production and evaluation of new botulinum toxoids, the rapid detection of botulinum toxins, and the successful treatment of personnel poisoned with these toxins present many problems. Toxoids are available to only 5 (A-E) of the 7 (A-G) immunologically distinct types of botulinum toxin. Rapid detection of toxin and rapid diagnosis of poisoning are essential to the implementation of specific prophylactic and therapeutic countermeasures. Equine antitoxins currently available for the treatment of botulism induce adverse reactions in 21% of recipients. These antitoxins neutralize free toxin, but are unable to reverse the toxin-induced blockage of acetylcholine release that occurs within poisoned nerves. Certain chemicals, such as the aminopyridines, greatly enhance acetylcholine release from botulinum-poisoned nerve terminals, and thus may be useful in reversing the clinical signs of botulism.

Much of the work reported herein was initiated by this investigator during the past 2-1/2 years under USAMRIID Work Unit 871 BA 121 (old no. 841 00 020) and 871 BD 143 (old no. 841-00-001).

Progress:

Toxoid evaluation: A study entitled "Evaluation of the Human Response to the Administration of Botulinum Toxoid, Adsorbed, Pentavalent (ABCDE), MDPH IND 161", which was designed to determine in volunteers the immunogenicity and reactogenicity of 2 new [Michigan Department of Public Health (MDPH)] investigational lots (A-2 and B-1) of botulism toxoid, and to compare the immunogenicity and reactogenicity of these new lots to that elicited by the currently used investigational Parke Davis pentavalent (ABCDE) botulinum toxoid (BB-IND 161), was completed. Fifty-two volunteers consisting of professional staff, medical research volunteer subjects, and at-risk laboratory workers participated in the study.

There was no significant difference in the incidence of none, mild, or moderate local reactions in volunteers immunized with either of the 2 MDPH toxoids. However, the incidence (A-2, 17.9%; B-1, 15.2%) of moderate reactions in volunteers immunized with either of the MDPH toxoids was greater than occured (2.8%) in those volunteers immunized with the Parke Davis toxoid. No systemic reactions occurred in any volunteers. The lack of difference in reactogenicity between the MDPH lots is notable in that MDPH Lot A-2 contains considerably less formalin, 0.022%, than does MDPH Lot B-1, 0.039%, and had been anticipated to be less reactogenic locally.

International units of neutralizing antibody to 3 (ABE) botulinum toxins were determined for sera collected 14 days after the third immunization. There were no statistically significant differences between the immune response elicited against type A toxin in volunteers immunized with either of the MDPH toxoids and those immunized with the Parke Davis toxoid.

The immune response elicited in volunteers by both the MDPH Lot A-2 and MDPH Lot B-1 to type B toxin was significantly ($P < .01$) greater than the response elicited in volunteers immunized with the Parke Davis toxoid. There was no significant difference demonstrated between the immunogenicity of the 2 MDPH toxoid lots for type A, B, or E toxin.

There were no significant differences among the immune responses elicited to type E toxin in volunteers immunized with any of the 3 toxoids. Two thousand five hundred vials (10 doses/vial) of MDPH Lot A-2 and 1,750 vials of MDPH Lot B-1 are now stockpiled at USAMRIID.

Under the auspices of two new human-use protocols entitled "Evaluation of Immunologic Response to Booster Administration of Botulinum Toxoid Adsorbed Monovalent (B) Lot 91" and an addendum to "Evaluation of Immunologic Response to Booster Administration of Botulinum Toxoid, Adsorbed, Pentavalent (ABCDE) (IND-161)", the human testing of a new type B botulinum toxoid was begun.

Toxin detection: An enzyme-linked immunosorbent assay (ELISA) using the "double-sandwich" technique was utilized to detect and quantitate both crude and partially purified type A botulinum toxin added to chlorinated tap water, pasteurized whole milk, and human urine. The technique employed the protein A IgG fraction of rabbit antiserum against purified (150,000 MW) type A neurotoxin, Cohn-method fractionated human immune globulin to types A, B, C, D, and E botulinum toxins and alkaline phosphatase-conjugated, rabbit antihuman IgG. As few as 20 $MIPLD_{50}$ of toxin were detected and quantitated within 5 h of receipt of samples. The technique is specific and provides a rapid means of detecting and quantitating nanogram amounts of type A botulinum toxin in suspect fluids.

Antitoxins: Five hundred liters of Botulism Immune Plasma (Human) collected during the past year in Arkansas were screened, sorted, and shipped to the MDPH to be ethanol-fractionated for the recovery of approximately 10 L of immune globulin suitable for i.v. administration. To date, approximately 1200 L of plasma have been collected in Arkansas under contract with Pine Bluff Biological Products. Inc.

The relationship between the quantity and titer to type A botulinum toxin of immune plasma administered, the predicted recipient titer, and the actual passively acquired recipient titer was determined in human volunteers infused with Botulism Immune Single Donor Plasma (Human) Pentavalent (BIP) [Medical Division Protocol 79-7]. The total number of international units (IU), an *in vivo* measure of BIP antitoxin activity, infused into each recipient ranged from 2,965-13,962 IU. In 4 of 5 volunteers the actual period of protection, expressed as the number of days the recipient's titer remained above 0.25 IU/ml of serum, equaled or exceeded the predicted period of protection, thus indicating the feasibility of making such predictions. Half-life values for the neutralizing activity of infused BIP to type A toxin ranged from 11-17 days to 42-49 days, with a group ($n = 5$) average of 21-27 days. Two of the volunteers were infused with BIP which contained a PRN_{80} Rift Valley fever titer of 1:640. Both

recipients maintained an RVF titer $\geq$ 1:20 through post-plasma infusion day 21, a titer thought to denote protection against RVF viremia. A pilot lot of Botulism Immune Globulin (Human), fractionated from a 52-L pilot lot of BIP, was evaluated and shown to have an anthrax titer of 1:512, an RVF PRN_{80} titer of 1:640, as well as neutralizing titers of 300 IU to type A, 100 IU to type E, and 10 IU to type B toxins.

As part of an effort to evaluate the purity of pilot lot #1 of Botulinum Immune Globulin (Human) Pentavalent (ABCDE), produced by the MDPH, aliquots of this product were examined by counter-immunoelectrophoresis. As evaluated by this technique, the immune globulin contained 2 small peaks of nonimmune globulin protein, as well as a trace of IgM and IgA.

The MDPH-prepared pilot lot of immune globulin and 4 botulinum immune globulin pilot lots prepared by ion exchange chromatography and SiO_2 or clot techniques at the University of Minnesota were examined by the automated immunoprecipitin system for content of 10 specific serum proteins. Only trace amounts of IgA and IgM were present in the MDPH product. Neither IgA nor IgM were detected in the products treated with SiO_2. However, trace amounts of IgM, C_3 complement, haptoglobin, α_1-antitrypsin, and α_2-macroglobulin were evident in the clot-treaced products.

The SiO_2 technique appears to yield a very pure immunoglobulin product. This technique, unlike the long and laborious fractionation method used by MDPH, is much less time consuming, less expensive and adaptable to small lots of valuable hyperimmune plasma.

Chemotherapeutics. Botulinum toxin blocks the release of acetylcholine from motor nerve terminals, causing long-lasting muscle paralysis and death. 3,4-Diaminopyridine (3,4-DAP) is known to enhance the release of acetylcholine in isolated botulinum toxin-poisoned muscle. Seventy Swiss mice were poisoned with a lethal dose of type A botulinum toxin. Fifteen mice died before treatment was begun at 16 h after poisoning. In the remaining 55 partially paralyzed and dying mice, i.p. administration of 3,4-DAP at 1 and 4 mg/kg body weight induced alertness, improved muscle tone, and restored mobility for periods lasting 2-3 h. A series of retreatments at 3-h intervals relieved paralysis with each usage. Fifty-seven percent (8/14) of the mice treated with 4 mg/kg and 27% (4/15) treated with 1 mg/kg were alive after 48 h of chemotherapy, as compared to the death within 12 h of 100% (10/10) of the untreated and 88% (14/16) of the placebo-treated mice. Used as a chemotherapeutic agent, 3,4-DAP was effective in temporarily restoring muscle tone and stimulating mobility, thus prolonging survival of mice poisoned with a lethal dose of type A botulinum toxin.

Presentations:

1. ELISA technique for the rapid detection of botulinum toxins. Presented, Workshop on Rapid Identification of BW Agents, 5-7 May 1980, USAMRIID, MD.

2. Lewis, Jr., G.E., S.S. Kulinski, D.W. Reichard, and R.J. Miller, Jr. Enzume-linked immunosorbent assay for detection and quantitation of type A botulinum toxin in water, milk, and urine. Presented, 20th Intersci. Conference Antimicrobial Agents and Chemotherapy, New Orleans, LA, 22-24 Sep 1980. (Abstract 440).

3. Lewis, Jr., G.E., J.F. Metzger, and R.M. Wood. Therapeutic activity of 3,4-diaminopyridine in mice poisoned with type A botulinum toxin. Presented, 20th Intersci. Conference Antimicrobial Agents and Chemotherapy, New Orleans, LA, 22-24 Sep 1980 (Abstract 449).

Publications:

Lewis, Jr., G.E., and J.F. Metzger. 1980. Studies on the prophylaxis and treatment of botulism, pp. 601-606. In Natural Toxins (D. Eaker and T. Wadström, eds.), Pergamon Press, Oxford.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OG2599	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. GROUP SNSTVTY	8a. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
80 02 26	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	072
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Include Security Classification Code)

(U) Role of microbial toxins in human disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
80 02	80 09	DA	C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR 80 (PREVIOUS)	1.5	88
FISCAL YEAR 81 (CURRENT)	0	0

19. RESPONSIBLE DOD ORGANIZATION

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TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

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Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Include Area Code if U.S. Autovon prefix)
NAME: Siegel, L. S.
TELEPHONE: 301 663-7211
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Lewis, G. E., Jr.
NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Include Exact Code and Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Microbial toxins; (U) Toxoids; (U) Botulism

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Include individual paragraphs identified by number. Include Security Classification Code.)

23 (U) Produce highly purified neurotoxoid from each of 7 types (A-G) of Clostridium botulinum in order to produce a multivalent toxoid for human immunization. If successful, this toxoid will significantly improve military medical defenses against botulism as a biological warfare threat agent. The toxoid available in 1980 protects against only five strains of botulism, has significant side effects, and is low potency. Improvement is badly needed.

24 (U) After developing production methods produce in large quantities, purify and toxoid each neurotoxin. Combine and test for efficacy.

25 (U) 80 02 - 80 09 - Maximal toxin production by the bean strain of Clostridium botulinum type B was obtained within only 48 h in a 50-L fermentor with yields of 400-500 thousand median lethal doses/ml. (Incubation times of 4-5 days are necessary to obtain similar quantities of toxin in statically grown cultures of this organism). Optimum conditions for toxin production in the fermentor include a nitrogen overlay at a rate of 5 L/min, an agitation rate of 50 rpm, a temperature of 35 C and an initial glucose concentration of 0.5 or 1.0%. Under these conditions, the Okra strain of type B produced a toxin concentration of 1 million median lethal doses/ml in 24 h.

A purification scheme previously developed for type A toxin has proved useful for the partial purification of type B toxin. Studies are continuing to develop suitable methods for the production of highly pure neurotoxin in adequate yields.

Publications: Appl. Environ. Microbial 38:606-611, 1979; 40: in press, 1980; Abst, Annu. Mtg. - 1980, Am. Soc. Microbial, p. 190;

Terminated for management efficiency. Continued in W.U. 871 BA 150. (DAOG3810)

26. Available to contractors upon originator's approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 69 (FOR ARMY USE) ARE OBSOLETE.

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BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BA: Prevention of BW Diseases Caused by Microbial Toxins

Work Unit No. 871 BA 124: Role of Microbial Toxins in Human Disease
(841 00 072)

Background:

Protein toxins elaborated by microorganisms play a role in a variety of human infectious diseases. The symptoms of diphtheria, tetanus and botulism are caused solely by a toxin produced by the infecting bacteria. Therefore, immunity to the toxin protects against the disease.

Botulism is caused by a protein toxin produced by Clostridium botulinum. There are 7 types of C. botulinum, designated A-G, each producing an immunologically distinct but pharmacologically similar neurotoxin. An amount of botulinal toxin sufficient to be immunogenic in a susceptible species would far exceed the lethal dose. Therefore, botulinal toxoid (toxin which has been chemically modified such that it is no longer toxic but is antigenic) is used as an immunogen. The botulinal toxoid currently in use for human immunization is derived from formalin-inactivated types A-E toxins and was produced by Parke-Davis, under contract to the U.S. Army in 1958. For type A, the preparation contains only about 10% neurotoxoid; similar values are to be expected for the other types. This toxoid produces sustained measurable antibody levels only after a series of 4 injections over a period of 1 year. Mild side reactions, including tenderness, redness, heat and swelling at the site of injection, are common. A new product, prepared from highly purified neurotoxins and including types F and G, is required.

Progress:

The growth and nutritional conditions required for maximum toxin production by the bean strain of C. botulinum type B have been investigated using a 50-L fermenter. Toxin concentrations of 4-5x10^5 LD_{50}/ml were attained within 48 h using the fermenter system. In contrast, incubation times of 4-5 days are necessary to obtain similar quantities of toxin in statically grown cultures of this organism.

Studies were initiated to determine the optimum culture medium formulation for maximum toxin production by the bean strain of type B. The use of a medium containing 1% lactalbumin hydrolysate, 2% yeast extract, 0.2% calcium lactate, 0.15% cysteine-HCl and 0.5% glucose resulted in a toxin concentration of 2x10^5 LD_{50}/ml in 48 h. In a medium composed of 2% casein hydrolysate, 1.5% yeast extract, 0.2% $CaCl_2$ and 0.5% glucose, a toxin concentration of 5x10^5 LD_{50}/ml was attained within 36 h. The addition of 0.2% $CaCl_2$ or calcium lactate to the medium did not increase yields in the fermenter system, in contrast to previous results obtained with static cultures.

The effect of glucose concentration, using 1.5, 1.0, 0.5 or 0.2% glucose, as well as no added carbohydrate was determined. The medium const of 2.0%

casein hydrolysate and 1.5% yeast extract + glucose as indicated above. Significant lysis of the culture occurred with 0.25, 0.5 and 1.0% glucose. Increasing the glucose concentration prolonged the time at which lysis of the culture began. The amount of toxin in the culture fluid was similar in cultures supplemented with 0.5 and 1.0% glucose, maximum toxin concentrations occurring in 48 h. (Prolonged incubation, up to 120 h, did not increase toxicity.) Cultures supplemented with 0.25 and 1.5% glucose and those to which no additional glucose was added produced less toxin.

Temperature was varied in the range of 25–40°C to determine its effect on growth and toxin production. Growth occurred at all temperatures tested, but 40°C was apparently optimum. The optimum temperature for toxin production, however, was 35°C, with maximum toxin concentrations attained in 48 h. Incubation temperatures of 25 or 40°C dramatically reduced toxicity.

The effect of nitrogen sparging (5 L/min), nitrogen overlay (5 L/min) and CO_2 sparging (1 L/min) was determined. Maximum growth was obtained in 12 h in all cases. The use of a nitrogen overlay resulted in the greatest toxin yields.

The possibility of increasing toxin yield by controlling pH of the culture was investigated. The pH, which after inoculation was 6.9, was uncontrolled until pH 6.0 was reached. This occurred after approximately 6.5 h of growth in the presence of 0.5% glucose. The pH was then maintained at 6.0 for the duration of the experiment. pH control had no effect on growth rate. The amount of toxin in the culture fluid increased more rapidly with pH control than without, but the values attained at 48 h were similar.

To date, optimum conditions for toxin production by the bean strain of C. botulinum, type B are: nitrogen overlay at a rate of 5 L/min, an agitation rate of 50 rpm, a temperature of 35°C and an initial glucose concentration of 0.5 or 1.0%. Under these conditions, the maximum toxin concentration ($4\text{-}5\times10^5$ LD_{50}/ml) was attained with 48 h.

The bean strain of C. botulinum type B has consistently produced a maximum toxin titer of $4\text{-}5\times10^5$ LD_{50}/ml in the fermenter. This concentration is marginal for large-scale production of a toxoid. Studies on toxin production by the Okra strain of type B in the fermenter system were therefore begun. In a medium composed of 2% casein hydrolysate, 1.5% yeast extract and 0.5% glucose, a toxin concentration of 1×10^6 LD_{50}/ml was attained in 24 h. This level of toxin production is adequate for large-scale production of toxin for purification and toxoiding studies. Further studies with the fermenter were precluded by mechanical difficulties; it was nonfunctional for 3 months.

Methods for the rigorous purification of types A, B, E, and F neurotoxins from culture fluid have been published. However, these methods were developed for the purification of small lots. The suitability of these methods for the purification of large batches of toxin (from 50 L or more of culture) is being evaluated.

The purification scheme previously developed for type A neurotoxin has been used for type B. The toxin is precipitated from 50 L of culture fluid by adjusting the pH to 3.5 with 3 N H_2SO_4. After washing the precipitate with distilled water, the toxin is released from the precipitate by repeated extractions with

0.2 M phosphate buffer, pH 6.0. The extract is dialyzed against 0.05 M citrate buffer, pH 5.5, and is then applied to a 10 x 100-cm column containing DEAE cellulose equilibrated in citrate buffer. The column is eluted with citrate buffer; the toxin emerges at the void volume. This single column chromatographic step allows the removal of nucleic acid from the equivalent of up to 100 L of culture. For type B, this scheme results in a 50% recovery of toxin from the culture fluid and approximately a 200-fold increase in specific activity (LD_{50}/mg protein). However, separation of the neurotoxin from the hemagglutinin has proved difficult. Studies are continuing to develop suitable methodology to produce neurotoxin of high purity in adequate yields.

Presentation:

Siegel, L.S. and J.F. Metzger. Toxin production by Clostridium botulinum type B under various fermentation conditions. Presented, Annu. Mtg ASM, Miami Beach, FL, 11-16 May 1980 (Abstracts, P7, p. 190).

Publication:

1. Siegel, L.S., and J.F. Metzger. 1979. Toxin production by Clostridium botulinum type A under various fermentation conditions. Appl. Environ. Microbiol. 38:606-611.

2. Siegel, L.S., and J.F. Metzger. 1980. Effect of fermentation conditions on toxin production by Clostridium botulinum type B. Appl. Environ. Microbiol. 40: in press, 1980.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OG2792	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. HANDLING	9A. SPECIFIC DATA - CONTRACTOR ACCESS	9B. LEVEL OF SUM
80 05 16	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	073
b. [illegible]				
c. [illegible]	STOG 80-7.2:3			

11. TITLE: (U) Evaluation of hemostatic derangement in infectious diseases of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
80 05 16	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS ($ Thousands)
80 (DIRECTORS)	0.3	282
81 (CURRENCY)	0	0

19A. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
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19B. PERFORMING ORGANIZATION
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ASSOCIATE INVESTIGATORS
NAME:
NAME: POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS: (U) Military medicine; (U) BW defense; (U) Infectious diseases; (U) Hematology; (U) Coagulation factors

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide individual paragraphs identified by number. Provide level of work with security classification code.)

23 (U) Determine the mechanism of fatal bleeding in hemorrhagic fevers of military significance which are natural threats to U.S. Forces in various parts of the world, and against which medical defenses will be required should they be used as biological agents; examples are Korean, Congo-Crimean, Bolivian, and Argentine hemorrhagic fevers, Lassa fever, Ebola and Marburg virus diseases, and Rift Valley fever. Once the mechanism of hemorrhage has been established in animal models, attempts will be made to devise treatments which interrupt what is often a fatal progression. Examine coagulation defects in a guinea pig using Pichinde virus infection. Determine pathogenesis of disseminated intravascular coagulation in other infectious diseases of military importance and the nature and mechanism of the hematologic toxicity of ribavirin.
24 (U) Animal models are used to simulate human infection. Detailed studies of the coagulation pathway are carried out. Studies of interacting pathways such as the complement and kinin pathways are also performed.
25 (U) 80 05 - 80 09 - Much of the time has been spent setting up the laboratory, including establishment of methods for numerous assay procedures. The laboratory also established control values for hematologic and coagulation parameters on the various animals which will be used in its studies. A detailed study of the effects of vaccination with dengue-2 virus has also been completed. Unfortunately, only 2 volunteers responded to the vaccine. Ribavirin studies are in progress and should be completed in the next year. Terminated for management efficiency. Continued in W.U. S10 AQ 197. (DAOG1529)

* Available to contractors upon originator's approval

DD FORM 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

BODY OF REPORT

Project No. 3M162770A871; Prevention of Military Disease Hazards (U)
(3M162776A841)

Task No. 3M162770A871 BC; Prevention of Viral Diseases of Potential BW Importance

Work Unit No. 871 BC 142; Evaluation of Hemostatic Derangement in Infectious
(841 00 001) Diseases of Military Importance

Background:

This work unit was established to investigate the pathophysiology of disturbances of hemostasis in diseases of military importance. This institute offers the unique opportunity of studying many of these diseases, including viral hemorrhagic fevers, rickettsial infections, and malaria. It is hoped that this research will lead to insights into therapy which will decrease morbidity and mortality, and which will increase the military's ability to deal with these disorders, both in terms of naturally acquired disease and as BW agents.

Progress:

During the last year a major effort has gone into making the coagulation laboratory functional. This has involved establishing numerous assays, obtaining necessary supplies, putting a quality control system into effect, and seeing that the laboratory meets the Good Laboratory Practices standards.

In addition to this effort, two studies were undertaken. The first, evaluation of changes in coagulation associated with vaccination with living dengue-2 vaccine, has been completed. Because only 2 volunteers had any response to vaccination as measured by antibody titers, and only one of these developed a high titer response, it is difficult to draw any conclusions. The single volunteer with a high titer to vaccination had a significant increase in platelet factor III activity, but there were no other changes of significance (Table I). As there are almost no data on the effects of vaccination on coagulation parameters, such information remains of interest but clearly the first necessity for such a study is the development of a subclinical response to vaccination.

The coagulation laboratory is presently involved in an on-going study of the hemotologic effects of ribavirin in rhesus monkeys. To date, the study has shown that the drug causes significant anemia in this model when given at toxic doses. The anemia appears to be due to both decreased red cell production and increased destruction. Following withdrawal of ribavirin, red cell production is resumed and anemia is quickly corrected.

TABLE I. LABORATORY DETERMINATIONS IN VOLUNTEERS IMMUNIZED WITH DEN-2 VACCINE

GROUP	NO.	VALUE BY DAY 0	7	11	14	21	60	NORMAL RANGE
Platelet count.								
Controls	1	319	324	-	309	-	248	(150-400/mm^3)
	2	321	316	-	316	366	301	
Non-converters	1	290	239	-	308	-	234	
	2	240	205	234	261	-	234	
	3	346	330	-	356	-	311	
	4	306	314	286	334	-	239	
Converters	1	-	282	233	239	-	217	
	2	-	242	208	198	-	232	
Circulating platelet aggregates. Ratio = Formalin-fixed count / Nonfixed count								
Controls	1	1.16	0.8	1.2	0.57	0.97	1.34	(0.62-1.35
	2	0.51	0.39	0.92	0.78	1.0	1.64	for n=5)
Non-converters	1	0.78	0.82	1.17	0.85	1.03	1.91	
	2	1.04	0.62	0.71	0.7	0.66	0.67	
	3	0.92	0.82	0.96	1.39	0.96	0.95	
	4	1.0	0.64	1.0	0.98	0.83	-	
Converters	1	1.61	0.61	0.74	0.83	1.03	-	
	2	0.99	1.82	0.69	0.94	0.83	1.1	
Platelet factor-3 (%).								
Controls	1	14	7.4	10	11	16	47	(4.5-35 for
	2	5	6.6	14	10	13	12	n=15)
Non-converters	1	10	11	14.5	9	7.3	6.2	
	2	8.4	14.2	16	18	46	15.5	
	3	16	14	8.8	12	17	11.5	
	4	12.5	5.1	18	18	18	8.6	
Converters	1	27	42	40	42	55	24	
	2	4.1	3.9	7.2	6	8.8	4.7	

Factor V (% normal).

Controls	1	88	88	100	108	78	104	(40-150)
	2	68	54	50	57	55	63	
Non-converters	1	-	98	100	98	74	94	
	2	74	74	90	100	94	100	
	3	88	108	98	100	84	100	
	4	104	104	124	136	114	126	
Converters	1	80	80	98	100	104	116	
	2	90	90	82	80	76	74	

Factor VIII (% normal).

Controls	1	78	40	46	54	-	68	(40-150)
	2	76	78	66	72	-	62	
Non-converters	1	64	47	74	88	-	100	
	2	40	56	56	82	-	60	
	3	80	62	78	82	-	94	
	4	38	64	56	-	-	34	
Converters	1	84	108	82	-	-	94	
	2	38	66	62	56	-	36	

Factor IX (% normal).

Controls	1	132	58	100	108	92	100	(40-150)
	2	86	84	92	100	112	70	
Non-converters	1	168	59	90	124	108	108	
	2	88	50	90	94	92	100	
	3	116	58	96	108	100	100	
	4	86	98	96	114	90	59	
Converters	1	132	58	100	108	92	100	
	2	86	84	92	100	112	70	

Fibrinogen (mg/dl).

Controls	1	290	235	195	217	190	230	(200-400)
	2	352	240	250	265	257	205	
Non-converters	1	-	240	250	280	230	205	
	2	216	257	210	230	272	210	
	3	241	155	138	186	265	222	
	4	440	352	410	445	380	290	
Converters	1	280	300	270	280	310	320	
	2	290	210	257	270	310	242	

Prothrombin time (sec).

Control	1	11.8	11.6	12.0	12.0	11.9	12.0	(11-13)
	2	12.4	12.6	12.6	12.8	12.7	13.1	
Non-converters	1	-	12.2	12.2	12.4	12.4	12.7	
	2	-	-	12.5	12.7	12.2	-	
	3	-	12.8	12.7	13.0	12.4	12.8	
	4	11.8	11.7	11.8	11.8	11.8	12.2	
Converters	1	11.6	-	12.0	12.0	11.9	11.9	
	2	-	12.0	12.3	12.5	12.4	12.8	

Partial thromboplastin time (sec).

Controls	1	39.8	41.7	44.0	32.7	28.8	35.6	(26-39)
	2	29.7	29.5	31.9	31.3	34.4	30.6	
Non-converters	1	-	38.0	39.3	30.1	31.1	33.0	
	2	-	-	34.0	31.0	30.6	-	
	3	-	30.6	33.6	29.1	28.8	32.2	
	4	40.6	41.2	38.9	31.6	33.6	33.2	
Converters	1	25.6	-	29.4	27.0	31.5	26.0	
	2	-	38.0	37.8	29.4	30.8	33.9	

Mononuclear cell tissue factor (sec).

Controls	1	25.5	34.1	36.4	30.2	38.0	52.4	(27-53
	2	32.3	26.4	32.3	29.8	43.2	62.7	for n=5)
Non-	1	29.6	41.2	36.1	46.9	34.2	55.1	
converters	2	35.4	32.2	-	58.2	48.8	58.6	
	3	26.4	51.8	42.8	56.1	60.5	64.2	
	4	44.2	23.3	29.6	52.9	49.3	60.7	
Converters	1	29.2	-	55.2	37.8	52.2	67.5	
	2	26.9	28.3	27.6	30.7	44.1	45.4	

Ribavirin administration was also associated with significant thrombocytosis in treated monkeys, although most of the rise in platelet count occurred following drug withdrawal. Thrombocytosis was associated with a significant shift to the right in the size distribution curve of the platelets. Platelet function abnormalities, characterized by a prolonged lag phase on collagen-induced aggregation were also noted, but again this occurred after drug withdrawal. At present we are looking at the effects of ribavirin in smaller doses and on the bone marrow, and are doing additional tests to determine its effect on platelet function.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6418	80 01 16	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. RSRCH SYSTEM	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	001
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE: (U) Effects of suppressor and helper T cell activities on the efficacy of immunization

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
76 10	79 12	DA		C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 80	1.0	74
FISCAL YEAR CURRENT 81	0.3	20

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Provide MACR with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Immunization; (U) Immune response regulation during infection; (U) Tularemia

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

23 (U) Determine the role of immunoregulatory functions (especially suppressor and helper T cells) during infection or vaccination. Identify these responses and apply them to optimizing immunity to BW agents.
24 (U) Develop techniques to quantitatively and qualitatively assay immunological regulatory functions in a model infectious system postvaccination. Examine and compare their effects in normal and vaccinated animals in an effort to identify major mechanisms influencing immunologic capacity of in vivo immunoregulatory phenomena to maximize immunizing effects of vaccines.
25 (U) 79 10 - 80 01 - Further work was done to define the mechanisms responsible for immune suppression in mice inoculated with F. tularensis (LVS). Major differences were noted in suppression of immune induction, weight change and bacterial titers of spleen and blood, when the 2 routes of inoculation, IV and IC, were used. There was greater total weight loss in SC-inoculated than in IV-challenged mice. Viremia was later in the SC group. The number of spleen cells committed to an immune response in the IV group correlated with splenomegaly; no such difference was observed in SC-inoculated mice. No further work will be done due to transfer of the investigator.

*Available to contractors upon originator's approval

DD FORM 1498 MAR 88 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 84 AND 1498-1 1 MAR 85 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. S03 00 001: Effects of Suppressor and Helper T Cell Activities on the Efficacy of Immunization

Background:

Aside from the invasive properties of pathogenic intracellular bacteria a less well-characterized effect may be the ability of the bacteria to alter normal immunoregulatory processes. These processes depend on the amount of antigen being presented to critical immune subpopulations. The number of bacteria in an inoculum is relatively easily determined, but it is much more difficult to determine the dose "seen" by particular lymphoid tissues. The importance of these dose effects has been shown in studies on nonproliferating antigen. The effects of dose variation on antibody titer have long been known (1).

Recent research has shown that these effects are operative also at the level of cellular subpopulations (2). In fact it is clear that cellular communication via lymphokines and contact interactions at the cell surface are sensitive to dose phenomena.

In light of these observations the major focal points were the study of the kinetics of bacterial proliferation and the correlation of bacterial mass with the ability of the immune system to respond to unrelated antigens during infection by an intracellular pathogenic bacterium.

Progress:

Experiments were performed to compare the effects of IV and SC Francisella tularensis LVS strain inoculation in AKR/J mice.

One type of experiment showed that there was a statistically significant weight loss among SC inoculated mice. The loss was greatest at day 7. It was not observed in IV inoculated animals. The weight loss was approximately 10% of total body weight. The literature suggests that weight loss can stress the immune system causing altered responses.

A second type of experiment was used to identify weight change in spleen and liver, and kinetics of bacterial recovery in these mice. Weight changes in liver were remarkably similar, while the weight of spleen of IV inoculated mice slightly exceeded that of SC inoculated mates. Differences were evident in bacterial recovery. Bacteria were recovered from IV inoculated mice from day 1, while bacteria were not recovered from SC inoculated mice until day 2 or 3. The rate of change of bacterial recovery from IV inoculated mice was also much less dramatic due to this early appearance. The later appearance of bacteria results in a greater rate of change to peak recovery at day 5. These observations are very significant because they point out differences in rate and dose of antigen presentation to spleen cells. Changes in these parameters are often responsible for subsequent initiation of immunoregulatory phenomena.

A third type of experiment was performed to compare the immune response potential during infection with LVS between SC and IV inoculated mice. Results showed that little or no suppression occurred in IV inoculated mice. Furthermore, an increase in immune inductive ability was observed that correlated well with cell number increase in splenomegaly. In other words, the number of cells committed to an immune response in the spleen of IV inoculated mice varies as a function of splenomegaly. However, no such correlation could be made in SC inoculated mice, even though splenomegaly was nearly identical. Therefore, the response decreased due to dilution or suppression in SC inoculated animals. This observation not only can be used in formulating hypotheses of mechanism of suppression, but clearly suggests that suppression can be controlled.

The concept behind "mixing" experiments is to see how a particular subpopulation of lymphoid cells, when placed with other whole populations of lymphoid cells, when placed with other whole populations of lymphoid cells in culture, will affect the primary in vitro induction ability of the normal cells. In these experiments adherent cells from either infected or normal mice, at various times after SC LVS inoculation, were placed into culture with either whole splenic populations or non-adherent subpopulations from normal or infected mice. All combinations were tested. Preliminary results showed that adherent splenic cell populations from infected mice placed in culture at day 7 effected suppression of the hemolytic plaque assay at day 11. That is, these data essentially duplicate what has been seen in vivo. This data suggests that an active suppressive process is going on and that the adherent cells (macrophage subpopulation) play a direct role in it. This work unit was terminated in January 1980, due to transfer of the Investigator.

Publications:

None.

LITERATURE CITED

1. Eisen, H. N. 1974. Immunology: an introduction to molecular and cellular principles of the immune responses. Harper & Row, Hagerstown, MD.

2. Pierce, C. W., and J. A. Kapp. 1976. Regulation of immune responses by suppressor T cells. Contemp. Top. in Immunobiol. 5:91-143.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6429	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DGN/N ENTR'S	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	006
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Procede with Security Classification Code):

(U) Enzymatic and chemical alteration of microbial proteins for toxoid production

12. SCIENTIFIC AND TECHNOLOGICAL AREAS:

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
71 08	80 09	DA			C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING: 80	1.0	38
FISCAL YEAR CURRENT: 81	0	0

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede each with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Amino acids; (U) Enzymes; (U) Toxoids; (U) Vaccines; (U) Staphylococcus; (U) Laboratory animals

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Precede each with appropriate paragraph identification by number. Precede text of each with Security Classification Code.)

23 (U) Prepare more effective immunogens against militarily important diseases in which proteins of bacterial and viral origin have significant biological effects. The immediate goal is the identification of the elements of the protein structures containing antigenic determinants and toxic sites. The enterotoxins produced by Staphylococcus aureus are now being studied. These toxins are potential agents for biological attack and are responsible for many outbreaks of food poisoning.

24(U) Enzymatic modification of enterotoxins is the major modification mechanism. Enterotoxin C-1 (SEC-1) undergoes a rapid, limited digestion by trypsin. Studies are concerned with the chemical and physical characterization of the fragments and determination of their role in the serological, emetic, and mitogenic activity of the whole molecule.

25 (U) 79 10 - 80 09 - Curve resolution of the circular dichroic (CD) spectra of staphylococcal enterotoxins A, B, and C-1 between 260 and 300 nm indicated that they possess 6 CD bands located at very nearly the same wavelengths. Three of these bands are attributed to tyrosyl residues, 1 to phenylalanyl residues, 1 to tryptophanyl residues and 1 to both phenylalanyl and tyrosyl residues. CD and chemical modification disclosed subtle but important differences in the environment of the chromophoric groups. Thus, the tryptophanyl residues of SEA are readily oxidized and have a positive CD band, while the tryptophanyl residues of SEB and SEC-1 are resistant to reduction and have a negative CD band. The disulfide of native SEC-1 is not reduced, and although the disulfides of both SEA and SEB are readily reduced the effects of this conversion upon structure and CD are notably disparate. The SEB disulfide makes a significant contribution to the CD spectrum and the molecule; the alterations in CD of reduced SEA are conformational in origin and are irreversible. Publications: Biochim. Biophys. Acta 621:233-240, 1980; Infect. Immun. 30: in press; in Methods Enzymol., in press.

Terminated for management efficiency. Continued in W.U. 510 AN 200 (DAOG1519)

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BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M161102BS03)

Task No. 3M161102BS10-AN: Characterization of Microbial Toxins of Potential Importance

Work Unit No. S10-AN-161: Enzymatic and Chemical Alteration of Microbial
(S03 00 006) Proteins for Toxoid Production

Background:

The staphylococcal enterotoxins are simple proteins elaborated by certain strains of Staphylococcus aureus, which cause emesis and diarrhea in a limited number of mammalian species. The several types are identified on the basis of individual immunological reactivity, e.g., enterotoxins A, B, and C do not cross-react in classical measurements of immunodiffusion or quantitative precipitin reaction. Even so they all appear to have the same basic chemical structure, a single polypeptide chain of about 240 amino acid residues containing one disulfide bridge. A comparison of the structures of SEA, SEB, and SEC_1 by means of their circular dichroic (CD) spectra revealed that the CD of SEB and SEC are very similar but differ from the CD of SEA. SEA retained most of the same bands with respect to both location and sign but with significant difference in intensity. It seems probable that all 3 enterotoxins fold in basically the same manner. The structures contain little α-helix.

Progress:

These studies have centered on a comparison of the structural properties of SEA, SEB, and SEC_1 as revealed by their CD spectra and the effect of chemical modification upon these spectra. We have empirically resolved on a computer the CD spectra of the 3 enterotoxins from 260-300 nm into a series of Gaussian curves (1). Each Gaussian curve corresponds to a CD band and one attempts to achieve a fit with the minimum number of curves. Three parameters are selected to define each curve: The wavelength of maximum ellipticity, the ellipticity at that point, and the standard deviation. Four components are obvious with each toxin as indicated by clear maxima or a well-defined shoulder. It is necessary, however, to incorporate 2 additional curves to obtain satisfactory correspondence with the experimental data. Clearly these solutions are not unique, but several important conclusions are nevertheless apparent: (a) for all 6 of the curves the maximum ellipticity is located at very nearly the same wavelength for all 3 enterotoxins; (b) CD theory predicts that CD bands correspond to absorbance bands at or near the same wavelength, and although no attempt was made to force-fit the data, it is noteworthy that each Gaussian curve does correspond to the absorbance and the CD band of an aromatic amino acid; (c) the 3 strongest CD bands, at 269, 279-280, and 285-286 nm, clearly arise from tyrosyl residues; (d) a weak CD band at 262-263 nm is produced by phenylalanyl residues; (e) a band at 269 nm also corresponds to a phenylalanyl transition. However, it is considerably stronger than the 262-263 band and since in most instances the 262 and 269 CD bands from phenylalanine are of nearly equal intensity (2) and there is a tyrosyl absorbance at 268 nm, it is likely that this band is a composite of transitions from both kinds of residues; and (f) a band at 290-293 nm

surely represents tryptophanyl residues. It is striking that the sign of the CD of this band is positive for SEA but negative for SEB and SEC_1. Tryptophan model compounds have a positive CD suggesting that the environment of the single residues in SEB and SEC_1 is significantly different from that of the tryptophans of SEA and from that of the free amino acid in aqueous solution. This correlates nicely with the availability of the tryptophans of SEA to oxidation and the refractory behavior of the tryptophanyl residues in the other enterotoxins.

The tryptophanyl residues of native SEB and SEC_1 are not oxidized by N-bromosuccinimide (NBS) (3). SEA is, however, readily oxidized by this reagent. Two residues per molecule are modified in agreement with the tryptophan content of the toxin found by amino acid analysis. Gel electrophoresis in SDS showed that no cleavages in the peptide chain occur. The CD spectrum in the near-UV is considerably different from that of the native toxin. However, when a difference spectrum is calculated it becomes clear that the changes are due to the loss of a positive CD band at 285-290 nm and the appearance of a negative band at about 255 nm. The former corresponds to the CD of tryptophan and the latter to the CD of the NBS oxidation product of tryptophan, β-3-oxindolyl-L-alanine (4), with good agreement as to location, sign and strength of both these bands. When the addition of NBS is restricted to that amount required for 50% oxidation (one cannot distinguish between the complete modification of one tryptophanyl residue and fractional modifications of both residues), the difference spectrum is altered proportionally.

Oxidation of SEA with NBS also affects the ellipticity in the far-UV. The major extremum at 216 nm is increased from -1400 to -5200 $deg \cdot cm^2 \cdot dmol^{-1}$ in the CD of the unmodified toxin; it is suggestive of an increase in β-structure. It should be noted, however, that an even greater change occurs simply upon going from pH 4 to pH 7 where the ellipticity is -2700 $deg \cdot cm^2 \cdot dmol^{-1}$.

The disulfide group is the only chromophore in the near-UV with an intrinsic chirality. The contribution of the disulfide to the CD spectra of the staphylococcal enterotoxins has been investigated. SEB is reduced by β-mercaptoethanol (βME) in the absence of denaturant (5). The conditions employed in the current experiments are 0.7 M βME for 3 h at room temperature in pH 8.6 Tris buffer containing 2 mg/ml EDTA. CD spectra in the UV cannot be determined in this solution because of the high absorbance of the reductant. Accordingly it is dialyzed against 0.05 M phosphate buffer at pH 6.0 containing 2 mg/ml EDTA and 0.01 M dithiothreitol (DTT) to remove the βME and maintain the toxin in its reduced state. The CD spectrum of reduced SEB is very similar to that of the native material. However, when a difference spectrum is calculated a smooth curve is obtained with a maximum near 273 nm. The contribution of the disulfide, $[\theta]_{M,273}$, is positive in sign with an intensity of approximately 8000 $deg \cdot cm^2 \cdot dmol^{-1}$. This magnitude is in the range generated by cystine in the crystalline state. Its sign suggests a right-handed chirality for the disulfide, but it is necessary to know the dihedral angles before a firm assignment can be made.

In order to extend this observation to the other enterotoxins, the reducibility of SEA and SEC_1 under the conditions employed with SEB was determined. SEA is completely reduced by βME, but SEC_1 is relatively resistant, less than 20% of the disulfide being affected. When the βME concentration is increased to 2.4 M SEC_1 is completely reduced but the protein is precipitated indicating denaturation. The refractory nature of the SEC_1 disulfide was not anticipated because this toxin is less stable to denaturing agents than SEB, implying a more open structure.

Reduced SEA in DTT and EDTA shows a decrease in ellipticity, but the maximum difference is blue-shifted to about 255 nm. The magnitude of the difference is considerably less than that seen with SEB so that the errors in the difference spectrum are greatly magnified.

It should be noted that while these data for both SEA and SEB are reproducible, the differences are small relative to the ellipticity generated by the aromatic residues. It is possible that the CD difference spectra are merely a reflection of minor conformational changes in other chromophores occurring as a result of the reduction. Reversibility upon reoxidation is one way of measuring this. When reduced samples of SEA and SEB are dialyzed free of reductant and EDTA to facilitate air oxidation of the molecules and after 1 week at refrigerator temperature in phosphate buffer at pH 6.0, substantially all the free SH disappears (In the presence of DTT and EDTA little reoxidation occurs.).

Striking differences are observed in the behavior of the 2 toxins. SEB gives, within experimental error, a CD spectrum indistinguishable from the native material. The CD of reoxidized SEA, however, is essentially the same as in the reduced state. Thus it is likely that the difference spectrum obtained with SEA is conformational in origin. Otherwise it would have to be postulated that the disulfide of the reoxidized SEA has its chirality and/or dihedral angles sufficiently altered with respect to the native toxin so as to completely negate a CD contribution. The reversibility with SEB is supportive of a real -S-S- contribution to the CD of the enterotoxin.

It was proposed a few years back by Bergdoll (6) that a 14 amino acid segment of the enterotoxin molecules starting with the second half-cystine residue composes the active site of these toxins. This was based on the high degree of homology between SEA and SEB in this region. The first 7 residues and 4 of the next 7 are identical. Sequence work carried out in our laboratory by CPT J. S. Cades on SEC_1 is supportive of this, in that a strong homology is found to occur in this region of its primary structure. In the first 7 residues the only difference is the conservative substitution of an isoleucine for a valine; 2 or 3 of the remaining residues are also identical. Our present chemical and CD work casts some question on this interpretation. These data demonstrate significant differences in the properties and environment of the disulfide loop in the 3 enterotoxins. Thus SEC_1 is largely unaffected by BME under conditions where the -S-S- loop of SEA and SEB is completely reduced; and, additionally, the dichroism of the loop of SEA and SEB is quite different. It is also noteworthy that sequence prediction methodology (7) places the 7 residues where the greatest homology exists in a B-pleated sheet structure. These structures are usually found in the interior of protein molecules and are not part of combining sites.

Publications:

1. Middlebrook, J. L., L. Spero, and P. Argos. 1980. The secondary structure of staphylococcal enterotoxins A, B, and C. Biochim. Biophys. Acta 621: 233-240.

2. Spero, L., and J. F. Metzger. 1981. Staphylococcal enterotoxin A (SEA). Methods Enzymol. (in press).

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2. Strickland, E. H. 1974. Aromatic contributions to circular iichroism spectra of proteins. CRC Crit. Rev. Biochem. 2:113-175.

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4. Strickland, E. H., M. Wilchek, and C. Billups. 1973. Circular dichroism of modified tryptophan residues 8-3-oxindolyl-L-alanine. Biochim. Biophys. Acta 303:28-35.

5. Dalidowicz, J. E., S. J. Silverman, E. J. Schantz, D. Stefanye, and L. Spero. 1966. Chemical and biological properties of reduced and alkylated staphylococcal enterotoxin B. Biochemistry 5:2375-2381.

6. Huang, I. Y., E. J. Schantz, and M. Bergdoll. 1975. The amino acid sequence of the staphylococcal enterotoxins. Jpn. J. Med. Sci. Biol. 28:73-75.

7. Middlebrook, J. L., L. Spero, and P. Argos. 1980. The secondary structure of staphylococcal enterotoxins A, B, and C. Biochim. Biophys. Acta 621:233-240.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6414	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. ORDER NUMBER	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	007
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code): (U) Therapeutic Reversal of Abnormal Host Amino Acid, Protein and RNA Metabolism during Infectious Disease of Unique Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 07	80 09	DA	

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR (PRECEDING) 80	1.0	351
FISCAL YEAR (CURRENT) 81	0	0

19. RESPONSIBLE DOD ORGANIZATION
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NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Amino acids; (U) Protein synthesis; (U) RNA synthesis; (U) Volunteers; (U) Gluconeogenesis

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

23 (U) Changes in the concentrations and pattern of amino acids in blood and tissues during various infectious diseases will be correlated with alterations in RNA, protein and carbonydrate metabolism. The data obtained will be utilized to develop nutrient therapy to prevent the body wasting of convalescence for these illnesses. In addition, alterations in the concentration or ratio of some blood amino acids may be a useful biochemical tool in the early detection of infectious diseases that pose a potential BW threat to this country. Studies are done in rodents, monkeys, and man.

24 (U) Free amino acid concentrations are determined by ion-exchange chromatography in plasma and tissue of experimental subjects and animals infected with bacterial or viral organisms. Radioactive, nonmetabolizable and metabolizable metabolites will be utilized to study amino acid flux, RNA and protein synthesis, glucogenesis, glucose turnover and oxidation, and rates of total body catabolism.

25 (U) 79 10 - 80 09 - A tethering system has been developed for maintenance of chronically catheterized monkeys in a cage. With this system, it has been demonstrated that intravenous or oral (enteral) infusion of amino acid plus calories will prevent wasting of body protein during extracellular gram positive or gram negative sepsis, as well as intracellular bacterial and viral infections that cause hepatocellular damage. In the latter infections, a 25% dextrose and amino acid infusion compounded the severity of hepatic lesion and caused glucose intolerance and insulin resistance. This complication could be preventd by increasing the branched-chain amino acid content to 48% and decreasing dextrose to 8%.

Publications: In Primates in Nutritional Research, 1979; J. Clin. Invest. 64:1565-1572, 1979; Metabolism 29:201-212, 1980; Feedstuffs 52 (53):16-18, 20, 24, 25, 1980; JPEN 3:524,1979, 4:277, 1980; Biochem. J. 190:663-671, 1980. Terminated for management efficiency. Continued in W.U. S10 AQ 197. (DAOG1529)

*Available to contractors upon originator's approval

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BODY OF REPORT

Project No. 3M161102BS10: (3M161102BS03) Military Disease, Injury and Health Hazards (U)

Task No. 3M161102BS10 AQ: Enhancement of Host Defense Against Agents of Potential BW Importance

Work Unit No. S10 AQ 175: (S03 00 007) Therapeutic Reversal of Abnormal Host Amino Acid, Protein and RNA Metabolism during Infectious Disease of Unique Military Importance

Background:

Catabolic effects of infectious disease are characterized by marked wasting of body protein, alterations in utilization of energy substrates and increases in anaerobic process associated with host defense (1). Thus, even in a mild infectious illness, this marked catabolic response can result in a reduced work capacity for 2-3 weeks after lysis of fever. Therefore, a therapeutic procedure which could prevent the wasting of body protein and reduce the time of recovery would be of value to a troop commander whose personnel were exposed to BW attack or had entered an epidemic area. Previous investigation (2, 3) has demonstrated that IV infusion of amino acids and calories can prevent the wasting of body proteins during gram-positive sepsis. The present studies have extended this observation to gram-negative sepsis as well as intracellular bacterial and viral infections causing hepatocellular damage. Also investigation was initiated to find amino acid mixtures and caloric substrates more effective in preventing protein wasting during infectious illness.

Progress:

Protein-sparing effect of IV administered amino acids or dextrose during sepsis in cynomolgus monkeys. Following surgery or during trauma, it is common practice to maintain a patient on 5% dextrose solution. Normally this would supply approximately 400 cal/day (about 1/4 the resting requirements). In the anorectic patient this can result in the depletion of body protein stores. Recently, it has been shown that in the ketone-adapted patient infusion of 3.5% amino acid solution spares body proteins as compared to dextrose infusion (4). In more severely stressed or traumatized patients, the combination of amino acid and dextrose infusion is required to prevent body protein depletion. Therefore, studies were initiated in the cynomolgus monkey to compare the effects of amino acids or dextrose infusion on protein wasting during minor surgery of catheter implantation or gram-negative (Salmonella typhimurium) and gram-positive (Streptococcus pneumoniae) sepsis.

To test this concept, a previously described parenteral nutrition model in the monkey (3, 5) was utilized to evaluate the protein-sparing effects of amino acid and dextrose infusions. After chair-adaptation, cynomolgus monkeys had indwelling catheters implanted in the jugular and femoral veins and carotid arteries. On the morning after surgery they were infused via the jugular vein with a solution that supplied either 32 cal/kg/day of dextrose or an amino acid solution that supplied 0.55 g of nitrogen and approximately 13 cal/kg/day. Both solutions supplied an equivalent amount of electrolytes, trace elements and vitamins. Daily blood samples and

complete urine and fecal collections were made throughout the study. On day 1 at the start of nutritional support, the monkeys received an IV injection via the femoral vein with either live or heat-killed 3 x 10^8 CFU of S. pneumoniae or 1 x 10^8 Cl. S. typhimurium. The monkeys given the live organisms rapidly became febrile; all had septicemia by day 2. At this time the monkeys were treated with antibiotics for the next 4 days.

Control monkeys injected with the heat-killed organisms did not develop a fever, clinical illness, or a septicemia, and were utilized as controls to measure changes during minor surgery. In the monkeys infused only with dextrose, nitrogen balance was negative throughout the 6 days of the study, but urinary nitrogen excretion significantly decreased on day 2 and was progressively reduced the rest of the study. During minor surgery, monkeys on dextrose lost approximately 5% of their body protein. Monkeys infused with the amino acid solution were in negative balance but at a significantly lower amount compared to those infused with dextrose. Cumulative nitrogen loss was significantly less in monkeys infused with amino acids compared to dextrose, losing only 2.3% of their body protein, which is about 1/2 of the loss of dextrose infused group. Thus, during minor surgery, the amino acid infusion with lower caloric intake spared body protein when compared to higher calorie dextrose infusion.

Sepsis following inoculation of S. typhimurium organisms was associated with a marked increase in urinary nitrogen, which remained elevated throughout the study. Negative nitrogen balance was greater in monkeys infused with dextrose compared to those receiving amino acids. Cumulative nitrogen balance losses increased at a significantly greater rate in the monkeys infused with dextrose, which lost approximately 7.9% of their body protein compared to 5% during the 6-day experimental period in monkeys infused with 24% branched-chain amino acids (BCAA) (Table I).

TABLE I. SUMMARY OF EFFECTS OF NUTRIENT SUPPORT ON WASTING OF BODY PROTEIN DURING SEPSIS IN MONKEYS

	% CHANGE IN BODY PROTEIN	
NUTRIENT SUPPORT	S. pneumoniae	S. typhimurium
Dextrose (8%)	-10.52 ± 0.41	-7.91 ± 0.32
24% BCAA	- 7.59 ± 1.28	-5.07 ± 0.14[a]
48% BCAA	- 4.17 ± 0.15[a,b]	-2.70 ± 0.31[a,b]
48% BCAA + Dextrose	1.30 ± 0.44[a,b,c]	0.85 ± 0.58[a,b,c]
48% BCAA + Lipid	- 1.57 ± 0.83[a,b,c,d]	-0.75 ± 0.68[a,b,c,d]
48% BCAA + Lipid + Dextrose	0.91 ± 1.26[a,b,c]	2.37 ± 0.36[a,b,c,d]

[a] P < 0.05 to Dextrose
[b] P < 0.05 to 24% BCAA
[c] P < 0.05 to 48% BCAA
[d] P < 0.05 to 48% BCAA + dextrose

In monkeys that developed pneumococcal septicemia, a prompt febrile response was observed which was quickly reduced with antibiotic treatment. Total fever-hours for this infection were approximately twice those observed in the gram-negative infection. Again, while increases in urinary nitrogen and negative nitrogen balance were observed during sepsis, differences in nitrogen loss between the dextrose or amino acid group were only significant during the first 3 days of the study. Cumulative nitrogen balance was less in the amino acid group but no significant differences were observed in total body nitrogen loss over the 6-day experimental period in the 2 groups of septic monkeys (Table I). Therefore, amino acid infusion prevented protein wasting after minor surgery as compared to dextrose, but neither mixture could effectively prevent wasting during sepsis associated with the gram positive or gram negative infection.

Protein-sparing effects of IV administered BCAA during sepsis in cynomolgus monkey. Metabolism of BCAA (valine, leucine, and isoleucine) may play a key role in explaining alterations in metabolism of amino acids during sepsis (2, 3). BCAA are deaminated almost exclusively in skeletal muscle, and the keto acid can be utilized as a source of energy. The amino group can be transferred to pyruvate for the formation of alanine or to the α-ketoglutamate for the formation of glutamate and glutamine. Thus, it has been postulated that increasing the exogenous supply of BCAA could spare body protein during sepsis and/or trauma.

To test this concept, septic and surgical control monkeys were infused via the jugular vein with a solution that contained either 24 or 48% BCAA. The 24% BCAA mixture was a standard FreAmine II formulation which was infused at the rate of 0.55 grams nitrogen/kg/day and supplied approximately 13 cal/kg/day. In the 48% BCAA mixture, the concentration of isoleucine, leucine, and valine were all increased with resulting decrease in the other essential and nonessential amino acids in the FreAmine II mixture. Both solutions supplied equivalent amounts of electrolytes, trace elements and vitamins.

In both 24% and 48% BCAA groups of surgical monkeys injected with the heat-killed microorganisms, urinary nitrogen excretion slightly exceeded intake, resulting in negative balance. The 48% BCAA group tended to excrete slightly less urinary nitrogen, but the difference was not significant. In surgical control monkeys, the 24% BCAA group lost 2.3% body protein, while those given the 48% mixture lost 1.4% body protein, and is not a significant difference. As reported by others in man and rats (4), increasing BCAA content of the amino acid mixture did not have a marked protein-sparing effect during calorie deprivation in mildly-stressed monkeys.

In contrast, nitrogen loss, as measured by negative nitrogen balance, was significantly less during S. typhimurium sepsis in monkeys infused with 48% BCAA compared to the 24% group. Septic monkeys infused with 24% BCAA lost approximately 5% of body protein over a 6-day experimental period, while those infused with the 48% branched-chain mixture had a significantly lower rate of cumulative nitrogen loss, representing only 2.7% of their total body protein (Table I). Similar beneficial effects of the higher BCAA mixture were observed during pneumococcal sepsis. Fever-hours were almost doubled compared to S. typhimurium septic monkeys and led to a much more severe negative nitrogen balance. In monkeys infused with 48% BCAA mixture, nitrogen loss as measured by daily nitrogen balance and cumulative nitrogen balance was significantly less than that observed in those infused with the 24% BCAA. This resulted in only a loss of 4.2% body protein over a 6-day experimental period in the monkeys infused with the 48% BCAA compared to 7.6% total body protein in the 24% group (Table I). These data indicate that increasing BCAA content during gram-negative or -positive sepsis in calorie-deprived monkeys represented a true sparing of body protein.

To determine whether the BCAA were sparing skeletal muscle or connective tissue protein, urinary 3-methylhistidine (3-MeH) and hydroxyproline were utilized to monitor rates of degradation of protein in these tissues. The pneumococcal septic monkeys had significant increases in the rate of excretion of urinary 3-MeH with the onset of sepsis, but the magnitude of response was significantly less in the monkeys infused with the 48% compared to 24% BCAA mixture. Urinary hydroxyproline was also significantly increased with the onset of sepsis, but no significant differences were observed between the 2 dietary groups. These data suggest that increasing BCAA content of the mixture spared skeletal muscle protein, but not the connective tissue protein during sepsis in the monkey. However, marked hypoglycemia developed in pneumococcal septic monkeys infused with 48% BCAA mixture. This effect was not observed in septic monkeys infused with 24% mixture or in the surgical controls infused with the 48% mixture. These changes may be due in part to a reduced supply of gluconeogenic substrates in the septic monkey. A slight decrease in plasma glucose has also been reported during therapeutic fast in patients given higher BCAA solutions (4).

With the addition of 32 cal/kg/day of dextrose to 24% BCAA solution, urinary nitrogen excretion was still significantly increased during illness, resulting in a more negative nitrogen balance and nitrogen loss (3). When 85 cal/kg/day from dextrose was combined with 24% BCAA solution, only slight increases in urinary nitrogen excretion were observed during the illness phase and no body protein losses were observed over the 6-day experimental period (2). In contrast, the addition of 32 cal/kg/day from dextrose to the 48% BCAA solution during sepsis from S. pneumoniae or S. typhimurium infection prevented wasting of body protein. During both infections, the monkeys remained essentially in positive nitrogen balance or nitrogen equilibrium and tended to gain body protein over the 6-day experimental period (Table I). Thus, only about 1/3 of the calories of dextrose were required to prevent wasting of body protein during sepsis when added to a 48% BCAA solution as compared to 24% mixture. This suggests more efficient utilization of dextrose calories when added to the higher amino acid infusion.

The addition of 50 cal/kg/day from Intralipid to 48% BCAA solution during sepsis from S. pneumoniae or S. typhimurium infection was not as effective in preventing wasting of body protein compared to addition of 32 cal/kg/day from dextrose. However, cumulative nitrogen loss was significantly less than with 48% BCAA alone (Table I). Addition of lipid and dextrose to 48% BCAA solution prevented wasting of body protein during gram-negative or -positive sepsis. This combined mixture was only slightly more effective than dextrose plus 48% BCAA solution (Table I).

These observations suggest that a high branched-chain mixture plus 8% dextrose is an effective support therapy to prevent wasting of body protein during infectious disease. A 48% BCAA mixture has been utilized with good success in patients with severe hepatic insufficiency (4). Further, 10% dextrose solution can be safely administered to patients with hepatic damage. Currently studies are underway to determine if this mixture can be used in infectious diseases causing hepatocellular damage. Studies also have been initiated to determine whether this type of nutrient support will adversely alter host defense responses to infectious organisms.

Use of enteral support model to prevent protein wasting during pneumococcal sepsis in monkeys. When a patient has a functional GI tract, the preferred route of nutrient support is by infusion via a nasogastric tube (enteral nutrition). A model has been developed in the monkey for substrate support by an enteral nutrition model (3, 5). This model involves the use of a lactose-free 300 mOsmolar commercially available solution (Osmolite) which supplied 0.55 g protein nitrogen/kg/day, as casein and soy protein, and 100 cal/kg/day, with 54.6% of the calories from complex

carbohydrates and 37.4% from medium and long chain triglycerides. The Osmolite solution was infused for 12 h and the catheter maintained patent by the infusion of 4 ml of water/h over the next 12 h. The monkeys were adapted to this cyclic procedure by increasing the infusion rate of Osmolite from 25 ml/kg/day to 95 ml/kg/day during the first 4 days in the metabolic chairs. At the end of the 4 day adaptation, the monkeys had indwelling catheters placed in the femoral vein and carotid artery. Complete urine and fecal collections as well as blood samples from the femoral and carotid catheters were obtained throughout the study. On day 2 after surgery the monkeys were inoculated via the femoral vein with either live or heat-killed 3 x 10^8 CFU of S. pneumoniae or 1 x 10^6 CFU of live or heat-killed S. typhimurium. Monkeys given the live organisms rapidly became febrile and all were septicemic by day 2. At that time the monkeys were treated with the antibiotics for 4 days.

Control monkeys injected with heat-killed S. pneumoniae and S. typhimurium organisms did not develop fever, clinical illness or septicemia. Both groups of monkeys were in positive nitrogen balance throughout the 6-day experimental period. During this time period they retained nitrogen equivalent to 6% of their original total body protein.

During pneumococcal sepsis, urinary nitrogen excretion was significantly increased on days 3-5 as compared to preexposure values, but nitrogen balance was only slightly negative on day 3. As a result, the monkeys with pneumococcal sepsis retained nitrogen equivalent to 1.4% of the original body protein compared to 5.5% in controls. Urinary nitrogen excretion was significantly increased in the monkeys inoculated with S. typhimurium, but still remained in positive nitrogen balance throughout the study. Cumulative nitrogen balance over the 6-day experimental period was only slightly reduced during S. typhimurium sepsis.

These data support the concepts that enteral nutrition with combined protein, carbohydrate and lipid calories prevents protein wasting during a gram-positive or -negative sepsis in the cynomolgus monkey. Slightly poorer nitrogen retention during pneumococcal or salmonellosis sepsis suggests a caloric requirement in excess of 100 cal/kg/day. Similar observations were made for IV nutritional support during pneumococcal sepsis (2).

Development of a caged model for chronically catheterized monkeys. The chaired-model for use with chronically catheterized monkeys places stress and discomfort on a monkey. Further, the chair-restraint model has been used successfully to evaluate nutrient support during the 8-day course of a bacterial infection but studies with some viral infections require chair-restraint for 21 days or more. Since this longer period has not proven to be practical in our experience, a jacket-tethering system was developed in which the chronically catheterized monkey can be maintained in a cage. Indwelling catheters are placed in the jugular vein and femoral vein by aseptic surgical procedures. The catheters are tunneled SC to an incision in the upper lumbar region in the back of the monkey and are passed, along with a temperature probe, through a flexible tethering cable. The jacket is then fitted to the monkey and held in place with brass rivets. The flexible cable from the jacket is attached to a swivel located in the upper portion of the cage. The catheters and temperature probe are passed through this swivel and the jugular vein catheter is attached to a second small swivel located on top of the larger one. The jugular vein catheter is then utilized for constant infusion of nutrient support. A plastic carrier is attached to the lower swivel, in which the catheter, which is utilized for blood sampling, is maintained with a heparin lock and stored in iodine soaked pads. The temperature probe is placed in another section of this plastic carrier. The monkey has

complete freedom of movement within the cage, is capable of lying down, can be fed ad lib., and has complete access to water. This model has an additional advantage of being able to compare nutrient support via IV or gastric catheter with oral nutrition consisting of biscuits in monkeys which have undergone similar surgical procedures and restraint conditions. The tethering system has been utilized in over 50 monkeys and has proven to be superior by far to chair-restraint. Several other investigators at USAMRIID have expressed interest in this system and are using it in their research projects. In addition, the model should overcome some of the humane objections raised to the use of the chaired-monkey.

Therapeutic value of nutrient support during yellow fever infection in the African green monkey. Previous studies have demonstrated that in extracellular systemic infections such as S. pneumoniae and S. typhimurium, IV support prevents wasting of body proteins in infected monkeys (2, 3). Similar observations in critically ill patients have also suggested beneficial effects of nutrient support during sepsis, mainly during gram-negative extracellular infections (4). Recently Murray and Murray (6) suggested that the severity of intracellular bacterial, parasitic or viral infections would be enhanced by oral nutrient support. In an attempt to evaluate this concept, an experimentally induced yellow fever (YF) infection in the African green monkey was evaluated during hyperalimentation and ad lib. feeding of biscuits.

Previous observations (3) suggested that following inoculation of 100 PFU of the Asibi strain of YF in the African green monkey a nonlethal clinical illness develops. Therefore, the tethering system was utilized to study 4 monkeys at a time which received either total IV nutrient support or oral nutrient support with IV electrolytes. In each replicate study, 2 monkeys received the standard "hyperal" solution (amino acid plus 25% dextrose) at the rate of 100 mg/day, while the other 2 received the IV electrolytes at the same infusion rate and were allowed to eat biscuits ad lib. The monkeys were started on nutrient support immediately after surgery, and 3-4 days later were injected SC with 100-8000 PFU of YF virus. By day 2, all monkeys developed fever; viremia was usually present by day 3-4.

By day 3 after exposure to YF, the monkeys receiving oral biscuits developed severe anorexia with resulting negative nitrogen balance for the next 11 days, and a 230-g weight loss. In contrast, those infected monkeys receiving the hyperal solution remained in positive nitrogen balance throughout the study, and after 24 days had gained 200 g. Thus, IV nutrient support prevented wasting of body protein during this viral infection.

Plasma albumin was decreased in both groups following surgery and subsequent tethering. In the hyperal group, plasma albumin concentration tended to remain constant following exposure to YF, while it tended to decrease slightly in the monkeys on oral support. Hematocrits fell slightly during infectious illness in the hyperal monkeys, but returned to normal during recovery. In contrast, hematocrits in orally supported monkeys did not return to preexposure values by termination of the experiment. Both groups of monkeys developed significant neutralizing antibodies by day 6 but were not different from each other. Thus, hyperalimentation maintained visceral protein synthesis during YF.

Only one of the 6 hyperal monkeys and 3 of 6 orally supported monkeys survived this infection. This is not a significant difference as measured by Fisher's exact test. However, in the monkeys on total IV support, the mean time to death was significantly longer, fever hours were reduced, and incidence of viremia was decreased

compared to oral feeding. Thus, the severity of infectious illness may be decreased by IV nutrient support, but survival from infectious illness is not improved compared to oral support. This suggests that the hyperalimentation solutions may have had a synergistic effect on pathogenesis of YF.

Since YF virus infection causes marked hepatocellular damage, plasma LDH was monitored in these monkeys. Plasma LDH was elevated on day 1 after surgery in both groups, and was subsequently shown to be related to the metathane anesthesia utilized during surgery. However, at the time of exposure to YF, plasma LDH concentrations had returned to presurgery concentrations. In monkeys receiving oral nutrition, plasma LDH was markedly elevated by day 5, which was followed by death of 2 monkeys. In contrast, plasma LDH was not markedly elevated in the hyperal monkeys until day 9, and was associated with the death of 4 of the monkeys. These data suggest that hyperalimentation decreased the onset of the YF-induced hepatocellular lesion, but once induced, the severity of hepatic damage was greater than that observed in orally fed monkeys.

In the monkeys receiving hyperalimentation, hepatocellular damage was associated also with a marked glucosuria. In contrast, urinary glucose was < 0.02 g/kg/day in monkeys on oral nutrition. Glucosuria in the hyperalimented monkeys was associated with marked hyperglycemia and elevated plasma insulin concentration, especially in the terminal stages of the infection. Glucose intolerance and insulin resistance had not been observed in the monkeys infected with extracellular bacterial organisms or in noninfected controls infused with similar amounts of hyperalimentation solution. Thus, it is possible that the 25% dextrose infusion compounded the severity of the reported hepatic lesion in this infection. Recently, dextrose infusion > 25% has been shown to produce hepatic damage in critically ill patients (4). Further, dextrose infusion > 10% caused glucose intolerance and insulin resistance in patients with hepatic damage. Therefore, infusion of > 10% dextrose in infections complicated by hepatocellular damage should be done with greater care and monitoring for glucose intolerance. From these observations, it was suggested that the high BCAA (48%) with 8% dextrose be tested as a possible IV nutrient support therapy during the YF infection in the African green monkey. In preliminary studies 6 monkeys infused with this solution developed viremia, mild clinical illness but no glucose intolerance. The monkeys survived SC injection of 1000 PFU of the Asibi strain YF, and maintained nitrogen equilibrium throughout the study. Thus, the 48% BCAA mixture with 8% dextrose may prove to be an effective form of nutrient support in infections that cause some hepatocellular damage.

Nutrient support during infection with a live vaccine strain of Francisella tularensis in the cynomolgus monkey. A cynomolgus monkey had catheters implanted in the jugular vein and carotid artery, was placed in a metabolic jacket and set up on the tethering system. Two weeks later the monkey was given a SC injection of 1×10^6 CFU of live vaccine strain of F. tularensis (LVS); 24 h later body temperature was slightly elevated, reaching a maximum of 38.8°C at 38 h and returning to normal by 48 h. Plasma LDH and SGOT were both elevated by day 2, reaching a 3-4-fold increase on day 3; they did not return to baseline concentrations until day 10. Food intake was decreased on days 7-10. On day 14 the monkey had a HI titer against LVS of 1:2048. The data on the cynomolgus monkey suggest that this organism causes a mild self-limiting infection, which is associated with relatively severe, transient liver damage. Since this organism is utilized to immunize at risk personnel and can be utilized in a class 2 facility, the LVS model of the cynomolgus monkey will prove helpful in development of nutrient support therapy for infections that develop hepatocellular damage.

In preliminary studies, some monkeys infused IV with hyperalimentation solution (amino acid mixture plus 25% dextrose) developed glucose intolerance 2-3 days after inoculation of the LVS. This glucose intolerance was characterized by plasma glucose concentrations of 250-350 mg/dl and 4+ glucosuria. Two of the monkeys required insulin treatment; and the third died during hyperglycemia. No difficulty was observed in monkeys injected with the LVS and maintained on biscuits. This is apparently another example of glucose intolerance during high dextrose infusions (25%) in monkeys with mild hepatocellular damage. Further, on day 21 plasma HI titers against LVS were almost a log lower in the monkeys receiving hyperalimentation compared to those fed biscuits. Whether this represents reduced immune response or decreased bacterial growth is unknown at this time.

Presentations:

1. Wannemacher, Jr., R. W. The biological immune response: effect of dietary amino acids. Presented, 1979 Animal Nutrition Research Council, Arlington, VA, 18 Oct 1979.

2. Wannemacher, Jr., R. W. The metabolic response of the host during infectious disease and various substrate support. Presented, The Boston Metabolic Club, Boston, MA, 20 Dec 1979.

3. Wannemacher, Jr., R. W. Use of branched-chain amino acids during trauma and sepsis. Presented, 4th Clin. Congr. Am. Soc. Parenteral Enteral Nutr., Chicago, IL, 30 Jan-2 Feb 1980.

4. Wannemacher, Jr., R. W. and R. E. Dinterman. Use of enteral nutrition to prevent protein wasting during sepsis in the cynomolgus (CM) monkeys. Presented, 4th Clin. Congr. Am. Soc. Parenteral Enteral Nutr., Chicago, IL, 30 Jan-2 Feb 1980 (JPEN 3:524, 1979).

5. Wannemacher, Jr., R. W., R. E. Dinterman, J. M. Bryant, and E. L. Stephen. Use of metabolic infusion jacket model to evaluate the effects of hyperalimentation (HA) on host defense against yellow fever virus (YF) infection in African green monkeys (M). Presented, 64th Annu. Mtg., FASEB, Anaheim, CA, 13-18 Apr 1980 (Fed. Proc. 39:888, 1980).

6. Wannemacher, Jr., R. W., R. E. Dinterman, J. M. Bryant, and G. A. McNamee. Amino acid and/or dextrose infusion for preventing nitrogen wasting resulting from minor surgery or sepsis in monkeys. Presented, Annu. Mtg. Am. Soc. Clin. Nutr., Washington, DC, 9-11 May 1980 (Clin. Res. 28:602A, 1980; Am. J. Clin. Nutr. 33:917, 1980).

Publications:

1. Wannemacher, Jr., R. W., J. G. Pace, F. A. Beall, R. E. Dinterman, V. J. Petrella, and H. A. Neufeld. 1979. Role of the liver in regulation of ketone body production during sepsis. J. Clin. Invest. 64:1565-1572.

2. Wannemacher, Jr., R. W., C. L. Hadick, Jr., and W. R. Beisel. 1979. Nutrition and infection interrelationships in monkeys, pp. 315-340. In Primates in Nutritional Research (K. C. Hayes, ed.), Academic Press, New York.

3. Wannemacher, Jr., R. W., F. A. Beall, P. G. Canonico, R. E. Dinterman, C. L. Hadick, and H. A. Neufeld. 1980. Glucose and alanine metabolism during bacterial infections in rats and rhesus monkeys. Metabolism 29:201-212.

4. Beisel, W. R., and R. W. Wannemacher, Jr. 1980. Gluconeogenesis, ureagenesis and ketogenesis during sepsis. JPEN 4:277-285.

5. Wannemacher, Jr., R. W. 1980. The biological immune response -- a review of the effect of dietary amino acids. Feedstuffs 52:16-18, 20, 24, 25.

6. Wannemacher, Jr., R. W., and R. E. Dinterman. 1980. Diurnal response in endogenous amino acid oxidation of meal-fed rats. Biochem. J. 190:663-671.

LITERATURE CITED

1. Wannemacher, Jr., R. W. 1978. Basic changes in protein metabolism during stress, pp. 202-209. In Western Hemisphere Nutrition Congress V, Nutrition in Transition (P. L. White, and N. Selvey, eds). American Medical Association, Chicago.

2. Wannemacher, Jr., R. W., M. V. Kaminski, Jr., H. A. Neufeld, R. E. Dinterman, K. A. Bostian, and C. L. Hadick. 1978. Protein-sparing therapy during pneumococcal infection in rhesus monkeys. JPEN 2:507-518.

3. U.S. Army Medical Research Institute of Infectious Diseases. 1 October 1979. Annual Progress Report, FY 79. USAMRIID, Fort Detrick, Frederick, MD, in press.

4. Blackburn, G. L., J. D. B. Miller, B. R. Bistrian, J. P. Flatt, and H. Y. Rienhoff. 1977. Amino acids -- key nutrient in the response to injury, pp. 305-330. In Nutritional Aspects of the Care of the Critically Ill (J. R. Richards, and J. M. Kinney, eds.). Churchill Liningstone, Edinburgh.

5. Wannemacher, Jr., R. W., C. L. Hadick, Jr., and W. R. Beisel. 1979. Nutrition and infection interrelationships in monkeys, pp. 315-340. In Primates in Nutritional Research (K. C. Hayes, ed.). Academic Press, New York.

6. Murray, M. J., and A. B. Murray. 1979. Anorexia of infection as a mechanism of host defense. Am. J. Clin. Nutr. 37:593-596.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0[illegible]5412	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY ACTY	6. WORK SECURITY	7. REGRADE	8. HNDLG INSTR'S	9A. SPECIFIC DATA-CONTRACTOR ACCESS	9B. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10A. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	008
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (U) Therapeutic correction of energy metabolism alterations during infection of unique importance in military medicine

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 07	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE	PREVIOUS/CURRENT FY	A. PROFESSIONAL MAN YRS	B. FUNDS (In thousands)
FISCAL YEAR	80	1.0	213
	81	0	0

19. RESPONSIBLE DOD ORGANIZATION
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ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
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20. GENERAL USE
Foreign intelligence considered

20. PERFORMING ORGANIZATION
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ASSOCIATE INVESTIGATORS
NAME: Beall, F. A. POC:DA
NAME:

21. KEYWORDS (U) Military medicine; (U) BW defense; (U) Inflammation; (U) Infection; (U) Metabolism; (U) Ketone bodies; (U) Lipids; (U) Trauma; (U) Stress

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

23 (U) Maintain by appropriate therapy body stores utilized as a source of energy during infectious diseases of unique importance in military medicine and biological warfare. During periods of decreased food intake, the body uses its own fat and protein to supply energy. Decreased ability of the host to use its fat stores during infectious disease could explain observed marked protein wasting. Understanding these metabolic changes can lead to effective nutrient therapy, reducing protein-wasting and promoting rapid recovery.

24 (U) Microanalytical methods are applied to study of various metabolites and alterations caused by infection.

25 (U) 79 10 - 80 09 - A striking metabolic change which occurs during a stress of inflammatory or infectious nature is the failure of a host to produce adequate amounts of ketone bodies during the fasting or anorexia. Using bacterial and viral infections and noninfectious inflammatory stresses, studies continued on the nature of the inhibition. 2 endocrine involvements with this metabolic alteration have been identified; in infectious or inflammatory stress the fasting insulin level is raised; and second, when infection is imposed on hypophysectomized rats, infection-induced inhibition of ketonemia is abolished. Work concentrated on 2 distinct areas to attempt to: define a direct role of insulin and identify the mechanisms by which the pituitary affects the pancreas. Progress has not been rapid. It was reported previously that the vagus nerve is not involved; this has been confirmed. The function of the sympathetic venous system is under study. Studies are underway using hypophysectomized rats and replacement hormone therapy to attempt to identify the nature of the relationship between pituitary and pancreas.

Publications: Fed. Proc. 39:2115, 1980; Endocrinology 107:596-601, 1980.

Terminated for management efficiency. Continued in W.U. S10 AQ 197. (DAOG1529)

DD FORM 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.

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U).
(3M161102BS03)

Task No. 3M161102BS10 AQ: Enhancement of Host Defense Against Agents of Potential BW Importance

Work Unit No. S10 AQ 176: Therapeutic Correction of Energy Metabolism Alterations
(S03 00 008) During Infection of Unique Importance in Military Medicine

Background:

Previous reports from this laboratory, in particular, have shown that the reduced ability to produce ketone bodies during fasting associated with acute infection is a primary metabolic dysfunction. The primary effort during the past 2 years has been to identify the primary mechanism which may explain this metabolic aberration. It has now been established that variations in normal endocrine function occur during the inflamed or infected state and are probably at the cause of the dysfunction. A major effort is now being expended to identify exactly the nature of this endocrine response, its implications, and means to correct it.

Progress:

Last year we reported that during the anorexia accompanying any infectious or inflammatory stress imposed upon the rat, normal ketogenesis was drastically impaired. The ketone body phenomena appeared to be closely related to an endocrine response involving both the pancreas and the hypophysis. The major effort expended this year has been an intensive study into the interrelationship between endocrine function and the onset and progress of an infectious or inflammatory process.

In infectious or inflammatory stress the following endocrine dysfunctions have been identified: (a) during the anorectic state there is an elevation of insulin; (b) an elevation of glucagon; and (c) there is no inhibition of fasting ketosis or elevation of insulin and glucagon if the hypophysis is not present.

The role of insulin seems to be predominant although a direct relationship between insulin and the depression of ketone bodies has yet to be established. The following are the implications of the role of insulin in this important metabolic event accompanying infection or inflammation. (a) In all rat models of inflammation or infection so far examined, there has been a rise in the plasma level of insulin approaching the fed value despite the fact that the rats were deprived of food; (b) when an infection has been imposed upon rats made diabetic (administration of streptozotocin) there is no depression of plasma ketone bodies during fasting; (c) hypophysectomized rats do not show a ketone depression during infection. Insulin values in such rats are extremely low; (d) administration of insulin to noninfected fasting rats whose ketone bodies are high, causes an immediate depression in plasma ketone bodies; and (e) administration of a bolus of glucagon to fed rats whose plasma ketones are low causes an immediate rise in plasma ketones which is short-lived because within several minutes plasma insulin

becomes elevated and the ketones are depressed.

All these represent indirect evidence for the role of insulin; direct evidence is still lacking. It has not been possible to demonstrate a direct relationship between insulin and the ketone effort. For example, if insulin is added during a rat liver perfusion study, no effect on ketone bodies can be demonstrated. Work is now progressing using isolated hepatocytes in an effort to see if an effect can be identified.

Additionally, it has been demonstrated that if an infection is given to a doubly vagotomized rat, inhibition of ketone bodies occurs. This indicates that the parasympathetic nervous system is not involved. Experiments are currently initiated to block α- and β-adrenergic receptors in order to evaluate the role of the sympathetic nervous system.

A series of experiments were performed in collaboration with Dr. Jemski (Aerobiology Division) in order to ascertain whether the same metabolic alterations would occur to rats made ill by aerosol exposure. Rats were exposed to Francisella tularensis, SCHU S-4 strain and the blood plasma analyzed for the usual parameters. In all studies, the metabolic variations were identical to those seen in the laboratory with nonaerosol induced infection.

Presentations:

1. Neufeld, H. A., Chairman, Carnitine Symposium, Cutter Laboratories, Chicago, IL, Feb 1980.

2. Neufeld, H. A. The effect of inflammatory stress on ketone bodies and glucoregulatory hormones. Presented, Hood College, Symposium for Gifted Students, Frederick, MD, Mar 1980.

3. Neufeld, H. A. The effect of inflammatory stress on ketone bodies and glucoregulatory hormones. Presented, Department of Biochemistry, Dental School, University of Maryland, Baltimore, MD, Mar 1980.

4. Neufeld, H. A. The effect of inflammatory stress on ketone bodies and glucoregulatory hormones. Presented, Department of Physiology, Medical School, Louisiana State University, New Orleans, LA, Jun 1980.

5. Neufeld, H. A. Chemiluminescence and bioluminescence in rapid detection. Presented, Workshop on Rapid Identification of BW Agents, Fort Detrick, MD, May 1980.

6. Neufeld, H. A., and Pace, J. G. The effect of stress in ketogenesis and glucoregulatory hormones. Presented, Annu. Mtg., Am. Soc. Biol. Chemists, New Orleans, LA, 1-5 Jun 1980 (Fed. Proc. 39:2115, 1980).

Publication:

1. Neufeld, H. A., J. G. Pace, M. V. Kaminski, Jr., D. T. George, P. B. Jahrling, R. W. Wannemacher, Jr., and W. R. Beisel. 1980. A probable endocrine basis for the depression of ketone bodies during infectious or inflammatory state in rats. Endocrinology 107:596-601.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0E6422	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION U		U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M161102BS03	00	013
b. [illegible] /				
c. [illegible] /	STOG 80-7.2:2			

11. TITLE (Include with Security Classification Code)

(U) Changes in leukocyte function during the course of viral and bacterial infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
73 07	80 09	DA			C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PREVIOUS 80	1.0	159
	CURRENT 81	0	0

19a. RESPONSIBLE DOD ORGANIZATION

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TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

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PRINCIPAL INVESTIGATOR (Provide SSAN if U.S. Academic participation)
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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Provide KEYWORDS with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Leukocytes; (U) Polymorphonuclear leukocytes; (U) Laboratory animals; (U) Bacterial; (U) Viruses

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

23 (U) Examine metabolic changes which occur in circulating leukocytes induced by exposure to potential BW agents and evaluate the usefulness of these physiological alterations as an aid in the early detection, diagnosis and prognosis of infectious illnesses of interest in BW defense.

24 (U) Using appropriate experimental animal models for bacterial, viral, and rickettsial infections determine leukocyte functions as measured by chemiluminescence, phagocytosis and chemotaxis.

25 (U) 79 10 - 80 09 - Results indicate polymorphonuclear (PMN) chemiluminescence (CL) may be extremely valuable as an aid in the differentiation of bacterial and viral infections in the host. Stimulation of PMN by endogenous pyrogen during bacterial infections may be partially responsible for the elevated CL response. Following PMN stimulation, reactions of the microbicidal superoxide anion and hydrogen peroxide with luminol directly or indirectly contribute to the CL phenomena. Initial results indicate antibody as well as complement are required for opsonization of bacteria and virus to stimulate PMN CL in vitro.

Publications: Fed. Proc. 39:5913, 1980; Proc. 2nd Int. Symp. Chemiluminescence Infect. Immun. 30: in press, 1980.

Terminated for management efficiency. Continued in W.U. 870 BC 068. (DAOG3811)

(If available to contractors upon originator's approval)

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BODY OF REPORT

Project No. 3M162770A870: Risk Assessment of Military Disease Hazards (U)
(3M161102BS03)

Task No. 3M162770A870 BC: Prevention of Viral Diseases of Potential BW Importance

Work Unit No. 870 BC 047: Changes in Leukocyte Function During the Course of Viral
(S03 00 013) and Bacterial Infections

Background:

Circulating peripheral polymorphonuclear leukocytes (PMN) constitute the primary defense against foreign agents introduced into the host by routes other than the digestive tract. Numerous studies have shown that light, termed chemiluminescence (CL), can be measured from PMN stimulated in vitro by particulate matter (zymosan), bacteria, viruses, and soluble agents (e.g., NaF). Activation of the PMN results in increased metabolic activities known as the respiratory burst, which include oxygen consumption, hexose monophosphate shunt activity, enzymatic activity and the production of microbicidal factors.

The microbicidal factors released during the respiratory burst include singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydroxyl radical ($\cdot OH$) and H_2O_2. Allen et al. (1) have proposed that the direct decay of $^1O_2$ to ground state or the transfer of energy from $^1O_2$ to products generated during microbicidal activity and their subsequent release of energy may be responsible for the PMN CL phenomena. Subsequent studies by Stevens et al. (2) demonstrated the CL response could be amplified by the addition of the cyclic hydrazide, luminol, to the PMN suspension. Luminol is proposed to react with O_2^-, $\cdot OH$, $^1O_2$, and H_2O_2 by forming a high energy aminothalate moiety, which upon decay, releases energy as light. Chemical amplification of the CL phenomenon reduces the number of PMN required for the measurements.

This work unit has been evaluating the use of PMN CL as an aid in the early detection, diagnosis and prognosis of militarily relevant infectious diseases. Studies during FY 1978-1979 resulted in the initial observation of enhanced endogenous CL measured from PMN isolated from bacteria-infected or endotoxemic rats. During the past fiscal year research was performed to clarify the mechanism(s) responsible for the elevated PMN CL response. To further evaluate PMN CL as an aid in the diagnosis of infectious diseases, experiments were performed to determine the CL response from immune and nonimmune guinea pigs challenged with Pichinde (PIC) virus. Studies were also initiated to determine the value of PMN CL as a tool for the identification of BW agents in vitro.

Progress:

Studies examining the mechanism(s) of the PMN CL response. Male, Fisher-Dunning rats were injected IP with 10^6 LVS Francisella tularensis organisms/100 g body weight; PMN CL was measured 24 h after injection. Control animals were injected with equal volumes of support medium. In some cases 200 ug of superoxide dismutase (SOD) or catalase (CAT) were added to the assay vial to determine the contributions of O_2^- and H_2O_2, respectively, to the enhanced endogenous PMN CL. Equal weight of bovine serum albumin (BSA) was used as a control for the enzymatic studies. SOD and

CAT depressed the PMN CL response 65 and 45%, respectively, compared to BSA control values. These results suggest that O_2^- and H_2O_2 contribute either directly or indirectly to the CL response measured from PMN stimulated in vivo during a bacterial infection.

Since our research had resulted in the initial observation of enhanced endogenous PMN CL during infection in the rat, no knowledge concerning the mechanism(s) of the in vivo stimulation of PMN was available. Kampschmidt et al. (3) reported that pretreatment of rats with rabbit leukocytic preparations (EP/LEM) increased survival following Salmonella typhimurium challenge; however, the mechanism of protection remained undefined. Studies were initiated to determine whether IP administration of humoral mediators such as EP/LEM, released by stimulated phagocytes, affected PMN CL in rats. PMN CL increased significantly 5 h after IP inoculation of EP/LEM (0.5 ml/100 g BW) and remained elevated for 48 h reaching an apparent maximum at 24 h. To correlate the elevated PMN CL response with protection rats were challenged IP with 2.4×10^9 LVS, 3×10^9 S. typhimurium or 2.4×10^1 Streptococcus pneumoniae/100 g BW 24 h following IP inoculation of EP/LEM (0.5 ml/100 g BW). Controls received an equal volume of heat-inactivated EP/LEM. Pretreatment of rats with EP/LEM significantly enhanced survival after challenge with the 3 organisms.

While these studies were in progress, Klempner et al. (4) reported that purified human leukocytic pyrogen (EP) stimulated the oxidative metabolism of human PMN in vitro and rabbit PMN in vivo as measured by glucose oxidation, superoxide production or NBT reduction. With the assistance of CPT Critz studies were performed to measure the in vitro effect of partially purified EP (PPEP) on rat PMN CL. PPEP also significantly stimulated (Ca. 10-fold) PMN CL in vitro.

These studies suggest that elevated PMN CL measured during infection may be partially attributed to stimulation by the humoral modulator EP. However, the phenomenon appears to be complex, since enhanced PMN CL has been measured in the absence of fever.

In vivo and in vitro effect of virus on PMN CL. Studies were conducted to further evaluate PMN CL as a diagnostic aid for infection in the host. Results from earlier studies showed enhanced endogenous PMN CL as measured in rats during various bacterial infections. Studies were performed to determine the PMN CL response to challenge by a model virus in immune and nonimmune animals. The PIC infection had been well-documented in guinea pigs by Dr. Jahrling (Virology Division) and was chosen for these studies. Initially, experiments were designed to determine if enhanced endogenous PMN CL occurred during a bacterial infection in the guinea pig. Guinea pigs were injected IP with 10^7 LVS/100 g BW; and PMN CL was measured 24 h later. Significantly enhanced (Ca. 30-fold) endogenous PMN CL was measured from infected guinea pigs compared to control values; however, the CL response from both the infected and control groups was lower compared to respective groups in similar rat studies. These results demonstrated that the enhanced endogenous PMN CL response measured during bacterial infection was not species specific. Variations noted in the CL response between species may be attributed to differences in the PMN oxidative metabolism. Guinea pigs were then inoculated SC with 40,000 PFU of PIC virus; PMN CL was measured on days 3, 8 and 11. Although significant fever and viremia were measured, no significant increase in PMN CL occurred during the study. Immune guinea pigs were also challenged SC with 40,000 PFU of PIC and CL was measured 12, 24, 48, 72, and 96 h later. No enhanced PMN CL was measured. Results from these studies suggest PMN CL may be a valuable aid for a differentiation between bacterial and viral infections in the host.

Evaluation of the Picolite Luminometer. The recently procured Picolite Luminometer was evaluated for implementation in PMN CL measurements. Studies were performed measuring PMN CL during LVS infection in rats and compared to results obtained using the Tricarb Liquid Scintillation Counter (TLSC) used in earlier studies. Results suggest the Picolite is not as sensitive as the TLSC for these measurements; however, this does not appear to affect significantly the ability to detect enhanced CL in the system studies. The Picolite also offers the advantage of compact size, easy mobility and a temperature controlled chamber essential for the PMN CL measurements. This new design will help to standardize the methods presently employed in PMN CL studies.

In vitro stimulation of PMN CL by virus and bacteria. Research was initiated to evaluate the PMN CL response as a tool for the early identification of BW agents in vitro. Initial studies examined the differentiation of opsonization of antigens using immune versus normal serum. Results indicate that opsonization of bacteria and virus is necessary to stimulate a significantly enhanced PMN CL in vitro. The studies also indicate the PMN CL response may correlate directly with the concentration of antibody as well as with protection against challenge in the immune host.

Presentations:

1. Sobocinski, P. Z., J. P. McCarthy, W. J. Critz. Stimulation of granulocyte (PMN) chemiluminescence (CL) by endogenous pyrogen (EP); possible relationship to protection against lethal bacterial infection. Presented, Annu. Mtg. Biol. Chemists, New Orleans, LA, Jun 1980 (Fed. Proc. 39:5913, 1980).

2. McCarthy, J. P., P. Z. Sobocinski, P. B. Jahrling, and D. W. Reichard. Differential effects of bacterial and viral infections on granulocyte chemiluminescence (CL). Presented, 2nd Int. Symp. Chemiluminescence, LaJolla, CA, Aug 1980.

Publication:

McCarthy, J. P., R. S. Bodroghy, P. B. Jahrling, and P. Z. Sobocinski. 1980. Differential alterations in host peripheral polymorphonuclear leukocyte chemiluminescence during the course of bacterial and viral infections. Infect. Immun. 30: in press.

LITERATURE CITED

1. Allen, R. C., R. L. Stjernholm, and R. H. Steele. 1972. Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Commun. 47:679-684.

2. Stevens, P., D. J. Winston, and K. Van Dyke. 1978. In vitro evaluation of opsonic and cellular granulocyte function by luminol-dependent chemiluminescence: utility in patients with severe neutrophenia and cellular deficiency states. Infect. Immun. 22:41-51.

3. Kampschmidt, R. F., and L. A. Pulliam. 1975. Stimulation of antimicrobial activity in the rat with leukocytic endogenous mediator. J. Reticuloendothel. Soc. 17:162-169.

4. Klempner, M. S., C. A. Dinarello, W. R. Henderson, and J. I. Gallin. 1979. Stimulation of neutrophil oxygen-dependent metabolism by human leukocytic pyrogen. J. Clin. Invest. 64:996-1002.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0H6411	80 01 09	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'S	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102B	3A161102BS03	00	015
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Provide with Security Classification Code): (U) Effects of infection/intoxication upon structure and function of cellular membranes

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
76 10	79 12	DA	

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING: 80	1.0	130
FISCAL YEAR CURRENT: 81	0.3	28

19. RESPONSIBLE DOD ORGANIZATION
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NAME: POC:DA

21. GENERAL USE

22. KEYWORDS (Provide KEYWORDS with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Lipogenesis; (U) Plasma membranes; (U) Enzyme regulation; (U) Macrophages; (U) Cytochemistry

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

23 (U) Determine the effects of infection upon the structure and function of liver cell membranes. The identification of infection-induced abnormalities will be useful in developing improved prophylactic and therapeutic measures aimed at the control of membrane-directed hormonal or metabolic activity for diseases of military importance.
24 (U) A variety of techniques, e.g., cell fractionation, biochemical and cytochemical analyses, and centrifugation analysis, are used to study effects of infection upon structure and function of cellular membranes. Bacteria and rickettsia are studied.
25 (U) 79 10 - 79 12 - Plasma membranes from livers of control and S. pneumoniae-infected rats were isolated. Their purity was determined. There was no difference in the amounts isolated between groups; however, differences were evident in relative activities of marker enzymes. Using tritiated leucine, it was determined that synthesis of liver plasma membranes was increased during infection. Total liver weight was greater in infected than in control rats. Coxiella burnetii in guinea pigs macrophages or in suspense could be inactivated by UV treatment. Such treatment for 15 sec had little effect on marker enzymes; although longer exposure did. Other studies were conducted on radiolabeled C. burnetii in guinea pig macrophages. Distribution of the label in a gradient did not correspond with lysosomes of the macrophage. Isolated and purified hepatic nuclei from S. pneumoniae-infected rats appeared to have increased numbers of insulin-binding sites. The insulin may stimulate these nuclei to synthesize RNA.
No further work will be done due to transfer of the investigator.

* Available to contractors upon originator's approval

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BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. S03 00 015: Effects of Infection/Intoxication Upon Structure and Function of Cellular Membranes

Background:

Maintaining the integrity of cell membranes is essential for normal cell function. Alterations in membrane structure may contribute significantly to cellular malfunction and ultimately enhance host susceptibility to infectious diseases. An understanding of the effect(s) of infection on both the structure and function of cell membranes will prove helpful in obtaining a more thorough understanding of the mechanism(s) involved in the pathogenesis of infectious diseases.

Progress:

The present study showed that phase I and II C. burnetii organisms either in suspension or growing within cultured guinea pig peritoneal macrophages were inactivated by brief exposure to UV light. Treatment of C. burnetii with UV decreased the risk to individuals working with this highly virulent species. Since certain procedures required for subsequent studies on the interaction of C. burnetii with macrophages could cause aerosolization, working with inactivated organisms is desirable. It must be noted, however, that photoreactivation of UV-inactivated organisms can take place. We have show that exposure of phase I or II C. burnetii to light under laboratory conditions for 3 days did not result in photoreactivation.

We have shown that exposure of macrophage cultures to UV light for 15 sec does not appreciably inactivate marker enzymes for macrophage organelles or their subsequent equilibration on linear sucrose gradients. These results suggest that UV treatment does not adversely affect the conformation of these proteins or alter the permeability to sucrose of lysosomes, microsomes or mitochondria. Since these enzymes retain most of their activity after UV treatment, they may be used confidently as markers for the localization of cellular constituents on linear sucrose gradients after fractionation of macrophage homogenates. It is now possible to determine the intracellular distribution and fate of C. burnetii in guinea pig peritoneal macrophages by using analytical methods of subcellular fractionation.

Finally, it has been shown that rickettsiae contain a number of different enzymes. Our results suggest that neither phase I nor phase II C. burnetii contains detectable amounts of the enzymes assayed in these experiments. However, it must be noted that these preparations were UV-treated before assay. The effect of this UV treatment on the rickettsial enzymes is unknown. The absence in phases I and II of detectable quantities of enzymes chose as markers for macrophage organelles greatly simplifies and facilitates fractionation studies. It eliminates the requirement for correcting macrophage enzyme activities for those due to rickettsiae.

The work unit was terminated in January 1980, due to transfer of the Principal Investigator.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. ENTER HISTORY	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	019
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE: (U) Mechanism of action of bacterial exotoxins

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 016800 Toxicology; 002300 Biochemistry

13A. START DATE	13B. ESTIMATED COMPLETION DATE	14. FUNDING AGENCY	15. PERFORMANCE METHOD
75 11	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE

		a. PROFESSIONAL MAN YRS	b. FUNDS
FISCAL YEAR	PRECEDING 80	1.0	184
	CURRENT 81	0	0

19A. RESPONSIBLE DOD ORGANIZATION
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ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Provide KEYWORDS with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Food poisoning; (U) Infected burns; (U) Pseudomonas; (U) Staphylococcus; (U) Prophylaxis; (U) Therapy

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

23 (U) Elucidate mechanisms of action of bacterial exotoxins in order to develop prophylactic or therapeutic measures, to augment or replace immunization. Agents will be sought to interfere at 1, preferably 2 or more steps in the mechanism. Since most toxins are enyzmes and many act intracellularly, present objectives are directed to blocking toxin-receptor interactions, toxin internalization or enzymatic activity.

24 (U) A well-defined model, diphtheria toxin (DE) is employed to obtain basic information. Various levels of effort are devoted to other militarily important toxins. The basic line of investigation used is to find and characterize cell line(s) or tissue(s) susceptible to a toxin; study toxin-induced biophysical and biochemical events leading to cell/tissue response; test drugs to find those which may protect the cell/tissue from the toxin; and test positive findings in laboratory animals to determine human therapeutic potential.

25 (U) 79 10 - 80 09 - Various cellular controls on DE receptors were elucidated. A ligand-induced "down regulation" was described, recovery from which is RNA- and protein synthesis-dependent, suggesting new receptor synthesis and little or no recycling. Certain metabolic inhibitors were shown to protect cells from DE by inducing a complete loss of toxin receptor. Continuous energy production seems to be required to maintain receptor at the cell surface. The lysosomotropic drug, chloroquine, both protected cells and inhibited degradation and excretion of radiolabeled toxin by cells, suggesting lysosomal involvement in delivery of DE to cell cytoplasm. Finally, ammonium chloride, a drug previously believed to protect cells from DE by preventing internalization, was studied. Several lines of evidence suggest that it acts intracellularly, not at the cell surface. Terminated for management efficiency. Continued in W.U. S10 AN 200. AO 199. Publication: J. Biol. Chem. 254:11337-11342, 1979, 255:2247-2250, 1980; Biochem. Biophys Acta 621:233-240, 1980; In Natural Toxins, pp. 463-470, 1980.

(DAOG1519 &DAOG1522)

DD FORM 1498 (1 MAR 80) PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M161102BS03)

Task No. 3M161102BS03-AN: Characterization of Microbial Toxins of Potential BW Importance

Work Unit No. S10-AN-162: Mechanism of Action of Bacterial Exotoxins
(03 00 019)

Background:

Bacterial toxins mediate the harmful effects of many bacterial infections. In some instances, morbidity results from preformed toxins, i.e., botulinum or staphylococcal food poisoning, while in others from toxin elaborated as the organism grows in the host, i.e., diphtheria or cholera. In either case, it is clear that successful treatment of, or protection from, the disease must deal with the toxin. In theory, this could be accomplished either prophylactically (immunization) or therapeutically (specific drug or antidote). While the former approach has been successful for several infections (diphtheria and tetanus, for example), there are enormous problems (logistical and medical) with immunizing an entire at-risk population. Moreover, it is by no means clear that all toxins will be amenable to toxoiding or that immunization will always confer adequate protection. It, therefore, seems prudent to make some efforts towards the development of therapeutic measures. To this end, it is our purpose to study the mechanisms of action of bacterial exotoxins and to test available drugs (or develop new ones) for their therapeutic potential.

At this project's inception, the available evidence indicated that each bacterial toxin was quite unique in its mode of action. Studies published since that time have proven that notion wrong. It now appears that many bacterial protein toxins are bipartite, one portion mediating the binding specificity and the other carrying out an enzymatic function. Moreover, it is of considerable interest that, of the 4 toxins whose molecular mechanisms of action are known, all have similar enzymatic (ADP-ribosylating) activities. Generally speaking, a microbial toxin's action can be divided into 3 stages: toxin-receptor binding, internalization of the toxin or toxin-receptor complex, and expression of enzymatic activity. In theory, one could develop drugs which intervene at any or all of these stages. During the past year, we have continued our efforts directed toward the first stage. One might obtain toxin analogues which would compete with authentic toxin for receptor occupancy. One might synthesize drugs which block binding for some other reason. Or one might induce the cell to "shed" or remove its toxin receptor thus conferring protection. Whatever approach is taken, it becomes critical to know how the cell controls its toxin receptor numbers. To our knowledge, no such information exists for any toxin. We therefore undertook such a study using our diphtheria toxin-Vero cell model system. The other main laboratory effort this year was the beginning of investigations into the second stage of toxin action, namely internalization.

Progress:

Continuing our investigations into how cells control toxin receptor numbers, we tested diphtheria toxin (DE) analogs to see whether a "down regulation" phenomenon is operative in Vero cells. Down regulation of receptor numbers is a well-documented phenomenon in hormone systems. However, such cellular controls have not yet been reported for toxin receptor systems. With DE, one has the added problem that the toxin itself is cytotoxic and very rapidly so. Thus, we found it necessary to use nontoxic analogs of DE to measure ligand-induced regulation of receptor numbers; most of our work was carried out with CRM-197. This protein binds normally to the DE receptor but, due to a point mutation in the enzymatically active A fragment, is not cytotoxic.

We first examined the effects of different CPM-197 concentrations on cell-surface DE receptor values after an incubation overnight at 37°C. We found that as the CRM-197 concentration increases the number of receptors remaining decreases. Moreover, the curve for receptor depletion was essentially a mirror image of the saturation curve for DE-receptor binding. This observation simply means that there is roughly one receptor lost for each toxin molecule bound. While this point may seem trivial, some hormone receptor systems have been shown to lose (down-regulate) 80-100% of the receptors when only 20-30% were occupied by ligand; such is obviously not the case with DE.

Next, the time course of CRM-197 induced DE receptor loss was determined. Using a saturating level of CRM-197, about 2 h were required to deplete binding capacity to very low values. We previously found that the halftime for prebound toxin to enter Vero cells is 25 min. Although we do not have detailed information about the rate constant for toxin-receptor binding at 37°C, the 1.5-2 hr time period for depletion is in the range expected for sequential toxin-receptor binding followed by internalization of the complex. In other words, receptor is probably depleted because it is taken inside the cell along with toxin.

Vero cells are capable of restoring the original number of DE receptors. If receptors are depleted and CRM-197 then washed out, the receptor numbers return to control values in 3-4 h. We asked the question - does the regain of receptor reflect synthesis of new receptor, or reutilization of that which accompanied CRM-197 inside the cell (recycling)? Because regain of receptor was blocked by inhibitors of either mRNA or protein synthesis, it would appear that new receptor synthesis is required and that receptor internalized with toxin is not recycled.

Some effort was devoted to a study of the effects of metabolic inhibitors on DE receptor numbers. These "drugs" are known to be very powerful protective agents from diphtheria toxin, although their mechanism of protection is obscure. We began by looking at the effects of several metabolic inhibitors after 1 or 2 h incubation with cells. As shown in Table I, cyanide and 2,4-dinitrophenol had little or no effect on DE receptor numbers. Deoxyglucose reduced receptors to about 40% of control values. Azide was even more effective, reducing receptor numbers by about 90%. Most powerful in this regard was salicylate and fluoride, both of which induced a complete loss of cell surface diphtheria toxin receptors (5% is considered the limit of detection).

TABLE I

EFFECT OF METABOLIC ENERGY INHIBITORS
OF DIPHTHERIA TOXIN RECEPTOR LEVELS

Agent (concentration)	% of Control Receptor After: 1 h	2 h
Cyanide (5 mM)	75	102
2,4-Dinitrophenol (0.5 mM)	95	81
2-Deoxyglucose (100 mM)	41	39
Azide (50 mM)	14	9
Salicylate (30 mM)	7	4
Fluoride (10 mM)	0	1

The kinetics of metabolic inhibitor-induced loss of toxin receptors were determined. Fluoride and salicylate induced a rapid and complete loss of receptors apparently at similar rates. The loss of toxin receptors after azide treatment was somewhat slower but ultimately complete. In contrast, receptor loss leveled out at about 40% of control even after prolonged treatment with the maximally effective, but nontoxic concentration of deoxyglucose.

By plotting the early time-point data of receptor loss in a semilog manner, it was possible to obtain an estimate of the rate constants for receptor inactivation by each drug. Good straight line fits were obtained in each case ($r \geq 0.93$). The apparent first-order rate constants for fluoride and salicylate inactivation of toxin receptors were not appreciably different, 8.5×10^{-2} and 7.8×10^{-2} min^{-1}, respectively. These values correspond to half-lives of 8.2 and 8.9 min. The rate constant for azide inactivation was 2.4×10^{-2} min^{-1} (half-life), 29 min) while for deoxyglucose the value was 1.7×10^{-2} min^{-1} (half-life, 40 min).

When used at sufficient concentration, metabolic inhibitors are potent cellular poisons. To demonstrate that their effects on diphtheria toxin receptor numbers were not due to nonspecific toxicity of the drugs, reversibility of the receptor loss was assessed. After a preincubation period to inactivate toxin receptors, the drug-containing medium was removed, fresh medium added, and incubation continued. Cells maintained at 37°C promptly and completely regained their toxin binding capacity; control receptor levels were usually obtained within 2 h. If cells were incubated at 4°C after the medium change, no increase in receptor was observed.

After binding to its receptor, the next stage of many toxin's action is internalization. Several studies were carried out to gain some insight into this process. One line of investigation involved the lysosomal drug, chloroquine. In collaboration with Drs. Leppla and Dorland (Pathology Division), it was determined that chloroquine protects cells from DE and that the drug probably acts at the level of the lysosome. This observation suggests that DE is targeted to the lysosome, a general process similar to many hormones. Binding to a receptor and transport of the ligand-receptor complex to the lysosome is a process now being

called "receptor-mediated endocytosis." This concept is at considerable odds with other ongoing theories as to how toxins enter cells. However, the evidence supporting this route of entry is just as good as the evidence supporting other theories, so only time and further study will reveal the truth.

Another line of investigation arose from a question raised at the International Conference on Toxins I attended in August, 1979. At that time, there was a good deal of discussion about the effects of NH_4Cl on the toxicity of several toxins, including diphtheria toxin. The majority view was that NH_4Cl protects cells by maintaining DE at the cell surface, i.e., by blocking internalization. The basis for this view resides in the results of experiments run by 2 laboratories (Groman's and Bonventre's) some time ago. In both cases, it was observed that cells incubated with DE in the presence of NH_4Cl were completely protected. If the NH_4Cl was removed, the cells died. If antitoxin was added immediately after NH_4Cl removal, the cells survived. The conclusion reached by both laboratories was that NH_4Cl held DE at the cell surface where it could not enter the cell. If NH_4Cl was removed from the medium, receptor-bound toxin was free to enter the cell and express its toxicity. If antitoxin was added soon enough, the cell-surface bound toxin was neutralized before it had the opportunity to enter the cell and the cell was protected. All these data notwithstanding, we had studied the effects of NH_4Cl in our Vero cell-CD system and obtained results incompatible with the above interpretation. Using the assay for determining the fraction of cell surface vs. internalized DE that she developed, Dr. Dorland showed that the DE internalization rate was not affected by NH_4Cl. Moreover, with the help of Dr. John White, we were able to confirm this observation by an autoradiographic approach. Our conclusion was, therefore, that NH_4Cl did not maintain the DE-receptor complex at the cell surface.

How could we reconcile these 2 lines of contradictory evidence? The first clue was found in a review by Drs. Gill, Pappenheimer, and Uchida (1) in which they mentioned the problem of DE sticking to tissue culture surfaces. It occurred to us that DE initially sticking, then coming off at a later time, might explain the earlier antibody protection data. In order to test this possibility, a series of experiments were carried out. First, we added radiolabeled DE to wells with no cells, but containing the same medium and serum complement employed in the previously published antibody experiments. After a few hours, the wells were washed thoroughly and treated with 0.1 M NaOH to solubilize any remaining protein, including, of course, toxin. These samples were then assayed for radioactivity and, employing the specific activity of the preparation, a straightforward calculation was made to measure the tissue culture surface-bound DE as a function of added DE concentrations. We found DE stuck to the tissue culture surface in a linear relationship. Significantly, a simple calculation showed that Bonventre used concentrations of DE in the range 1-3 μg/ml for his antibody experiments. From our data, this concentration should result in the "sticking" of approximately 0.3-0.8 ng. Since the 48-h LD_{50} for the cell line used by Bonventre (HEp-2) is 0.3 ng/ml, it is clear that the potential for artifactual results exists and his data should be viewed cautiously.

To demonstrate that there is more than just potential for artifacts, we ran the following type of experiment. Tissue culture plates without cells were incubated for 1 h with various DE concentrations of NH_4Cl-containing medium. The wells were then carefully washed and lightly trypsinized Vero cells were seeded in the wells. Besides control cells, aliquots which contained 1 μg/ml

of CRM-197 (a nontoxic variant of DE) or highly avid horse anti-DE were also seeded. After 48 h, the usual cytotoxicity assay was employed to assess toxicity. Cells added in medium alone were killed in a dose-dependent manner. Cells in medium containing antitoxin were completely protected. These are, of course, exactly the same results obtained by both Groman and Bonventre. Clearly, however, NH_4Cl did not retain the toxin at the cell surface because the cells were never incubated with DE in solution, only with the washed wells. Obviously, the toxin stuck to the surface was coming off and interacting with the cells, ultimately leading to death. Antitoxin was able to confer protection not because toxin was on the cell surface but was on the tissue culture surface. Finally, CRM-197 was also a good protector under this protocol, almost certainly due to its competition with authentic toxin for receptor sites. This offers even more proof that the earlier workers were in error in their interpretations. Similar results were obtained with BHK-21 cells, a line with essentially the same sensitivity to DE as the HEp-2 cells employed by Bonventre.

Although we have shown that Bonventre and Groman worked in artifactual ranges of DE concentrations, this does not prove whether or not NH_4Cl keeps DE on the cell surface. Thus, we repeated their experiment; we employed levels of DE below that at which sticking presented a problem. Vero cells were incubated with various concentrations of DE, all in the presence of NH_4Cl (1 mg/ml). After 1 h at 37°C, the monolayers were washed well and medium containing 1 mg/ml CRM-197, or medium containing antitoxin, were added back. After 48 h, cytotoxicity was assessed; the results were quite informative. At a concentration of 10-30 ng/ml, virtually complete protection was observed after washing out the toxin and NH_4Cl. This concentration is equivalent to 30-100 LD_{50}, as shown by a control incubation without NH_4Cl. Clearly, antibody was not required to protect the cells, giving no support to the notion that NH_4Cl maintains toxin at the cell surface. At concentrations > 30 ng/ml, we found antitoxin was required for complete protection, but in this concentration range, there was a biologically significant amount of DE stuck to the tissue culture surface. Cells were also protected by CRM-197. It is hard to understand how CRM-197 could work except by blocking toxin not yet bound to receptor, i.e., "stuck" toxin coming off the tissue culture surface. We would, therefore, suggest that NH_4Cl probably protects cells from DE by a mechanism involving effects on vesicles or lysosome. Such actions of NH_4Cl have been reported by many other laboratories working with other systems.

Presentations:

1. Middlebrook, J.L. Studies on the mechanism of action of diphtheria toxin. Presented, Invited seminar, at the Uniformed Services University of Health Sciences, Feb 1980.

2. Middlebrook, J.L., and R.B. Dorland. Receptor-mediated binding and internalization of diphtheria toxin. Presented, Conference on Receptor-Mediated Binding and Internalization of Toxins and Hormones, USAMRIID, 24-26 March, 1980.

Publications:

1. Dorland, R.B., J.L. Middlebrook, and S.H. Leppla. 1979. Receptor-mediated internalization and degradation of diphtheria toxin by monkey kidney cells. J. Biol. Chem. 254:11337-11342.

2. Middlebrook, J.L., L. Spero, and P. Argos. 1980. The secondary structure of staphylococcal enterotoxins A, B and C. Biochim. Biophys. Acta 721:233-240.

3. Middlebrook, J.L., R.B. Dorland, S.H. Leppla, and J.D. White. 1980. Receptor-mediated binding and internalization of Pseudomonas exotoxin A and diphtheria exotoxin by mammalian cells, pp. 463-470. In Natural Toxins (D. Eaker and T. Wadström, eds.). Pergamon Press, Oxford.

4. Leppla, S.H., R.B. Dorland, and J.L. Middlebrook. 1980. Inhibition of diphtheria toxin degradation and cytotoxic action by chloroquine. J. Biol. Chem. 255:2247-2250.

LITERATURE CITED

1. Gill, D.M., A.M. Pappenheimer, Jr., and T. Uchida. 1973. Diphtheria toxin, protein synthesis, and the cell. Fed. Proc. 32:1508-1515.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OH6414	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	9a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	021
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code) (U) Regulation and involvement of acute-phase proteins in infections of BW importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 04	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 80	1.0	85
	CURRENT 81	0	0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701
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TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Physical Sciences Division
ADDRESS: USAMRIID
Fort Detrick, MD 21701
PRINCIPAL INVESTIGATOR
NAME: Thompson, W. L.
TELEPHONE: 301 663-7181
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Wannemacher, Jr., R. W. POC:DA
NAME:

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Laboratory animals; (U) Protein synthesis; (U) RNA synthesis; (U) Early detection

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

23 (U) Study the hepatic regulation of acute-phase proteins during various types of infectious diseases. Determination of the effect of these proteins on the host's defense mechanism will enable evaluation of their importance during early stages of infection. Additional studies based on these findings would deal with approaches toward controlling these processes to the optimum benefit of the host in dealing with infectious diseases of BW importance.

24 (U) Quantitative and qualitative measurement of hepatic RNA in both in vivo and in vitro systems using control and infected animals will be made. Purification and development of specific antibodies to certain acute-phase proteins will be utilized to determine the type of products made by RNA from infected hepatic cells. The involvement of these proteins in humoral or cell-mediated immune response will also be studied. Various drugs will be tested for their effect on RNA and acute-phase protein production.

25 (U) 79 10 - 80 09 - Early increase in the production of acute-phase proteins in response to infection has led to the study of their regulation and function. Past studies on RNA regulation have shown that infection stimulates an increase in production of ribosomal and messenger RNA which are subsequently involved in the production of extracellular protein; this effect is dependent upon circulating levels of hydrocortisone. Endotoxin appears to stimulate a very early redistribution of RNA from bound to free ribosomes. Isolation and purification of antibodies to several acute-phase proteins continues in order to provide material for studies of their regulation and function. The assay systems currently being developed for this purpose are: ribosome binding, in vitro cell-free translation, lymphocyte transformation and antibody production. Terminated for management efficiency. Continued in W.U. S10 AQ 197(DAOG1529)
Publication: Am. J. Physiol. 238:G303-G311, 1980.

*Available to contractors upon originator's approval

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

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COPY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U).
(3M161102BS03)

Task No. 3M161102BS10 AQ: Enhancement of Host Defense Against Agents of Potential BW Importance

Work Unit No. S10 AQ 177: Regulation and Involvement of Acute-phase Proteins in
(S03 00 021) Infections of BW Importance

Background:

Numerous metabolic alterations occur in the host animal during the early stages of infection. Many of these demonstrate a sequential, interrelated series of events such as the cellular uptake of amino acids, increase in RNA transcription and the subsequent increase in production of certain serum proteins termed acute-phase proteins. These processes take place as an apparent host defense mechanism against the invading organism. Although it has been established that there is an increase in production of RNA which is directed to the bound ribosomes, presumably for the production of acute-phase proteins (1), further studies are needed to determine the mechanism(s) for the regulation of specific mRNA and their products. The recent finding that endotoxin causes a very early redistribution of cytoplasmic RNA from bound to free ribosomes (2) increases the possibility of additional cytoplasmic translational regulators other than just the available mRNA.

In addition to regulation of acute-phase protein production, it is also of interest to determine their involvement in the host's defense mechanisms. As stated previously, they have been implicated in such processes as antiproteolytic activity, wound healing, protection against free hemoglobin, transport of metal ions and regulation of the immune response. The study of the involvement of several acute-phase proteins in some of these processes can provide valuable information as to their specific function in the host response to infection and hopefully provide a general picture of the host's overall defense mechanisms.

Progress:

Work continues on the isolation of several rat serum acute-phase proteins. These are: α_1- and α_2-macroglobulin, haptoglobin, albumin and α_1-acid glycoprotein. The first step for all of them, except haptoglobin, is separating serum from turpentine-inflamed rats into 4 fractions on a gel filtration column. By knowledge of the MW range of the proteins in each of these fractions, it is possible to determine in which of the fractions a protein of interest should be located. Since we have found haptoglobin in 3 of the 4 fractions, a better yield results from its isolation using total serum rather than one of the fractions from the column. Changes in the properties of an ion exchange material (DEAE-cellulose) used in the isolation of most of these proteins has caused a delay in the purification procedures. The techniques originally used to isolate the 4 acute-phase proteins were no longer effective due to the use of DEAE-cellulose. However, a newer type manufactured by the same company was found to have about the same properties as the original material and has been used as an effective substitute since then.

Improvement in the yield and purity of the macroglobulins has been accomplished by making small adjustments in their step-wise elution off the final step ion exchange column. Testing of the resulting fractions on immunoelectrophoresis plates show that the α_2-macroglobulin is pure and the α_1-macroglobulin has only a trace of contamination. This can be removed by running through a CNBr-Sepharose affinity column with α_2-macroglobulin antibody bound to it.

Although albumin can be isolated using an affi-gel blue affinity column (BioRad), it is the only purified rat serum protein which has now become commercially available. Testing of the commercial product using immunoelectrophoresis and electrofocusing demonstrated its purity as a homogenous protein free from other contaminating serum proteins. Therefore in order to save time, it will be used in our assay systems and for developing antibodies in rabbits.

Development, purification and accumulation of antibodies for future use in assay systems, to each of these acute-phase proteins, as well as total antibodies to both normal and inflamed rat serum, is in progress using rabbits rather than goats. The advantage in the use of rabbits is in their accessibility and the fact that their antibodies can be isolated on protein A sepharose column, since this material binds rabbit IgG much better than goat. The use of CP 20,961 and lipid emulsion provided by Animal Assessment Division as adjuvants for development of these antibodies has worked well with all but the α_1-acid glycoprotein; however, its low titer may be due to the instability of the protein with storage or a poorly responding rabbit, rather than problems with the adjuvant.

IgG antibodies from the rabbit serum are routinely isolated by $(NH_4)_2\ SO_4$ precipitation, which prevents disruption of the protein A column by removing albumin, and subsequent purification on protein A sepharose columns. The use of affi-gel blue affinity columns which is used specifically to remove albumin, rather than $(NH_4)_2\ SO_4$ was unsuccessful, since the resulting IgG from the protein A column, although comparable in amount, showed very low antibody titer on Ouchterlony plates. The purified antibodies are either stored in the refrigerator or lyophilized when long-term storage is anticipated.

Studies have begun on the regulation of several of these acute-phase proteins using the ribosome binding assay of Taylor and Tse (3) and their involvement in the immune response using the procedure described by Murgita and Tomasi for mitogen-induced lymphocyte transformation and antibody production (4).

The ribosome binding assay involves the isolation at various times after infection of *in vivo* labeled free and bound ribosomal populations from rat liver. Aliquots from infected and paired control rats are treated with antibodies to several of the acute-phase proteins. They are then treated with a second antibody to allow precipitation of the ribosome-antibody complex. The radioactivity of the precipitate is determined to estimate the quantity of the specific protein being made at various stages of the infection. Preliminary studies have shown that there may be a problem with sensitivity of the assay, particularly when working with proteins normally found at low concentrations in the rat. However, these problems may be worked out by increasing the amount of radioactivity in the system and making alterations in the technique. Another approach to regulation studies being developed is the use of the *in vitro* cell free translation assay followed by isolation of the products on affinity columns having the specific antibodies bound to them. The success of this procedure depends on obtaining isolated mRNA from

bound ribosomes to translate proteins in the in vitro assay.

The involvement of several of these proteins, particularly the α-macroglobulins, in the immune response is being studied using the lymphocyte transformation and antibody production assays. Initial attempts at the transformation assay using whole rat blood and testing albumin and α_2-macroglobulin have shown a possible stimulation of the lymphocytes by the α_2-macroglobulin with no response to the albumin. When α_2-macroglobulin is incubated with the mitogen, concanavalin A (ConA), the activity is approximately half that seen with ConA treatment alone, but at about the same level of response as seen with α_2-macroglobulin treatment alone. Additional assays will be done to verify these results. Also refinements in the system, such as the use of spleen cells rather than whole blood, and dialysis of the sample against the assay buffer will be made to eliminate the possibility of nonspecific interactions causing the observed responses. The use of the antibody production assay using the Jerne plaque technique awaits the arrival from a commercial source of sheep RBC which will work in this assay.

An additional technique for the identification of acute-phase proteins is the 2-dimensional protein mapping procedure described by O'Farrel (5). The technique has been used in the fractions from the gel filtration column, but requires an extensive amount of work with available equipment. Therefore, part of the apparatus needed for running multiple samples at a time has been constructed in the model shop, and the rest of the equipment ordered through a commercial source. When operative it will provide a valuable tool not only for this work project but for anyone interested in a visual analysis of the proteins present in samples from serum, urine or tissue.

Publication:

Thompson, W. L., and R. W. Wannemacher, Jr. 1980. Effects of infection and endotoxin on rat hepatic RNA production and distribution. Am. J. Physiol. 238:G303-G311.

LITERATURE CITED

1. Thompson, W. L. and R. W. Wannemacher, Jr. 1973. Effects of infection with Diplococcus pneumoniae on synthesis of ribonucleic acids in rat liver. Biochem. J. 134:79-87.

2. Thompson, W. L. and R. W. Wannemacher, Jr. 1980. Effects of infection and endotoxin on rat hepatic RNA production and distribution. Am. J. Physiol. 238:G303-G311.

3. Taylor, J. M. and T. P. H. Tse. 1976. Isolation of rat liver albumin messenger RNA. J. Biol. Chem. 251:7461-7467.

4. Murgita, R. A. and T. B. Tomasi, Jr. 1975. Suppression of the immune response by α-fetoprotein. I. The effect of mouse α-fetoprotein on the primary and secondary antibody response. J. of Exp. Med. 141:269-286.

5. Murgita, R. A. and T. B. Tomasi, Jr. 1975. Suppression of the immune response by α-fetoprotein. II. The effect of mouse α-fetoprotein on mixed lymphocyte reactivity and mitogen-induced lymphocyte transformation. J. Exp. Med. 141:440-452.

6. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007-4021.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0H6420	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. ENTER SYM'S	8a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
80 07 23	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	022
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Structure-function relationships of potential pathological agents

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 012300 Biochemistry

13a. START DATE	13b. ESTIMATED COMPLETION DATE	14. FUNDING AGENCY			15. PERFORMANCE METHOD
77 08	80 09	DA			C. In-house

16. CONTRACT/GRANT

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

17. RESOURCES ESTIMATE

	PRECEDING	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	80	1.0	99
	CURRENT 81	0	0

18. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
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TELEPHONE: 301 663-2833

19. GENERAL USE

Foreign intelligence considered

20. PERFORMING ORGANIZATION

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Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Funded R&D at U.S. academic institutions)
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TELEPHONE: 301 663-7211
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME: POC:DA

21. KEYWORDS (Precede each with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Pseudomonas; (U) Staphylococcal enterotoxin C; (U) Amino acids

22. TECHNICAL OBJECTIVE, 23. APPROACH, 24. PROGRESS (Furnish individual paragraphs identified by number, keyed to and in same order as blocks 23 and 24. Security Classification Code.)

23 (U) Determine the amino acid sequence (primary structure) of specific proteins which are the mediators of diseases of potential BW importance. Knowledge of the covalent chemical structure of the proteins will aid in the understanding of the biochemical basis of their toxic effects. This understanding will improve our ability to treat diseases in military personnel in which these toxins contribute to the pathophysiology of the infection.

24 (U) Initially, proteins or fragments of proteins will be subjected to analysis on the Beckman 890C sequencer, with subsequent identification of the individual amino acid derivatives by gas chromatography and other standard methods. It will be necessary to establish a peptide fractionation system in order to separate peptides resulting from the fragmentation of the polypeptide chain. This is necessary in order to determine the complete amino acid sequence of such high molecular weight proteins as bacterial toxins.

25 (U) 79 10 - 80 09 - A limited tryptic cleavage of staphylococcal enterotoxin C (SEC) produces 3 large fragments, having approximate molecular weights of 4000, 6500, and 19,000. The last 23 residues of the 4K fragment have been sequenced and combined with data obtained earlier by CPT Cades, the primary structure of this fragment is now complete. The 6.5K fragment was cleaved with cyanogen bromide; resulting peptides were purified, which allowed the sequencing of 24 of the last 34 residues. Sequence analysis of the 19K fragment as obtained clarified several uncertainties in the partial sequence that had been determined previously. Further cleavage of this peptide and purification of the products will be necessary for determination of the complete structure.

Sequence analysis of whole Pseudomonas toxin was begun, but no reliable data were obtained after the first cycle. The most likely explanation is the presence of glutamine as the penultimate residue, which cyclized to pyroglutamic acid. Attempts are being made to remove this enzymatically, so that sequencing can proceed. Terminated for management efficiency. Continued in W U S10 AN 200 (DAOG15 79)

* Available to contractors upon requestor's approval

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BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M161102BS03)

Task No. 3M161102BS10-AN: Characterization of Microbial Toxins of Potential BW Importance

Work Unit No. S10-AN-163: Structure-Function Relationships of Potential
(S03 00 022) Pathological Agents

Background:

The principle objectives of these studies are the determination of the primary structures, antigenic determinants, and mechanisms of action of proteins associated with the pathogenicity of certain microorganisms. These include staphylococcal enterotoxin C (SEC), the exotoxins of Pseudomonas aeruginosa and Corynebacterium diphtheriae, and certain viral-coat proteins.

SEC is 1 of 6 related protein toxins produced by Staphylococcus aureus. The various types are classified on the basis of antigen-antibody reactions (1). While intoxication from ingestion of foods contaminated with staphylococci is rarely fatal, the purified toxins are extremely effective at low concentrations, and must be handled with suitable caution. Structural analyses have thus far been limited to those purified toxins that are available in large quantities. While the primary structure of the B variant has been published (2), about 75% of the SEC remains unsequenced.

The major exotoxins of P. aeruginosa and C. diphtheriae are thought to be involved in the pathogenicity of these organisms. Both inhibit protein synthesis in cells by catalyzing the transfer of ADP ribose from NAD to elongation factor 2 (EF-2). However, antisera to the 2 do not cross-react, and they have different cell receptors and mechanisms of internalization (3). Taken together, these results suggest an excellent opportunity for the study of structural factors involved in the catalysis of an enzymatic reaction and in the binding of toxins to cells.

It is expected that protein chemical studies of the toxins and of certain viral coat proteins will contribute to an understanding of the mechanistic and immunological properties of pathological agents. In addition, this may lead to new developments in prophylaxis and in treating acute toxemias.

Progress:

The current research plan was approved in July 1980, supplanting that of a predecessor. Some delays occurred due to laboratory move and locating and ordering supplies.

Results with peptides and with a standard protein indicated that the Beckman automatic sequencer was not functioning properly. Over the course of several months, the service technician performed what amounted to a general overhaul of the instrument, rebuilding solvent/reagent valves, replacing vacuum control

systems, etc. In addition, an entire lot of sequencer chemicals proved defective, and was replaced by the manufacturer. These extensive repairs have placed the instrument in an operational condition, but further evaluation will be necessary to bring its performance to the optimum level.

In February, 1980, a new high pressure liquid chromatography (HPLC) apparatus was received. Some time was spent in setting up this instrument and learning how to operate it. The equipment is now working well, and is being used for identification of phenylthiohydantoin (PTH) amino acids from the sequencer, and for peptide mapping and purification.

Most efforts in this time period were directed toward determining the sequence of SEC begun by CPT J. S. Cades. The basic approach takes advantage of the sensitivity of SEC to limited proteolysis by trypsin (4). Exposure of the native toxin to trypsin for 3 h, followed by reduction and carboxymethylation in a denaturing solvent, produced 3 relatively large fragments, with approximate MW of 4000, 6500, and 19,000.

The 4K fragment was further digested with trypsin to release the unsequenced carboxy terminal region of this peptide. Certain peptide bonds in this fragment proved highly resistant to trypsin, and the digest was more complex than expected. However, we were able to purify the desired peptide and place it in the sequencer. The following structure was found: lys.tyr.lys.asp.glu.val.val.asp.val.tyr.gly.ser.asn.tyr.tyr.val.asn.cys.tyr.phe.ser.ser.lys.

Together with data obtained by CPT Cades, the sequence of the 4K fragment of SEC was complete. However, this peptide is several residues shorter than the corresponding region of the homologous protein staphylococcal enterotoxin B (SEB). This has important implications in regard to the structure and function of this area since it contains the disulfide loop. Therefore, it will be necessary to ascertain whether or not a small peptide was overlooked during the original isolation of the 4K and 6.5K fragments of SEC.

The structure of the 6.5K fragment was also studied. This peptide contains the amino terminal region of SEC; it was partially sequenced by CPT Cades. The peptide was treated with cyanogen bromide and the products were purified on Sephadex G-50. The largest fragment, representing the carboxy terminal area of the 6.5K fragment, was then placed in the automatic sequencer. The following structure was obtained: lys.val.leu.tyr.asp.asp.his.tyr.val.ser.ala.thr.lys.val.lys.ser.val.asp.lys.phe.leu.ala.his.?.?.leu. At this point, results were no longer interpretable. However, amino acid analysis of the intact peptide show that only 9 or 10 residues remain to be placed. In order to obtain the sequence of these residues, the cyanogen bromide fragment was further cleaved with chymotrypsin. This should produce a fragment that begins with the sequence: leu.ala.his.etc. The digest was chromatographed on G-25 Sephadex; analysis is currently in progress.

The remainder of SEC is a large peptide MW = 19,000 and includes the carboxy terminal region of the intact toxin. This was partially analyzed by CPT Cades, but numerous residues at various points in the peptide were not definitely identified. A sample of the 19K peptide was placed in the sequencer; results have resolved several uncertainties. These include a lysine at residue number 5, a methionine at residue number 8, and the location of the second half-cystine.

Given the size of this fragment, cleavage of the peptide and purification of the fragments must precede any further sequencing. Therefore, the peptide was reacted with cyanogen bromide; amino acid analysis predicts a maximum of 6 peptides. Analysis and attempts to purify these peptides are underway.

Finally, a sample of Pseudomonas toxin (provided by Dr. Leppla) was reduced and carboxymethylated, and placed in the sequencer. The objective was to compare the structure of the amino terminal region of this toxin to the same area of diphtheria toxin, since both catalyze the same enzymatic reaction but have different cell receptor sites.

The first cycle of Edman degradation produced the amino terminal residue, alanine, in the expected amount. Thereafter, the degradation ceased entirely. This is the 3rd attempt to obtain a partial sequence of Pseudomonas toxin. The first 2 were hampered by mechanical failures of the sequencer, and the fact that quantitative data on the recovery of PTH amino acids were not available. However, further analysis of these earlier efforts suggest the same pattern of events. A somewhat different approach for sequencing this protein will have to be devised. The most likely cause for the cessation of sequencing is the presence of glutamine as the next residue after alanine. In some cases, glutamine has been shown to cyclize to pyroglutamic acid, a substance that will not react with the Edman reagent. This possibility will be investigated with leucine aminopeptidase, and with an enzyme that specifically removes pyroglutamic acid from polypeptides.

Publications:

None

LITERATURE CITED

1. Spero, L., and J. Metzger. 1980. Staphylococcal enterotoxin A. Methods Enzymol. (in press).

2. Huang, I.-Y., and M.S. Bergdoll. 1970. The primary structure of staphylococcal enterotoxin B. III. The cyanogen bromide peptides of reduced and aminomethylated enterotoxin B, and the complete amino acid sequence. J. Biol. Chem. 245:3518-3525.

3. Middlebrook, J., and R. Dorland. 1977. Differential chemical protection of mammalian cells from the exotoxins of Corynebacterium diphtheriae and Pseudomonas aeruginosa. Infect. Immun. 16:232-239.

4. Spero, L., B.Y. Griffin, J.L. Middlebrook, and J.F. Metzger. 1976. Effect of single and double peptide bond scission by trypsin on the structure and activity of staphylococcal enterotoxin C. J. Biol. Chem. 251:5580-5588.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0H6429	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	CO	024
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Provide with Security Classification Code)

(U) Diagnosis and pathology of Legionnaires' disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
78 03	80 09	DA			C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS ($ in thousands)
FISCAL YEAR	PRECEDING 80	2.5	426
	CURRENT 81	0	0

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

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NAME: Lowry, B. S.
NAME: Ristroph, J. D. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Provide KEY with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Laboratory animals; (U) Special containment facilities; (U) Legionnaires' disease

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

23 (U) Develop rapid serologic methods for diagnosis of L. pneumophila and Legionella-like organisms, develop appropriate DNA homology techniques for recognition. Determine optimal conditions for growth, survival and maintenance of virulence and define factors involved in the pathophysiology. Assist in the study of aerosol infectivity in appropriate laboratory animals.

24 (U) Starting with information, procedures and reagents from CDC, assess strain differences, develop appropriate antibodies for direct serologic recognition, develop DNA homology techniques, test a variety of growth media, isolate and characterize toxins

25 (U) 79 10 - 80 09 - Direct and indirect assays are available for Legionella and Legionella-like organisms. A diagnostic microagglutination assay has been set up for Legionella organisms; histopathology caused by this toxin in the AKR/J mouse model has been demonstrated. The effect of this toxin on human peripheral white cells has been demonstrated. A new chemically defined liquid media has been developed.

Terminated for management efficiency. Continued in W.U. S10 A0 199. (DAOG1522)

Publications: J. Clin. Microbiol. 11:19-21, 1980; In Analytical Chemistry Symposia Series, in press, 1980.

* Available to contractors upon originator's approval

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

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M161102BS03)

Task No. 3M161102BS10 A0: Bacterial and Rickettsial Diseases of Potential BW Importance

Work Unit No. S10 A0 168: Diagnosis and Pathology of Legionnaires' Disease
(S03 00 024)

Background:

Eight months following the dramatic outbreak of what is now termed Legionnaires' disease in the city of Philadelphia, in which 29 people died, a gram-negative bacterium now called Legionella pneumophila was isolated and identified as the etiologic agent by Drs. Fraser (1) and McDade (2).

Although Legionnaires' disease has been recognized in 43 states and the District of Columbia (3) the disease is not restricted to North America. In addition to Canada, cases have been reported in Australia, England, Israel, Scotland, Denmark, Spain and The Netherlands. The largest outbreak of legionellosis yet documented outside the United States occurred in Vasteras, Sweden, from August 28 to September 21, 1979, and involved 67 cases (4).

We now recognize 2 distinct clinical syndromes associated with this organism. The first termed Legionnaires' disease is the pneumonic form of the disease and has a 16% lethality in normal individuals and a 54% lethality in immunologically compromised patients. Death is associated either with respiratory failure or shock.

The second clinical syndrome presents as a nonpneumonic, nonlethal, debilitating flu-like illness popularly termed "Pontiac fever" named after the city in Michigan where the original outbreak occurred. Fraser (5) has pointed out that what determines whether L. pneumophila will cause Legionnaires' disease or Pontiac fever is entirely unknown. He suggested that the latter might result from a large dose of nontoxigenic organisms. At the time he made that suggestion no known toxins were recognized in any isolated strains of L. pneumophila.

In addition to our investigations on enhanced growth, early recognition and virulence studies, we have been able to isolate a low molecular weight toxin which we believe may have some relationship to the pathogenesis in legionellosis.

Progress:

Legionella pneumophila toxin. The presence of a toxin associated with L. pneumophila has long been suspected. We have demonstrated a 3400 MW intracellular protein which is lethal when injected IP into AKR/J mice. The techniques used in the isolation of this toxin included acid precipitation, gel filtration, and preparative isotachophoresis. The advantages of this last step conferred that it allowed the dissociation of a high MW protein entity (5,000,000) and that it organized the toxic activity under a single low MW protein peak (3400).

Legionnaires' disease and Pittsburgh Pneumonia are clinically similar but are caused by genetically unrelated gram-negative bacteria. The fact that we can show that both extracts are toxic in vivo and antigenically related opens up intriguing possibilities in understanding the pathogenesis of these 2 previously unrecognized bacterial agents.

Chemically defined media. A chemically defined liquid medium has been developed for the study of the pathophysiology of L. pneumophila. The medium contains inorganic salts, a mixture of 18 amino acids, rhamnose, choline and ferric pyrophosphate. The final concentrations of salts and amino acids were modeled after yeast extract (YE). Shake cultures at 37°C of L. pneumophila, strain Philadelphia, produced a lag phase of approximately 5 h. The logarithmic phase of growth proceeded for 31 h attaining a maximum cell population of 1.4 x 10^9 CFU/ml with an average generation time of 4 h. Stationary phase growth was noted for an additional 18 h. Cell population was determined to be 2.4 x 10^8 CFU/ml, 96 h postinoculation. A soluble brown pigment was observed at the onset of logarithmic growth, however the greatest increase in pigment was observed when the culture entered the death phase at 54 h. There was a steady increase in pH over the entire period of growth, up to a final pH of 7.3 at 122 h. Choline was found to stimulate growth, as were some carbohydrates (CHO). Rhamnose gave the best stimulation of the CHO tested thus far, an effect which was enhanced by the addition of choline. The amino acids arginine, serine, threonine, valine, cysteine and methionine were found to be essential for growth.

The nutritional requirements of L. pneumophila are not complex, since good growth was obtained in this simple medium of salts and amino acids. This organism has the ability to synthesize all the required vitamins and coenzymes, since none of these compounds were added to the medium. It is possible that enhancement of growth can be obtained by their addition. However, other investigators have not been able to demonstrate this in other defined media. It is of interest that we have shown that CHO does play a role in the metabolism of L. pneumophila, but this role is yet to be defined.

This chemically defined liquid medium is preferred for conducting physiologic studies and has many advantages over those previously described. Excretion products, antigens, and vaccines can best be produced and studied in this new medium. Moreover, this medium permits the detailed examination of both supplemental carbon sources and amino acids to optimize the growth of L. pneumophila.

In vitro growth and survival. All of the isolates of L. pneumophila obtained from the Center for Disease Control, Atlanta, in October 1977 and subcultured at that time on supplemented Mueller-Hinton agar (MHA) slants, covered with sterile glycerol, and stored at -10°C are still viable, as are all of the original cultures and subcultures suspended in 50% rabbit serum in tryptose saline and stored at -70°C. The Pontiac isolate, which apparently causes a clinical disease in humans quite different from Legionnaires' disease even though it is identical to L. pneumophila by established taxonomic criteria, continues to be the only isolate which does not produce the melanin-like pigment on our modification of Feeley-Gorman agar.

Outbreaks of Legionnaires' disease and Pontiac fever have been associated with environmental sources of L. pneumophila, such as aerosols from air conditioner cooling towers and airborne dust from earth-moving operations. Evidence indicates that L. pneumophila may be common in water and mud from lakes, rivers and streams;

therefore, a study was initiated to determine whether the organism can survive in local public water supplies, lakes, fountains and air conditioning systems which generate aerosols to which humans are exposed. The water samples were sterilized by filtration and sufficient L. pneumophila (Washington strain) were added to give a concentration of 6×10^5 organisms/ml in 100-ml aliquots. All samples were incubated at room temperature, and the number of viable organisms per ml determined at various intervals by standard plate count methods using MHA and charcoal YE agar incubated at 35°C in 2.5% CO_2 and 80% RH. There was no significant decrease in the number of viable L. pneumphila in 11 of 15 water samples after 24 h incubation; however, there was an approximately 10-fold decrease in water from 2 to 5 local public fountains, a 100-fold decrease in local tap water, and a 1000-fold decrease in USAMRIID air conditioner cooling tower water sampled 1 h after routine addition of a quarternary ammonium biocide called ENTEC 340 (there was no decrease in the USAMRIID cooling tower water taken 3-1/2 days after addition of the biocide). After 5 days incubation, there was still no significant decrease in viable L. pneumophila in cooling tower water samples from Buildings 1302 and 539, and from the pond; however, a 1000-fold decrease was detected in the tap water, and 10-fold decrease occurred in all of the other samples excepting the USAMRIID cooling tower water sample 1 h after treatment, which was sterile. By the 14th day of incubation, there was apparent growth of L. pneumophila in water from Culler Lake and only a slight decrease in the number of viable L. pneumophila in the Fort Detrick pond water sample, while the number of viable organisms in all other samples had decreased by 10- to 1000-fold, and 20 organisms/ml remained in chlorinated tap water. There was little change in the number of viable organisms surviving between 2 and 4 weeks; however, after 8 weeks the number surviving was too low to be detectable in most samples, with the following exceptions: there was no significant decrease from the number of viable organisms initially inoculated into water from Culler Lake, about 1-log decrease in air conditioner cooling tower water from Building 560 and the east fountain at Francis Scott Key Mall. By the 20th week of incubation, surviving organisms could only be detected in Culler Lake samples.

It should be noted that for practical purposes the lowest number of viable organisms detectable by standard plate counting methods using available media is probably about 20 CFU/ml under the best conditions; however, many batches of media require inoculation of $\geq 10^5$ organisms/ml before growth will be initiated, and then only confluent, veil-like growth results with few, if any, isolated colonies. The problem of inconsistent growth of L. pneumophila on media currently recommended for routine use continues to plaque laboratories. Development of selective media has been fruitless, since the organism is extremely sensitive to not only all the usual selective bacteriologic chemicals, but even to concentrations of salts, lipids and detergents tolerated by most organisms. Changes in composition of defined chemical ingredients during storage seem to be as much a problem as variations in batches of standard media from suppliers. Progress in developing a good medium which will permit rapid growth and identification of minimal numbers of L. pneumophila from clinical and environmental samples may well depend upon discovering and controlling the factors responsible for the variation in effectiveness of media currently employed; such is the goal of current studies.

Work in this laboratory and elsewhere indicate that L. pneumophila can survive for months in water samples from public supplies, lakes, fountains, and air conditioning systems; however, except for Culler Lake samples, no evidence of multiplication in terms of increased numbers of viable organisms was detected. A recent published report indicates that L. pneumophila actively grows in a natural aquatic environment when certain algal species are also present and will grow

rapidly and well experimentally in a uni-algal culture. All the water samples tested in this laboratory were from sources treated with algacides. Apparently, growth of L. pneumophila in public water can be prevented by preventing the growth of algae. Natural waters containing high concentrations of algae will be tested for suitability as growth media for L. pneumophila, in the hope that algal extracts or byproducts may be used to improve current culture methods.

As noted previously, development of a selective medium for L. pneumophila has been frustrated due to the high sensitivity of the organism to chemicals and antibiotics usually employed in such a medium. The chemically defined medium developed by MAJ Ristroph is relatively simple in composition, so it was hoped that this medium might be unsuitable for the growth of much of indigenous bacterial flora in potential natural sources of L. pneumophila. Samples of water from Culler Lake and the Detrick Pond were plated on the chemically defined medium as well as a number of standard and enriched media. There was no significant difference in the number and types of colonies developing on the media.

Antigenic differences in attenuated and virulent strains of L. pneumophila. Work with attenuated and virulent strains of L. pneumophila continues. There have been promising results in seeking an antigenic difference between these 2 strain types. We have reported that using immunoelectrophoresis we were able to demonstrate the loss of an antigen in the attenuated strain of Legionella. We have taken the same preparation and used SDS-gel electrophoresis and again demonstrated the loss of an antigen in the attenuated strain. This antigen appears to have a MW of approximately 20,000. Further work will continue in this area to characterize the antigen and determine its relationship to virulence.

Further AKR/J mouse studies. AKR/J mice were used for a morbidity-mortality curve, 5 mice per point, using a single IP injection of the Washington strain of L. pneumophila with counts ranging from 0.2 to 20 x 10^8; 8 x 10^8 organisms gives 100% lethality and there is some mortality at 4 x 10^8; if the latter animals live, they appear normal at 72 h. At $\leq$ 0.8 x 10^8 organisms illness can be missed if the animals are not examined at 12 h. Conjunctivitis and diarrhea are obvious marks of illness, with the former the more dependable sign, but disappearing before the diarrhea.

The effects of live vs. heat-killed and formalinized Washington strain were compared. At IP doses of 8 x 10^8 live organisms, all mice were dead in 36 h, while those given heat-killed or formalinized became ill, but recovered in 48 h. The heat-killed injected mice reached a peak of illness at about 12 h with recovery in 24 h, while the formalinized-organism injected mice showed some diarrhea at 12-24 h with recovery at 48 h.

HA titers for sera from L. pneumophila immunized mice have frequently been negative or reported as very low. These findings have not been consistent with associated laboratory results leaving interpretation of the tests uncertain. This problem led to a cooperative project with serology section for a better test for titrating sera for antibodies. All 6 serogroups of L. pneumophila were consequently grown in large quantities for test antigens. Formalinized antigen proved superior to the original autoclaved product and the laboratory now uses this antigen exclusively. AKR/J mice immunized IP one time with low (0.2 x 10^8) and high dosage levels (4 x 10^8) of the Washington strain and bled over a 2-month period provided inconsistent HA titers. However, the newly developed MA test, using the antigen described above, at both dosages showed titers that were first

present at 7 days and, as expected, disappeared quite rapidly between 38 and 49 days.

ALLO antisera. Antisera to ALLO organisms TEX-KL, Heba, Tatlock, WIGA, and OLDA have been raised in rabbits. Formalinized antigens and antisera for all of these have been made available to serology section with agglutination titers raised from 0 level to a minimum of 1:20,000 (WIGA) to $\geq$ 1:40,000 in all others.

Human sera for comparison studies. Thirty-nine sera (bled at 1979's convention of the Pennsylvania Division of the American Legion) from Legionnaires and guests who had participated at the Philadelphia Convention in 1976 (the original outbreak) were obtained to give our division human sera to compare the validity of test results obtained with our animal sera (only sera available to us up to this time). Nine of the sera have been requested of us by CDC to follow-up original and convalescent sera titers in a study to be published with the acknowledged help of USAMRIID.

Staining of organisms in tissue. In cooperation with Aerobiology's Dr. Berendt a study has been begun on the localization and progression of aerosolized L1 strain in guinea pigs. Animals killed at intervals after exposure ranging from immediately to 24 h had their chest and (with a longer elapsed time after exposure) abdominal organs dissected out and preserved in formalin. The plan is to identify the original deposition of the organisms and later sequestration or disposition of the organism.

Without an available cryostat for fresh-tissue comparison, FA studies on formalin-fixed paraffinized mouse and guinea pig tissue compared several techniques given in the literature. Both room temperature and 37°C incubator trypsinization procedures allow readable results in a half-day's time and seem comparable. Organisms can be readily identified in a cross-section of guinea pig trachea early after inhalation of the aerosolized bacteria. Positive tissue controls of the first 4 serogroups in which individual bacteria fluoresce are assembled since tissue which had been kindly made available as positive controls has individual cells which fluoresce, but no identifiable bacteria. These studies have yielded a technique that appears unique. Formalin-fixed guinea pig lung with L. pneumophila introduced for positive control slides, and cut at 3 μ, can be gram-stained up to the safranin stain ("3/4 gram stain"), then overlaid with fluorescein isothiocyanate-conjugated antiserum to the specific serogroup, resulting in a single slide stained both for fluorescent and light examination. Fluorescent microscopy identifies the presence of the organisms and by a switch to the light microscopy mode, one can identify the location of the bacterium in the same tissue on the same slide.

Giemsa stain. The refractoriness of L. pneumophila to stains routinely employed in histopathologic and bacteriologic examination of clinical specimens was a major reason cited for the long delay in discovery of the etiologic agent of Legionnaires' disease and Pontiac fever. In an attempt to find a simple method for staining L. pneumophila in exudate and tissue impression smears, it was observed in our laboratory that the classic Giemsa stain used after fixation in absolute methanol was superior to the Giménez stain. Smears of cultures from liquid or solid media and embryonated egg yolk sacs also stained well by this method. The advantage of the Giemsa stain is that it permits easy identification of tissue and exudate cells plus visualization of phagocytosed L. pneumophila, and excellent bacterial cytologic detail. Most individual organisms are seen as long thin rods containing deeply stained chromatin bodies, which are larger than in diphtheroids. Some of the organisms contain large unstained vacuoles, and appear swollen. Very

long curved, kinked and S-shaped chains are frequently seen in cultures which are very unusual. Many of the cahins seem to be made up of a syncytium of bacilli resembling nonseptate hyphae, and some contain unstained vacuoles which give them a "moth-eaten" appearance. When Giemsa-stained infected peritoneal exudates from guinea pigs were examined only pairs and short chains of L. pneumophila were observed, but the morphology of the organisms was very different from the various other bacteria present after post mortem incubation, so that L. pneumophila were easily distinguishable. Individual organisms with the characteristic morphology of L. pneumophila were observed within macrophages in Giemsa-stained impression smears of guinea pig lung tissue taken 48 h after exposure to aerosols containing L. pneumophila; however, no chains of organisms were observed. It may be that the morphology of this organism in Giemsa-stained clinical material from suspected Legionnaires' disease and Pontiac fever patients or victims is sufficiently unique to be pathognomonic, and permit a presumptive diagnosis to be made. Application of the classic Giemsa stain technique for demonstrating L. pneumophila in impression smears of human tissue specimens would be appropriate, and routine use for staining culture smears is recommended.

Presentations:

1. Hedlund, K. W. The toxin of Legionella pneumophila. Presented, Gordon Conference on Microbial Toxins, July 1980, New Hampshire.

2. Ristroph, J. D., K. W. Hedlund, and S. Gowda. A chemically defined medium for the growth of Legionella pneumophila. Presented, 80th Annu. Mtg., ASM, May 1980, Miami, FL (Abstracts, I42, p. 91).

Publications:

1. Ristroph, J. D., K. W. Hedlund, and R. G. Allen. 1980. Liquid medium for growth of Legionella pneumophila. J. Clin. Microbiol. 11:19-21.

2. Hedlund, K. W., and R. G. Larson. 1980. Legionella pneumophila toxin, isolation and purification. In Analytical Chemistry Symposia Series. Elsevier Scientific Publishing Co., Amsterdam, in press.

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1. Fraser, D. W., T. R. Tsai, W. Orenstein, W. E. Parkin, H. J. Beecham, R. G. Sharrar, J. Harris, G. F. Mallison, S. M. Martin, J. E. McDade, C. C. Shepard, P. S. Brachman, and the Field Investigation Team. 1977. Legionnaires' disease. Description of epidemic of pneumonia. N. Engl. J. Med. 297:1189-1197.

2. McDade, J. E., C. C. Shepard, D. W. Fraser, T. R. Tsai, M. A. Redus, W. R. Dowdle, and the Laboratory Investigation Team. 1977. Legionnaires' disease. Isolation of a bacterium and demonstration of its role in other respiratory disease. N. Engl. J. Med. 297:1197-1203.

3. Center for Disease Control. 1978. Legionnaires' disease -- United States. Morb. Mortal. Wkly Rep. 27:439-441.

4. Center for Disease Control. 1980. Legionellosis -- Västeras, Sweden Morb. Mortal. Wkly Rep. 29:206-207.

5. Fraser, D. W., D. C. Deubner, D. L. Hill, and D. K. Gilliam. 1979. Nonpneumonic, short-incubation-period legionellosis (Pontiac fever) in men who cleaned a steam turbine condenser. Science 205:690-691.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6413	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. GRP'N METH'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	026
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Include Security Classification Code)

(U) Cell surface expression of viral antigens during the infectious process

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
78 07	80 09	DA			C. In-house

17. CONTRACT/GRANT

a. DATE/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: NA d. AMOUNT:

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

18. RESOURCES ESTIMATE

	FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
PRECEDING	80	1.0	67
CURRENT	81	0	0

19a. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Barquist, R. F.

TELEPHONE: 301 663-2833

20a. PERFORMING ORGANIZATION

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PRINCIPAL INVESTIGATOR (Provide name if U.S. Academic institution)

NAME: Urbanski, G. J.

TELEPHONE: 301 663-7241

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Provide Each with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Vaccines; (U) Immunology; (U) Rift Valley fever; (U) Laboratory animals; (U) Viruses

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

23 (U) Determine the cell surface expression of viral antigen in Rift Valley fever (RVF) virus and arenavirus infections. Determine the nature of the humoral immune response to these infections, and available vaccines or vaccine candidates, in terms of quantity and specificity of the antibody elicited. These techniques will be applied to vaccine development for viruses of military importance.

24 (U) Develop solid phase radioimmuno assay (SPRIA) procedures for the quantitation of antiviral antibody and antigen. Develop immunoprecipitation techniques for the determination of antibody specificity and detection of cell surface antigens.

25 (U) 79 10 - 80 09 - A SPRIA for RVF virus antibody has been developed which demonstrates a 1:1 correlation with the 80% plaque reduction neutralization test (PRNT). This procedure is rapidly approaching the stage where it can replace the costly PRNT as the primary measure of anti-RVF virus antibody response. Studies on the antigenic specificity of the anti-RVF virus immune response have centered on the molecular structure of the RVF virion. Ambiguities in the data obtained thus far have not allowed for a clear separation of the G1/G2 complex; tryptic mapping data indicate that G1 and G2 may have the same or very similar amino acid sequences. This project will continue with a study of the basic molecular structure of this virus because an understanding of this structure is essential to an understanding of the immune response it elicits.

Terminated for management efficiency. Continued in W.U. S10 AP 198. (DAOG1526)

* Available to contractors upon requestor's approval

DD FORM 1498 (1 MAR 80) PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M161102BS03)

Task No. 3M161102BS10 AP: Biology of Viral Agents of Potential BW Importance

Work Unit No. S10 AP 169: Cell Surface Expression of Viral Antigens During
(BS03 00 026) the Infectious Process

Background:

The presence of virus-specific surface antigens on infected cells has been demonstrated for a large number of viruses of interest at USAMRIID, including VEE, EEE, Dengue, JBE, Pichinde and lymphocytic choriomeningitis. The virus induced specific antigenic changes in host membranes have been detected by a number of procedures: immunofluorescence, immunoradiolabeling, immunoelectronmicroscopy and assays employing antibody or lymphoid cells. However, the antisera used in a large number of these studies were obtained from convalescent hosts and it was not possible to determine whether the antigenic changes detected were the result of viral proteins expressed on the cell surface, altered or unmasked host proteins, or a combination (1-3).

The major objective in this project was to study the role of viral antigens expressed on the surface of infected cells during the evolution of viral infections of military importance. The major techniques to be used were immunoprecipitation of radiolabeled viral proteins (4) to qualitatively measure the recognized antigens and a system of solid phase radioimmunoassays to quantitate the immune responses.

The virus chosen for these studies was the agent responsible for Rift Valley fever (RVF), a disease of both domestic livestock and man, currently epidemic in Africa. The basic molecular information for this virus was available from previous studies at USAMRIID (5); however, as will be discussed, the studies were forced to take a more basic molecular approach.

Progress:

The approaches taken in this study have been 2-fold. First, to quantitate the antibody response to RVF virus infection and/or vaccination and to identify qualitatively the antigens recognized in this response. Second, to use the molecularly defined antisera resulting from the first study to probe antigen expression on the infected cell surface.

The quantitation of the immune response was accomplished through the development and use of a SPRIA. The methodology used was described last year. Because of the potential savings of large amounts of money due to reduced cost of material and reduction of man-hours over those now required for the plaque reduction neutralization (PRN) test 2 studies were undertaken to test the feasibility of adapting this assay for diagnostic use. The data from the first study are presented in Table I. This table shows a comparison of the titers obtained for 75 human sera using both SPRIA and the PRN procedures. Disregarding the 20 negative sera, 73% of all values obtained in the SPRIA fall

within a + 1 dilution range of the PRN_{80} titers. The second study, still in progress, will relate the observed RIA titers to not only the PRN_{80} titers but also to HI and CF titers. At the conclusion of this study, due consideration will be given to replacing PRN_{80} with the RIA as the standard measure of an anti-RVF antibody response at USAMRIID.

With the success of the RVF procedure, this technique has been applied with some success to respiratory melioidosis in conjunction with Dr. George Scott (Aerobiology Division) and attempts have been made to develop a procedure for Lassa fever in conjunction with Dr. Peter Jahrling.

TABLE 1. COMPARISON OF RVF RECIPROCAL PRN_{80} AND SPRIA, TITERS FOR 75 HUMAN SERA

RECIPROCAL RIA TITER	SPRIA > PRN_{80}			SPRIA-PRNT	SPRIA < PRN_{80}		
	3	2	1		1	2	3
>10	0	0	0	20	0	0	0
10	0	0	2	0	0	0	2
20	0	0	0	1	0	1	3
40	0	0	0	0	0	0	1
80	0	0	0	3	3	1	0
160	0	0	0	3	2	2	0
320	0	1	3	3	4	0	0
640	1	3	3	3	0	0	0
1280	0	0	3	2	0	0	0
2560	0	0	2	1	0	0	0
5120	0	1	0	2	0	0	0
TOTAL	1	5	13	38	9	3	6

The qualitation of the immune response against the RVF virus has proven more difficult than its quantitation. Serological cross-reactivity studies have placed RVF virus into the Phlebotomus fever group (6). Viruses of this group normally consist of a large MW protein around 180,000, two glycosylated proteins G1 and G2 around 65,000 and 55,000, respectively, and a non glycocylated core protein around 25,000. Molecular studies at the Institute have shown this to be the case for RVF virus (5). However, the separation of G1 and G2 in RVF virus has been intermittent and very elusive. A typical electrophoretic run of the purified RVF virus on a 15% acrylamide slab gel (using DATD from Bio Rad as the cross-linker in a ratio of 30:1.6, respectively) shows no clear

separation between G1 and G2, and a 43,000 mw protein that is transient in nature.

Data obtained thus far on the tryptic maps of G_1 and G_2 show no differences; in fact, the 100,000 and 43,000 mw proteins also have tryptic maps identical to those of the G1/G2 complex. These data were obtained in conjunction with CPT Erlick of USAMRIID and Dr. Allen of FCRC.

Immunoprecipitation of these virus preparations with various antisera again shows no separation of the G1/G2 complex, whether the virus was labeled intrinsically with ^{3}H or externally with ^{125}I. Currently we plan to attack the G1/G2 complex in a number of ways which will include: (a) further increasing the gel concentrations used, (b) use of high molar urea in the gels, and (c) modification of these glycoproteins by removal of their sugar moieties. An initial experiment using neuraminidase proved inconclusive due to protease contamination of the enzyme preparation used.

Presentations:

Urbanski, G. J., Rift Valley Fever virus RIA. Presented, Workshop for Rapid Identification of B. W. Agents, Sponsored by the Technical Cooperation Program Subgroup E Technical Panel 4, 5-7 May 1980, Fort Detrick, MD.

Publications:

None.

LITERATURE CITED

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3. Burns, W. H., and A. C. Allison. 1977. Surface antigens of virus. Virus Infections and the Cell Surface (G. Poste and G. L. Nicolson, eds). North Holland, Amsterdam.

4. Pink, J. R. L. and A. Ziegler. 1979. Radiolabeling and immunoprecipitation, pp. 169-179. In Immunological Methods (I. Lefkovits and B. Pernis, eds). Academic Press, New York.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6415	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCT	6. WORK SECURITY	7. REGRADING	8a. ENTER SYSTEM	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	027
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE: (U) Production and use of endogenous pyrogen antibodies in early detection of infections of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 08	80 09	DA	

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR 79 (PRECEDING) 80	1.0	124
FISCAL YEAR 80 (CURRENT) 81	0	0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
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20. PERFORMING ORGANIZATION
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21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Provide KWOC with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Immunology; (U) Endogenous pyrogen; (U) Radioimmunoassay

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

23 (U) Produce and purify antibodies to human endogenous pyrogen (EP). Use these antibodies to develop an effective radioimmunoassay for EP in serum, as a means of early detection of illness in military personnel exposed to BW agents or other sources of infection.
24 (U) Stimulate monocytes obtained from the blood of volunteers or tissue culture lines to produce EP. Inject this EP into goats or rabbits to produce antibodies, and purify these antibodies by immunoabsorption and column chromatography. Develop a radioimmunoassay for EP in human serum using the purified antibodies.
25 (U) 79 10 - 80 09 - Using a rat model of infection, 2 important assumptions were shown to be valid: differences in EP levels could be measured, and these EP levels change with course of the illness. For development of immunoassays for human EP, pyrogen was purified from tissue culture of human cells and injected into rabbits. Antibodies with demonstrable neutralizing activity against EP have since been obtained. The effort toward providing pure human EP for RIA included not only purifying the pyrogen preparations as produced in vitro, but also stimulation of tissue-culture cells and transference to media lacking fetal calf serum in order to produce EP in a relatively cleaner state.
Publications: Fed. Proc. 39:991, 1980; Proc. Soc. Exp. Biol. Med. 166: in press, 1981.
Terminated for management efficiency. Continued in W.U. S10 AQ 197. (DAOG1529)

* Available to contractors upon originator's approval

DD FORM 1498 1 MAR 80 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U).
(3M161102BS03)

Task No. 3M161102BS10 AQ: Enhancement of Host Defense Against Agents of Potential BW Importance

Work Unit No. S10 AQ 178: Production and Use of Endogenous Pyrogen
(S03 00 027) Antibodies in Early Detection of Infections of Military Importance

Background:

Endogenous pyrogen (EP), a 15,000-dalton protein that induces fever, is part of the defense mechanism a host animal utilizes to ward off bacterial or viral infection. It is made (by phagocytic cells of the host) in response to such stimuli as bacteria, viruses or endotoxin. Work with highly purified preparations suggest that EP itself also interacts with phagocytic cells, causing release of inflammation-related constituents (1).

Because EP induces fever in animals, it must appear in the bloodstream before the temperature increases. This suggests that the presence of increased concentrations of EP may be an early indication of infection. In order to detect these changes, this study was undertaken to develop a radioimmunoassay (RIA) for EP.

Development of a RIA requires pure EP and pure antibodies directed against it. Production of specific antibodies to EP is important for these studies both for the RIA themselves and for use in purifying the EP. This aspect of the project is being actively pursued.

Progress:

Detection of circulating EP. The EP bioassay procedure used in this project is intracerebroventricular (ICV) injection of rats, as detailed previously. Careful quantitation has placed its sensitivity 50-100 x greater than that of the conventional rabbit EP bioassay, and approximately equal in sensitivity to that of published EP RIA procedures. Bioassays are unlikely to be improved further, but immunoassays can be made more sensitive by various immunochemical amplification methods, so that RIA procedures should have a clear advantage in not only specificity but also sensitivity. However, these RIA methods require months of preliminary work to prepare purified antigen, and to produce and purify antibodies towards it. During the antibody production stage of this project, an experiment was undertaken with the available ICV bioassay to test the hypothesis that EP concentrations change detectably early in infection. EP is known to appear in plasma of infected animals while they are febrile, but little is known about the variation of its concentration with time of infection.

Male Fisher-Dunning rats were infected with the live vaccine strain (LVS) of Francisella tularensis; rectal temperatures and plasma levels of EP were monitored and compared to those of control rats receiving heat-inactivated LVS. F.-tularensis

was chosen because of its military importance, and because it does not contain endotoxin, so that endotoxin effects could be kept separate from the effects of bacterial infection alone.

In this experiment, groups of 5 experimental and 5 control rats were studied at various times after infection, filter-sterilized plasma was obtained from each rat and injected ICV into assay rats. The results of these assays indicate that EP could be detected, and that it began to appear in the plasma of the infected rats at least as early as the time of onset of fever. This animal model experiment gives hope that more sensitive RIA procedures could detect EP changes even earlier, and therefore be useful as a means of early detection of infection.

The experiment also showed that plasma EP levels decline before fever in infected rats. This may mean that a decline in plasma EP concentration signals the beginning of recovery.

Evidence that the pyrogen measured in the rat plasma is EP includes the facts that it was heat-labile, gave a negative Limulus test for endotoxin, did not pass through dialysis tubing, and bound to a cm-Cibacron blue affinity column in the same manner that EP does

Immunoassay. For successful immunoassay of human EP, a suitable source must be found, and used to obtain purified EP. Furthermore, the EP must be used to produce antibodies which must also be purified. These 2 steps are related since the cleaner the antigen (EP, in this case) injected, the easier it is to purify that particular antibody, and consequently the more specific the assay.

Cells that produce EP upon stimulation can produce purer initial preparations than do cells that produce it spontaneously, as the U-937 cell line normally does. This is because once stimulated, the cells can be put into (and produce EP in) growth media lacking fetal calf serum and other sources of contamination. A human monocyte cell line which could possibly be stimulated to produce EP was received from MAJ. James Anderson. Stimulation with a 30:1 ratio of Staphylococcus epidermidis to IM-9 cells gave encouraging results on a small scale (10^8 cells, 50 ml), producing the expected 2,500 pyrogen units. Larger-scale preparations would require adjustments in growth conditions, and further study to maximize EP production to usable quantities.

Rabbit EP has been used as a model system for familiarization when handling EP preparations, and for antibody production. As discussed in earlier annual reports, rabbit EP is greatly purified by gel filtration followed by cm-Cibacron blue affinity chromatography. At present, batches are made only occasionally to assist other projects.

In this project, human EP has been obtained from tissue culture lines, mainly U-937 hystiocytes. This cell line produces EP spontaneously, at a slow rate, that is, without prior stimulation. Production of EP from this cell line has proceeded steadily, and the EP obtained has been used for inoculation of rabbits for antibody production. The scientific literature indicates that it takes 7-9 months for antibodies against EP to appear (2, 3). Our results confirm this, but the immune serum from the rabbits inoculated have finally shown demonstrable, pyrogen-neutralizing activity (Table I); results indicate that anti-EP is present.

TABLE I. NEUTRALIZATION OF EP ACTIVITY BY ANTISERUM

TREATMENT	TEMP Δ ± SE (%°C)	NET Δ ± SE (%°C)	FEVER NEUTRALIZED BY ANTISERUM (± SE) (%°C)
EP + serum	0.97 ± 0.12	0.07 ± 0.37	0.88 ± 0.44
Serum control	0.90 ± 0.35		
EP + saline	1.10 ± 0.23	0.95 ± 0.24	
Heated EP control	0.15 ± 0.05		

Another cell line, the human monocytic IM-9 culture, has been tried as a source of EP. The advantage it has over the U-937 line is that it must be stimulated by phagocytosis before EP production begins. The production of EP itself can be carried out in media without fetal calf serum, so that EP is obtained in an initial state of greater purity than the EP obtained from U-937 cells. Initial results with this IM-9 cell line are encouraging and suggest that the EP can be produced efficiently from them at least on a small (100 ml) scale. Attempts at larger scale batches have resulted in changes in cell growth conditions, so that adjustments must be made to optimize EP production. The greater purity of the EP produced makes the effort worthwhile.

Immunoabsorption column. Now that anti-EP is available, immunoabsorption purification methods are feasible. Two such methods of purifying EP have been attempted and found successful on a small scale: in one case, anti-EP was bound to the column material and in another, anti-bovine serum was attached to the column material. This latter column was designed to remove fetal calf serum proteins from the U-937 EP preparations.

The procedure used is to couple the chosen antiserum to activated CH-Sepharose 4B, block any unreacted ligands and wash away excess proteins. The antigen-containing solution is applied and allowed to equilibrate with the antibody. Unbound protein is washed off the column; the antigen-antibody complexes formed are dissociated with high salt concentration and the bound antigen collected.

Presentation:

Critz, W. J. Endogenous pyrogen: its detection in plasma and partial purification by cibacron blue chromatography. Presented, FASEB meeting, Apr 1980, Anaheim, CA (Fed. Proc. 39:991, 1980).

Publication:

1. Critz, W. J. 1981. Intracerebroventricular injection of rats: a sensitive assay method for endogenous pyrogen circulating in rats. Proc. Soc. Exp. Biol. Med. 166 in press.

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2. Dinarello, C. A., L. Renfer, and S. M. Wolff. 1977. The production of antibody against human leukocytic pyrogen. J. Clin. Invest. 60:465-472.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6417	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. SMRY'S INSTR'N	8A. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	028
b. [illegible]				
c. [illegible]	STOC 80-7.2:2			

11. TITLE: (U) Cellular internalization of bacterial exotoxins

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense;

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGCY	16. PERFORMANCE METHOD
78 11	80 09	DA	C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 80	1.0	72
FISCAL YEAR CURRENT 81	0	0

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POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS: (U) Military medicine; (U) BW defense; (U) Exotoxins; (U) Therapy; (U) Pseudomonas aeruginosa

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Precede each entry with security classification code.)

23 (U) Determine the means by which bacterial exotoxins are internalized and processed by mammalian cells, with the goal of fully characterizing mechanisms of intoxication and proposing effective countermeasures.

24 (U) Conjugate selected toxins with various substances conducive to microscopic localization so as to study interactions with resistant and sensitive cell lines. New cell lines may be developed for toxin-resistance in order to study the interactions better. Diphtheria and Pseudomonas exotoxins are studied.

25 (U) 79 10 - 80 09 - A large number (230) of diphtheria toxin (DE)-resistant mutants were selected from Vero cells and the mode of resistance determined. All but one of the mutant lines possessed a mutant form of cytoplasmic elongation facter 2 (EF-2) and were totally resistant to both DE and Pseudomonas (PE) toxins. One of the mutant lines (LDV-20) appeared to be defective in toxin uptake or intracellular processing; it displayed an intermediate (50 X) degree of resistance to DE toxin and an altered uptake pattern as determined by PIHP assay. Scatchard analyses demonstrated that the mutant cells possessed the same toxin-specific receptor number and affinity as did the parent cells. Pharmacologic studies of the DE uptake process were continued. It was shown that inhibitors of transglutaminase had no effect in the Vero cell system, strongly suggesting that the internalization of DE does not require a transglutaminase-mediated clustering of ligand-receptor complexes. A wide range of organic amine compounds was found to prevent DE-induced inhibition of protein synthesis and block degradation of toxin in Vero cells, implying an intracellular locus of action.

Terminated for management efficiency. Continued in W.U. S10 AN 200. (DAOG1519)
Publication: J. Biol. Chem. 254:11337-11342, 255:2247-2250, 19791.

26. [illegible]

DD FORM 1 MAR 80 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

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M161102BS03)

Task No. 3M161102BS10-AN: Characterization of Microbial Toxins of Potential BW Importance

Work Unit No. S10-AN-164: Cellular Internalization of Bacterial Exotoxins
(S03 00 028)

Background:

The mechanisms by which various biologically active macromolecules are internalized by target cells are currently the subject of study in many laboratories. A number of such molecules, including human choriogonadotropin, insulin, low-density lipoprotein, and epidermal growth factor, have been shown to enter cells by a receptor-mediated process known as "adsorptive endocytosis" (1). More recently, it was demonstrated that 3,3',5-triiodo-L-thyronine (T3) (2) and nerve growth factor (3) are taken up by a similar mechanism. Previous work from our laboratory strongly indicates that the exotoxins of Corynebacterium diphtheriae (diphtheria toxin, or DE) (4) and Pseudomonas aeruginosa (Pseudomonas exotoxin A or PE) (5) are also taken up by receptor-mediated endocytosis. Furthermore, our studies suggest that some form of additional processing, probably by lysosomal enzymes, is necessary for expression of biological (cytotoxic) activity (6). However, the sequence of events leading from initial toxin-receptor binding to intracellular inactivation of elongation factor 2 (EF-2) are still largely unknown.

During the past year, we attempted to delineate the process of toxin uptake and activation in mammalian cell systems by a number of genetic, biochemical, and pharmacologic methods. Our ultimate aim is to elucidate fully the series of steps leading to cytotoxicity, thus facilitating the formulation of effective preventive therapies for various toxin-induced disease states. Results obtained from this research may also contribute to an understanding of how other types of macromolecules, such as hormones and viruses, enter mammalian cells and elicit biological responses.

Progress:

One approach to the study of bacterial toxin-induced cytotoxicity involved the use of toxin-resistant mutant cells blocked at various steps in the uptake or activation process. We have previously selected and characterized a number of resistant mutant lines from highly toxin-sensitive Vero cells: results demonstrated that resistance in all the mutant lines was attributable to a mutant toxin-resistant form of cytoplasmic EF-2. Genetic studies this year centered around efforts to isolate toxin-resistant lines with mutations at levels other than EF-2.

Initially, a microtiter screening technique was developed that permitted the rapid assessment of the relative sensitivities of mutant cell lines to DE and PE. Cells were seeded in 96-well microtiter plates and grown to near-confluency; toxin was then added to the desired concentration and cells were incubated in the

presence of toxin for 48 h. Wells were then rinsed with phosphate-buffered saline to remove detached (killed) cells; remaining cells were fixed with 2% glutaraldehyde and stained with 0.1% crystal violet.

Previous DE-resistant mutants were selected in very high toxin concentrations, ranging from 20- to 10,000-fold the parental median tissue culture lethal dose ($TCLD_{50}$). We hypothesized that selection in lower toxin doses (1-20-fold the parental $TCDL_{50}$) might allow survival of mutant cell lines of intermediate resistance, with mutations at some step in the intoxication pathway other than EF-2. Under these conditions, the mutation frequency was about 0.6×10^{-4}. Resistant colonies developed in about 3 weeks; 230 were picked by scraping and cultured for further characterization. The mutants were screened using the microtiter assay technique; 229 were totally resistant to both DE and PE and were therefore assumed to be EF-2 mutants. Twelve of these lines were grown up for more detailed studies. All were > 10,000-fold more resistant to DE and > 1000-fold resistant to PE than the parent. Saturation experiments using ^{125}I-labeled diphtheria toxin showed that these lines possessed only 25-30% the parental level of toxin-specific cell surface receptors, suggesting that these mutants were identical to the EF-2 mutants previously isolated.

Of the 230 clones studied, one, designated LDV-20, was apparently defective in diphtheria toxin uptake or intracellular processing. This mutant was approximately 50-fold more resistant to diphtheria toxin and 200-fold to PE by 48 h cytotoxicity assay, and 10-fold resistant to diphtheria toxin by an inhibition of protein synthesis assay. Uptake kinetics of ^{125}I-diphtheria toxin at 37°C were somewhat different from those of the parent Vero line; although LDV-20 also exhibited a biphasic pattern, the peak reached was about 5 times greater than that of the parent cells; no decrease in cell-associated radioactivity was observed up to 3.5 h after toxin addition, as opposed to 1.5-2 h in the parent line. The uptake kinetics at 4°C were apparently identical to those of the parent line. Scatchard analyses of saturation data showed that the LDV-20 cells possessed about 100,000 receptor sites/cell with an affinity constant (K) of 1.6×10^4 L/mole; these values correspond closely to those obtained with the parent Vero line. Toxin-cell association in both cell lines was blocked by ATP (1 mM) and enhanced by concanavalin A (100 µg/ml), indicating receptor similarity. Crude EF-2 extracts were prepared from Vero parent and LDV-20 cell populations and analyzed for the ability to be ADP-ribosylated by DE fragment A in an *in vitro* assay. EF-2 from both sources was ADP-ribosylated to the same extent by toxin, indicating that LDV-20 cells possess normal EF-2.

The toxin internalization pattern in the LDV-20 cells, as assayed by the previously described pronase-inositol hexaphosphate (PIHP) technique (4), was altered in some manner relative to that in the parent line. In the mutant, there was significantly less accumulation of PIHP-resistant (internalized) radioactivity. The reason for this is as yet unclear. The results could reflect a mutation at the level of the plasma membrane, thus altering the toxin transport mechanism, or a mutation at the level of intracellular (perhaps lysosomal) processing. However, toxin degradation, as measured by excretion of trichloroacetic acid-soluble radioactivity into the culture medium, proceeded at the same rate and to the same extent in parent and mutant cells. Furthermore, LDV-20 and Vero cells showed no difference in their ability to internalize another exogenous macromolecule, an ^{125}I-labeled complex of trypsin and α_2-macroglobulin (prepared by Dr. Stephen Leppla). This complex recently was shown in an alveolar

macrophage system (7) to be internalized by receptor-mediated endocytosis and subsequently degraded in lysosomes.

Pharmacologic studies of the DE uptake process have also been continued. Recent studies with α_2-macroglobulin and epidermal growth factor have indicated that internalization is preceded by a transglutaminase-mediated clustering of ligand-receptor complexes (8). Results in our system suggested that DE internalization does not require such a clustering step, since a number of known transglutaminase inhibitors, including bacitracin, bleomysin, and dansylcadaverine, had no effect on toxin-induced inhibition of protein synthesis.

It has also been shown that ammonia and a number of organic amine compounds block the internalization of epidermal growth factor by preventing the clustering of ligand-bound receptors. In the Vero cell system, we found that NH_4Cl completely protected the cells from DE-induced inhibition of protein synthesis without detectably affecting either toxin internalization or degradation. Methylamine also effectively protected the cells from DE without measurably altering toxin internalization; however, methylamine partially inhibited degradation. The more highly substituted organic amines, ethylamine, propylamine, butylamine, triethylamine, and tributylamine, all markedly inhibited both degradation and toxin-induced inhibition of protein synthesis, as did the substituted ethylenediamines. The parent compound, ethylenediamine itself, was without effect. None of the substituted organic amines blocked toxin internalization. The protective pattern displayed by ethylenediamine and its alkylated derivatives suggests that expression of the protective effect requires a degree of hydrophobicity and thus implies that the amines act by entering the cell. Furthermore, the correlation between protective potency and inhibition of toxin degradation suggests an intracellular locus of action for the organic amines. It has been demonstrated in other systems that ammonia or amines elicit a marked increase in the internal pH of lysosomes in living cells. It is possible that amine-mediated elevation of intralysosomal pH protects cells from DE by inactivating a lysosomal enzyme(s) essential for the generation of active toxin.

In contrast to the organic amines, NH_4Cl protected cells from DE without detectably affecting toxin degradation. Several approaches to determining the mode of action of NH_4Cl were taken this year. Results obtained in other systems suggest that NH_4Cl acts by maintaining DE in a position accessible to antibody neutralization, presumably at the cell surface. Though out previous work demonstrated that NH_4Cl has no effect on toxin internalization, as assayed by the PIHP technique, or on degradation, a series of experiments using cells prebound with diphtheria toxin at 4°C showed that, in the presence of NH_4Cl, a class of potentially lethal toxin molecules remained accessible to specific antibody. This may imply dual uptake mechanisms for DE: a bulk internalization pathway, measured by the PIHP technique, and an NH_4Cl-sensitive "productive" pathway that results in delivery of fragment A to the cytoplasm. Preliminary fluorimetric studies indicate that NH_4Cl does elevate lysosomal pH in Vero cells, suggesting a possible intracellular locus of action.

Ten mutant DE samples, designated tox 101-110, were analyzed in mammalian cell culture. The mutant toxins are immunologically homologous to normal DE (i.e., are cross-reacting materials or CRM), but are to varying extents less toxic. The 10 CRM were produced and purified by Dr. Leppla from mutant strains of C. diphtheriae supplied by Dr. Walter Laird. The CRM samples were compared to

normal DE in 48-h cytotoxicity, inhibition of protein synthesis, blocking of toxin-receptor binding, and in vitro ADP-ribosylation assays. Seven CRM defective in the A fragment and 3 defective in the B fragment were identified by these methodologies. We hope that these mutant toxin preparations will prove useful in studies of the toxin-receptor interaction, internalization, and intracellular processing.

Presentation:

Dorland, R.B., J.L. Middlebrook, and S.H. Leppla. Isolation of pleiotropic diphtheria toxin-resistant mutants: elongation factor 2 mutants with reduced receptor levels. Presented, ICN-UCLA Symposium on Molecular and Cellular Biology, Keystone, Colorado, February 1980.

Publications:

1. Dorland, R.B., J.L. Middlebrook, and S.H. Leppla. 1979. Receptor-mediated internalization and degradation of diphtheria toxin by monkey kidney cells. J. Biol. Chem. 254:11337-11342.

2. Leppla, S.H., R.B. Dorland, and J.L. Middlebrook. 1979. Inhibition of diphtheria toxin degradation and cytotoxic action by chloroquine. J. Biol. Chem. 255:2247-2250.

LITERATURE CITED

1. Goldstein, J.L., R.G.W. Anderson, and M.S. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature 279:679-685.

2. Cheng, S.Y., F.R. Maxfield, J. Robbins, M.C. Willingham, and I.H. Pastan. 1980. Receptor-mediated uptake of 3,3',5-triiodo-L-thyronine by cultured fibroblasts. Proc. Natl. Acad. Sci. U.S.A. 77:3425-3429.

3. Levi, A., Y. Shechter, E.J. Neufeld, and J. Schlessinger. 1980. Mobility, clustering, and transport of nerve growth factor in embryonal sensory cells and in a sympathetic neuronal cell line. Proc. Natl. Acad. Sci. U.S.A. 77:3469-3473.

4. Dorland, R.B., J.L. Middlebrook, and S.H. Leppla. 1979. Receptor-mediated internalization and degradation of diphtheria toxin by monkey kidney cells. J. Biol. Chem. 254:11337-11342.

5. Leppla, S.H., R.B. Dorland, J.L. Middlebrook, and J.D. White. 1981. Interaction of Pseudomonas exotoxin A with sensitive mammalian cells. Rev. Infect. Dis. (in press).

6. Leppla, S.H., R.B. Dorland, and J.L. Middlebrook. 1980. Inhibition of diphtheria toxin degradation and cytotoxic action by chloroquine. J. Biol. Chem. 255:2247-2250.

7. Kaplan, J., and M.L. Nielsen. 1979. Analysis of macrophage surface receptors. II. Internalization of α-macroglobulin-trypsin complexes by rabbit alveolar macrophages. J. Biol. Chem. 254:7329-7335.

8. Davies, P.J.A., D.R. Davies, A. Levitzki, F.R. Maxfield, P. Milhaud, M.C. Willingham, and I.H. Pastan. 1980. Transglutaminase is essential in receptor-mediated endocytosis of α_2-macroglobulin and polypeptide hormones. Nature 283: 162-167.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6422	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SEC.	6. WORK SECURITY	7. REGRADING	8A. GRADE'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 05	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	031
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Provide with Security Classification Code)

(U) Role of bacterial exotoxins in disease pathogenesis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
75 07	80 09	DA			C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE

	PROFESSIONAL MAN YRS	FUNDS (In thousands)
FISCAL YEAR PRECEDING	80	1.0	114
FISCAL YEAR CURRENT	81	0	0

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS
NAME:
NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Provide KEYWORDS with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Exotoxins; (U) Pseudomonas aeruginosa; (U) Brun infections; (U) Laboratory animals; (U) Diphtheria

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

23 (U) Characterize potent bacterial exotoxins and identify roles in infection. The exotoxin used as a model, Pseudomonas aeruginosa, is believed to be responsible for high frequency and severity of infections of burn wounds. The bacterium and exotoxin are potential BW agents. Characterization will improve ability to prevent, diagnose, and treat and deal with novel uses of these and other pathogens.

24 (U) After characterizing and developing an assay for exotoxins in mice, examine biologic factors involved in toxin synthesis and pathogenesis.

25 (U) 79 10 - 80 09 - Large amounts of Pseudomonas exotoxin A (PE) were supplied to outside investigators. Chloroquine protects cells from diphtheria toxin (DE) and blocks intracellular degradation; thus, lysosomal processing is required for release of enzymatically active peptides. NH_4Cl protects cells but does not block degradation, evidence of 2 parallel uptake mechanisms for DE. Analysis of 125-I-DE degradation by Vero cells using slab gel electrophoresis showed a 28,000 MW and expected A and B peptides. A fully toxic fluorescent derivation of PE was taken up by L929 cells. Chemical modification studies were performed to identify essential amino acids, facilitate preparation of protein-protein conjugates, and make toxoids. Nitration of DE produces a derivative with 1-5% native toxicity and almost normal ability to bind to receptor. Oxidation of methionine residues with small amounts of chloramine T inactivates PE or A fragment of DE. Nontoxic, mutationally-altered DE proteins (CRM) were characterized. CRM 197 contains an insertion not a single amino acid substitution. 10 full size CRM were prepared and shown to include 7 A- and 3 B-region mutants; B had not been available before.

Publication: J. Biol. Chem. 225:2247-2250, 1980.

Terminated for management efficiency. Continued in W.U. S10 AN 200. (DAOG1519)

*Available to contractors upon originator's approval

DD, FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 69 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M161102BS03)

Task No. 3M161102BS10-AN: Characterization of Microbial Toxins of Potential BW Importance

Work Unit No. S10-AN-165: Role of Bacterial Exotoxins in Disease Pathogenesis
(S03 00 031)

Background:

Bacterial exotoxins are responsible for the pathogenesis seen in certain infections (diphtheria, cholera, tetanus, anthrax). Characterization of a number of these toxins has led to successful immunization against the toxin, with coincident protection from infection.

Pseudomonas aeruginosa infection is a frequent and serious complication in debilitated patients, such as those suffering extensive burn wounds. Of the toxic materials produced by this bacteria, the most potent is the ADP-ribosylating exotoxin A. This exotoxin (PE) is produced by nearly all clinical isolates of P. aeruginosa and is lethal to a variety of animals and cultured cells. Evidence suggests that antibody to PE is protective in human infections. This laboratory has developed a toxoid which effectively induces neutralizing antibodies in experimental animals. Test of this toxoid in a number of animal infection models should help determine the role of PE in infection.

Rational design of toxoids requires a detailed knowledge of the structure of toxin molecules and of how various portions of the molecule interact with sensitive cells to achieve the toxic action. We are conducting a broad ranging study using chemical modification and genetic manipulation to characterize the PE and diphthera (DE) toxins, and to elucidate how these proteins enter cells and become activated.

Progress:

PE was produced in the 50-L fermenter as required to maintain our stocks and to supply other laboratories. In one preparation, technical difficulties caused a delay of several days during chromatography on hydroxylapatite. The final product had a potency lower than normal batches, consistent with our earlier observation that the protein is subject to oxidative damage if kept for extended periods in the absence of reducing agents. A more rigorous examination of this phenomenon will be described. The purified toxin, goat or horse antitoxin, or both, were supplied to 8 investigators during FY 80. The largest amounts have been supplied to Dr. Paul Sigler of the University of Chicago. Using the toxin supplied in FY 79, Dr. Sigler determined conditions which cause crystallization of the protein. These crystals appeared suitable for X-ray diffraction work. Therefore, an additional 40 mg of toxin were sent to him, and used to prepare crystals from which diffraction data are now being collected. It can be expected that important information on the structure of the toxin will be obtained.

During FY 80 we continued work on the uptake and activation of bacterial toxins by eukaryotic cells. Previous data had strongly indicated that DE and PE enter cells by adsorptive endocytosis and are delivered to lysosomes. In the case of PE, endocytosis appeared to occur via coated pits. To test this hypothesis further, studies were performed with a number of drugs thought to inhibit specifically endocytosis or lysosomal functions. Chloroquine, previously shown in this laboratory to protect cells from DE, was studied in detail (Publication #1). It was found that this drug had no effect on the rate or extent of internalization of DE prebound to receptor. Chloroquine did, however, prevent the degradation of toxin which had become internalized. The parallel effects on toxin degradation and cytotoxicity provide support for the view that degradation in lysosomes is required for release of enzymatically active toxin peptides into the cytoplasm.

Tests on a number of other amines gave results which were in part like those with chloroquine (Table I). Monoamines all protected cells from both DE and PE, although higher concentrations were required for PE.

Table I. Relative Abilities of Amines to Prevent Toxin-Induced Inhibition of Protein Synthesis

	Concentration Of Amine (mM) Required To Achieve 50% Protection From PE Or DE		
	L-929	Vero	
Amine	20 ng/ml PE[a]	10 ng/ml PE[a]	2 ng/ml DE[a]
NH_4Cl	25	20	3
Methylamine	15	20	4
n-Propylamine	20	15	10
n-Butylamine	20		8
Triethylamine	(P)[b]	15	5
Ethylenediamine (ED)			None
Tetramethyl-ED	10	15	2
Tetraethyl-ED	3	3	2
N,N-Dimethyl-ED	2		2
N,N'-Dimethyl-ED	2		7
Chloroquine	(P)	0.15	0.1

[a]The toxin doses used led to $\geq$ 80% inhibition of protein synthesis in the absence of amines.

[b]Protection was observed, but protein synthesis did not reach 50% of control values at nontoxic doses of the amine.

Of the diamines, all were protective except ethylenediamine, which probably lacks sufficient lipophilicity to achieve penetration of the cell membrane. With the exception of NH_4Cl and methylamine, all the protective amines also block degradation of ^{125}I-DE. These compounds therefore act like chloroquine, and support the hypothesis that degradation is tightly coupled to activation of the toxin. However, the exception provided by NH_4Cl (and to a lesser extent, methylamine) introduces an additional complexity. NH_4Cl is highly protective of cells, but has no effect on the processing of ^{125}I-diphtheria toxin as measured in either internalization or degradation assays. Furthermore, experiments reported some

years ago combining NH_4Cl and antitoxin were most directly interpreted by concluding that NH_4Cl holds diphtheria toxin on the cell surface. We refined these experiments to exclude the possibility that toxicity results from toxin binding to the plastic dishes, and have also shown that NH_4Cl holds toxin in a location where it is susceptible to neutralization with antibody. These data can be reconciled most simply by invoking dual uptake routes, a bulk uptake pathway which processes > 90% of the toxin, delivering it to lysosomes for degradation, and a productive uptake route that efficiently transports a small fraction of the toxin molecules to the cytoplasm. NH_4Cl would act only on the productive uptake path, while chloroquine and other amines would act on both uptake processes.

Recent reports from Dr. Pastan's group at NIH provided a possible explanation for the protective action of amines (1). It was shown that a number of primary amines prevent clustering of receptors for α_2-macroglobulin (α_2MG) and epidermal growth factor. It was suggested that an amine-sensitive transglutaminase was required for cross-linking of receptors to each other or to cellular components of the endocytic machinery. Subsequent reports identified other, more potent, transglutaminase inhibitors that prevented clustering of α_2MG receptors. Effective compounds, listed in order of decreasing potencies, included dansyl cadaverine, bacitracin, n-butylamine, and other primary amines, and putrescine. To determine whether this putative transglutaminase was involved in uptake of either toxin, we tested all these compounds. None conferred significant protection, arguing against a role for transglutaminase. Furthermore, the ability of the secondary and tertiary amines to protect (Table I) cannot be explained in this way, since these amines are not expected to be transglutaminase inhibitors.

Although we have learned a great deal about internalization mechanisms using biochemical and pharmacologic tools, this information is largely indirect. In order to characterize more fully the internalization process we have begun to examine directly the patterns of intracellular degradation of ^{125}I-toxins. This work is being done in collaboration with Drs. R. Dorland and Robert Platz. While it is widely assumed that intracellular processing of DE produces fragment A, this has never been demonstrated. Therefore, the exact form in which the enzymatically active toxin exists in the cytoplasm is not known. In the basic protocol, Vero cells were incubated with ^{125}I-diphtheria toxin at 37°C, treated with pronase and inositol hexaphosphate to remove cell surface toxin, lysed in SDS, and electrophoresed on polyacrylamide gel slabs. When performed with the ^{125}I-toxin routinely employed for other experiments, the autoradiograms of the gels showed numerous radioactive peptides, with the A and B fragments most prominent. Since the ^{125}I-toxin was mostly nicked, the formation of A and B may merely reflect intracellular disulfide bond reduction. In order to determine whether cells proteolytically cleave toxin to A and B it was necessary to perform this experiment using radiolabeled, unnicked toxin. Preparation of unnicked diphtheria toxin will be described later. In the initial experiments with unnicked toxin, a prominent band of about 28,000 daltons was seen in addition to the A and B fragments. When conditions favoring production of this band are established, peptide maps will be used to determine its relationship to the A and B peptides.

Morphologic studies of toxin binding and internalization focussed principally on fluorescence techniques. Fluorescent derivatives of insulin, epidermal growth factor, and α_2MG have been used by Pastan and others to show clustering and uptake into cells. A highly sophisticated fluorescence microscope equipped with a silicon intensifier tube has been acquired. Rhodamine-labeled α_2MG was prepared and shown

to be internalized by fibroblasts, confirming Pastan's results and demonstrating the sensitivity of our instrument. Also available is a high quality spectrophotofluorimeter which allows quantitative measurement of fluorescence. The primary requirement for fluorescence studies is preparation of functional fluorescent toxin derivatives. A careful study was made of the effect of fluorescein substitution on the ability of DE to bind to receptor; affinity for receptor was assessed by measuring the ability of the fluorescein-labeled toxin to compete for binding of ^{125}I-labeled toxin to cells at 4°C. It was found that substitution of a single fluorescein molecule decreased the affinity for receptor by > 80%. Further substitution, up to 5 molecules of fluroescein, caused little additional decrease. It appears, therefore, that some particularly reactive amino groups are located in the receptor-binding portion of the DE molecule. Fluorescence studies on uptake of DE will have to wait until other methods for attachment of fluorescent molecules to this protein are developed.

More success was obtained in preparation of a fluorescent derivative of PE. It was found that this toxin may have at least 3 fluorescein residues attached without decreasing the toxicity toward L-929 cells. The unaltered toxicity indicates that this derivative is bound and internalized by the normal mechanism. Preliminary uptake studies were done in suspension L-929 cells, which are an advantageous material since large numbers of cells may be placed in the cuvette of the spectrophotofluorimeter. Calculations from fluorescein standards indicate that it should be possible to detect 2×10^5 molecules of fluorescein-labeled toxin associated with each cell. This is comparable to the number of receptors measured with iodinated toxin. In initial trials, cells incubated with the toxin for 4 h at 37°C gave a strong fluorescence signal corresponding to uptake of about 2×10^6 molecules/cell.

The other morphologic method used successfully employed colloidal gold. Affinity-purified goat antibody to PE was used to coat gold colloid particles. L-929 cells exposed to toxin at 4°C or control unexposed cells were incubated with the antibody-coated gold and examined by transmission electron microscopy. In the toxin-treated cells, ≈ 30% of the gold particles were above coated pits, while in control cells the localization to pits was < 5%.

An alternate route to characterizing toxin uptake involves selection and characterization of toxin-resistant mammalian cell mutants. This is a principal activity of Work Unit No. S10-AN-164. In previous work it has been found that the desired mutant class in Vero cells, those defective in internalization, are very rare. Therefore, future selections will need to screen hundreds of colonies to find the desired mutants. To facilitate simultaneous selection and clonal purification, a scheme was developed for replicate plating. Cells previously mutagenized and selected for toxin resistance in mass cultures are distributed into 96-well tissue culture plates at dilutions chosen so that the average well contains < 1 live cell. A beaded dextran material on which cells can attach and grow (Cytodex) is also added. When the individual cells grow and fill the wells, aliquots of the beads are transferred to other multi-well plates, in which screening for toxin resistance is performed. Use of the Cytodex beads replaces trypsinization of monolayers, which has been the operation limiting the number of clones that can be screened.

Previous selections successfully yielded one Vero isolate, LDV-20, which seems to be defective in internalization. This mutant has normal DE receptors

and elongation factor 2 (EF-2), but has increased resistance to both PE and DE. To determine whether these cells have a generalized defect in receptor-mediated endocytosis, uptake of a trypsin-α_2MG complex was measured. Trypsin was labeled with ^{125}I and then mixed with a slight excess of α_2MG. Fibroblasts are known to take up the complex at the same rate as free α_2MG. Uptake of the complex into LDV-20 cells proceeded at a rate equal to that in Vero cells, showing that the mutant's defect is probably not a generalized inability to perform endocytosis.

With the exception of LDV-20, prior efforts to obtain mutants in Veros yielded only strains altered in EF-2. Attempts to obtain a significant number of mutants with defective receptors or internalization mechanisms will be difficult because such mutants will be rare compared to those altered in EF-2. An alternative selection procedure offers a way to circumvent this problem. A conjugate of a toxin molecule lacking ADP-ribosylation activity with another toxic molecule could use the toxin receptor to enter cells, but would cause cell death through a mechanism independent of EF-2. Efforts were begun to prepare a conjugate having these properties by linking the toxic plant lectin, ricin, to DE inactivated by nitration. In the presence of lactate, which blocks binding of ricin to cells, such a conjugate should enter cells using the DE receptor and kill the cells through action of the ricin A subunit on ribosomes. Since ricin-resistant mutants altered in ribosomes have never been found, the only cells able to survive exposure to the conjugate will be the desired receptor or internalization mutants. Initial attempts to prepare this conjugate have used the fact that ricin is a glycoprotein and might therefore be conjugated by the procedure routinely used to prepare horse radish peroxidase conjugates, oxidation with periodate to produce aldehydes and subsequent reductive amination to a second protein. It was found that reductive methylation to block the amine groups of ricin decreased its toxicity and its affinity for galactose residues, probably by alteration of the ricin B subunit. Tests with [^{3}H]leucine showed that periodate oxidation generated aldehydes to which amines (leucine) could be attached. However, little or no conjugate was obtained when DE modified by nitration or by attachment of a diamine spacer molecule was used. Subsequently (as discussed later) it has been found that the toxin used in these experiments contains a substantial amount of aggregate. This fact may provide an explanation for the poor yield of conjugate.

As demonstrated in previous paragraphs, a number of experimental approaches to studying the action of toxins require chemical manipulation of the protein molecules. Identification of the essential amino acid side chains of a toxin allows selective modification so as to produce toxoids retaining essentially normal immunogenicity. Nontoxic analogues of PE or DE are valuable reagents for studying cellular uptake mechanisms. Knowledge of the essential regions of the toxin molecule would allow construction of protein-protein conjugates selectively retaining either receptor-binding or ADP-ribosylation activity.

We conducted an extensive study of the nitration of diphtheria toxin (2). Tetranitromethane has received wide use due to the high specificity with which it causes nitration of tyrosine residues. The report (2) claimed that one tyrosine in the enzymatically active site of DE was especially susceptible to nitration, and that this modification destroyed the enzymatic activity and rendered the protein nontoxic. Nitration of up to 5-6 tyrosines had little effect on binding to receptor, as measured by the ability of the derivative to block competitively the cytotoxic action of native DE (a Schild plot analysis). In our studies, it was confirmed that extensive nitration (5-6 nitrotyrosines/molecule) did not greatly decrease the affinity of the protein for receptor. This was demonstrated both in

Schild analysis and by directly measuring competition with ^{125}I-toxin for binding to Vero cells. However, in contrast to the prior report (2), this rather heavily modified toxin retained 1-5% of the toxicity of the native protein. Two explanations for this result have been considered. One of these suggests that the reported detoxification occurred through an unrecongized oxidation of an amino acid other than tyrosine. The susceptibility of DE to oxidation is discussed later. The other explanation holds that a fraction of the toxin molecules are in an altered form in which the essential tyrosine is unavailable for reaction. As discussed more fully in a later section, Lory and Collier (3) recently showed that about 1/2 the molecules in DE preparations have a firmly-bound nucleotide-like material blocking the NAD binding site. These protein molecules could be refractory to nitration. Our recently acquired ability to purify the nucleotide-free form of DE will allow a test of this hypothesis.

As discussed earlier, direct attachment of fluroescein to DE causes substantial inactivation. A number of other lines of evidence indicate that the amino groups of this protein are essential. Therefore, reaction schemes which achieve conjugation at other groups were sought. One promising approach was revealed when it was found that diamines can be attached to the carboxyl groups by a carbodiimide-catalyzed reaction without decreasing toxicity. Thus, reaction with 5 mM carbodiimide in the presence of 200 mM putrescine (1,4-diaminobutane) gave a preparation which had no material banding at the normal location on isoelectric focussing gels; all the protein had shifted to higher pI, indicating attachment of substantial amounts of the diamine. This preparation appears to retain full toxicity, and by implication must bind normally to receptor. The putrescine is expected to act as a "spacer," analogous to those used in construction of affinity chromatography resins, and should allow more efficient conjugation of proteins or fluorescent dyes to DE.

The chemical modification of PE most extensively studied was oxidation. We had previously noted that this toxin was sensitive to oxidation with Chloramine T, and that radiolabeling must be performed with lactoperoxidase to preserve toxicity. Through a more rigorous survey of reaction conditions, it was found that treatment with 1 mM Chloramine T for 60 min at pH 7 causes complete loss of toxicity. Electrophoretic analysis showed that both modest size and change heterogeneity is introduced by the Chloramine T treatment. Amino acid analyses revealed significant losses only in methionine, with concomitant appearance of methionine sulfoxide. UV spectra suggest that aromatic residues may also be destroyed. ADP-ribosylation activity was measured on samples treated with denaturants and reductants in combination expected to release enzymatically active fragments. Since none of these treatments produced enzymatically active material, it can be concluded that oxidation destroys an amino acid in the active site. Subsequently, it was found that this toxoid retains substantial ability to bind to receptor, perhaps 20% that of the native toxin. The affinity of the receptor for this toxoid was determined with a Schild plot, where toxicity (protein synthesis inhibition) is measured as a function of toxin concentration at several fixed concentrations of toxoid. While the results varied somewhat, it appeared that the binding constant of the oxidized PE for receptors on L-929 cells is 1-5 x 10^{-7} moles/L.

Though it was initially assumed that Chloramine T was a rather nonspecific oxidant, we later learned of a carefully performed study showing that this reagent does in fact have high specificity for methionine residues in proteins

(4). This report demonstrates that convincing evidence for an essential methionine can be obtained by showing that inactivation occurs at low molar ratios (1:5) of Chloramine T to protein. Since the active site is at least partially buried in the native, proenzyme form of PE, experiments of this type are less ambiguous if performed on activated toxin. Therefore, the protein was unfolded by denaturation and reduction and the new sulfhydryls blocked by either disulfide interchange with hydroxyethyldisulfide, reaction with N-ethylmaleimide, or reaction with iodoacetamide. These derivatives were treated with varying molar ratios of Chloramine T and then assayed for ribosylation activity. In several experiments 90% inactivation of the N-ethylmaleimide derivative was produced by < 10 equivalents of reagent, indicating the probable presence of an essential methionine. These experiments will be further refined by use of the enzymatically active peptide (MW 26,000) of PE. A controlled method for production of this material has just been published (3) and we have successfully performed this procedure and produced several milligrams of the peptide.

Since DE has the same enzymatic activity as PE, it seemed reasonable to hypothesize similarity in the active sites. Therefore, Chloramine T inactivation was tested on the purified, enzymatically active A fragment of DE; 99% inactivation was achieved at 5 equivalents of reagent, demonstrating the probable presence of an essential methionine.

An alternate way to obtain inactive derivatives of a toxin is by mutation of the appropriate gene. Work by Uchida et al. (5) led to a set of 5 nontoxic, serologically cross-reacting DE analogues (CRM). One of these, CRM 197, has been a particularly useful reagent for cell uptake and receptor studies because it appears to have a single amino acid change in the A fragment which renders it unable to catalyze ADP-ribosylation of EF-2. Since the B fragment appeared unchanged, CRM 197 was considered to bind normally to receptor, and it has been used in Schild plot analyses to measure receptor affinity. Because this material is so useful, and because it could not be obtained in significant amounts from other investigators, we developed the ability to produce and purify CRM 197. After repeated trials, methods were developed which reliably yield 5-10 mg of pure protein from 3 L of culture. In the course of examining such preparations on SDS gel slab electrophoresis, it was noted that the intact protein and the A fragment appeared slightly larger than the corresponding species of native toxin. Since a single amino acid substitution does not normally cause a detectable change in mobility on SDS gels, it seems unlikely that CRM 197 is a simple analogue of DE. Initially we suggested that the increase in molecular weight might be due to retention of the N-terminal signal sequence. However, Dr. James Schmidt (Pathology Division) found that the N-terminal sequence of CRM 197 is identical to that of native toxin. Therefore, if CRM 197 is in fact larger than native toxin, this must be due to insertion of additional amino acids within the A fragment sequence.

The recognition that CRM 197 might not be an accurate analogue of DE led us to search for other CRM. Laird and Groman (6) isolated a group of nontoxic CRM and determined their size, but no other characterization was performed. In hope of identifying a CRM altered only in the A region, we obtained the 10 strains from Laird which produce full size (62,000 daltons) CRM. These are designated tox 101 to tox 110. Using the methods developed for production and purification of CRM 197, several milligrams of each of these were prepared. Initial characterization by toxicity and ADP-ribosylation assays and electrophoresis on SDS slabs shows this group to be extremely interesting. Seven appear to be A region mutants, having

greatly reduced or undetectable enzymatic activity, and little or no toxicity. One of these is altered so that endogenous or trypsin-induced nicking produces peptides unlike the normal A and B fragments. The remaining CRM appear to be altered in B. Several of these are approximately 100-fold less toxic than native toxin, and bind less avidly to receptor, as determined in competition assays. CRM altered only in B have not been reported previously. These proteins should prove to be very useful in characterizing the binding of DE to cells and in isolating the toxin receptor.

Most of the studies with DE depend on use of a homogeneous toxin sample. Studies in FY 80 dealt with 3 types of heterogeneity which previously either had not been considered significant or had not been recognized. The first of these involves the presence in our current toxin preparation of a component that appears to be a stable toxin dimer. Chromatography of this preparation on DEAE Sepharose CL-6B gives 2 well-resolved peaks. Peak I, eluted first, contains approximately 1/4 of the protein applied to the column, and is highly toxic. Peak II is much less toxic. The 2 peaks have the same amount of enzymatic activity after activation, are nicked to the same extent (10-20%), have identical UV spectra, and are indistinguishable on SDS electrophoresis. Other workers have occasionally reported the existence of a toxin dimer and suggested that it is formed during precipitation with ammonium sulfate. Our peak II is apparently similar, and may have formed during precipitation or during storage at -70°C. The second type of heterogeneity involves nicking. Connaught Laboratories has come to recognize that many purchasers of toxin prefer samples with a minimal degree of nicking between the A and B fragments. Thus, through special arrangements, we were able to obtain a lot that is only 10-20% nicked. However, for experiments studying intracellular processing of ^{125}I-toxin even this level of nicking is unacceptable. It was found possible to prepare an unnicked species by incubation of diphtheria toxin with 400 mM dithiothreitol at 37°C, a condition which separates nicked molecules into A and B peptides and causes precipitation of the latter. Chromatography of the supernatant on DEAE Sepharose CL-6B yields a peak of pure, unnicked toxin. The final type of heterogeneity dealt with involves nucleotide binding. Lory and Collier recently showed that DE could be separated into 2 components on ATP-agarose. The first fraction contained a firmly bound nucleotide-like material and did not reversibly bind ATP, while the second did. Since ATP blocks binding of toxin to cells, it was suggested that ATP is blocking the site on the toxin which interacts with receptor. However, this hypothesis seems inconsistent with the fact that both fractions are equally toxic. We have confirmed the above results. Conditions have been optimized for binding of toxin to ATP- and NAD-agaroses, and toxin has been separated into the 2 components which are equally toxic to Vero cells, even when exposure to toxin lasts only 10 min. Therefore, the relationship between nucleotide binding to toxin and the ability of nucleotides to protect cells remains unclear. Our ability to recognize and control these 3 types of heterogeneity allows preparation of various species. In particular, we can now isolate the putative "native" form: monomeric, unnicked, nucleotide-free DE.

Presentations:

1. Leppla, S.H., R.B. Dorland, J.L. Middlebrook, and J.D. White. Interaction of *Pseudomonas* exotoxin A with sensitive mammalian cells. Presented, WRAIR Symposium on Pseudomonas Infections, Washington, DC, 6-7 Dec 1979.

2. Leppla, S.H., J.L. Middlebrook, J.D. White, and R.B. Dorland. Receptor-mediated internalization of diphtheria and Pseudomonas exotoxins by mammalian cells. Presented, Symposium Session 7, American Society of Microbiology, Miami Beach, FL, 11-16 May 1980.

3. Leppla, S.H., and R.D. Dorland. Uptake mechanisms of ADP-Ribosylating toxins. Presented, USAMRIID Conference on Internalization of Toxins and Hormones, Frederick, MD.

Publications:

Leppla, S.H., R.B. Dorland, and J.L. Middlebrook. 1980. Inhibition of diphtheria toxin degradation and cytotoxic action by chloroquine. J. Biol. Chem. 255:2247-2250.

LITERATURE CITED

1. Davies, P.J.A., D.R. Davies, A. Levitski, F.R. Maxfield, P. Milhaud, M.C. Willingham, and I.H. Pastan. 1980. Transglutaminase is essential in receptor-mediated endocytosis of α_2-macroglobulin and polypeptide hormones. Nature 283: 162-167.

2. Beugnier, N., and J. Zanen. 1977. Diphtheria toxin: the effect of nitration and reductive methylation on enzymatic activity and toxicity. Biochem. Biophys. Acta 490:225-234.

3. Lory, S., and R.J. Collier. 1980. Expression of enzymatic activity by exotoxin A from Pseudomonas aeruginosa. Infect. Immun. 28:494-501.

4. Schechter, Y., Y. Burstein, and A. Patchornik. 1975. Selective oxidation of methionine residues in proteins. Biochemistry 14:4497-4503.

5. Uchida, T., A.M. Pappenheimer, Jr., and A.A. Harper. 1972. Reconstitution of diphtheria toxin from two nontoxic cross-reacting mutant proteins. Science 175: 90-903.

6. Laird, W., and N. Groman. 1976. Isolation and characterization of tox mutants of corynebacteriophage beta. J. Virol. 19:220-227.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6414	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. CITE AUTH	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	032
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Include with Security Classification Code)

(U) Rift Valley fever virus infections: genetic and cellular aspects

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
78 08	80 09	DA			C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE

	PRECEDING	a. PROFESSIONAL MAN YRS	b. FUNDS ($ thousands)
FISCAL YEAR	80	2.7	200
	CURRENT 81	0	0

19a. RESPONSIBLE DOD ORGANIZATION

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20. GENERAL USE

Foreign intelligence considered

19b. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
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NAME: Erlick, B. J. POC:DA

21. KEYWORDS (Provide KEY with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Immunology; (U) Genetics; (U) Rift Valley fever; (U) Bunyaviruses; (U) Hemorrhagic fever; (U) Encephalitis

22. TECHNICAL OBJECTIVE, 23. APPROACH, 24. PROGRESS (Provide brief individual paragraphs identified by number. Proceed each with Security Classification Code.)

23 (U) Define antigenic and other laboratory characteristics of recent Rift Valley fever (RVF) virus isolates to ascertain if previously developed vaccine will be efficacious and to try to explain the emergence of severe forms of the disease as part of the clinical spectrum. Knowledge of this virus will help in medical defense to better understand Bynyaviruses, most of which could be considered of importance as potential BW offensive weapons against U. S. troops.

24 (U) Suitable animal models are developed, preferably using discrete genetic differences to highlight key steps in pathogenesis. Virus replication, antigen expression, and the immune response are monitored during infection to allow inferences about critical determinants which are tested by experimental manipulation.

25 (U) 79 10 - 80 09 - Host genetic models in inbred rats have been studied in rats genetically destined to develop hemorrhagic fever. Spreading infection occurs in the liver and leads to hepatic, adrenal and renal involvement with death in 2-3 days. In resistant rats virus replication in the liver is contained within the first few hours of infection implicating an early defense, such as macrophages or interferon. Antibody may play a role in consolidation since immunosuppression of resistant rats with cyclophosphamide leads to subacute hepatic and nervous system disease. Inbred rat strains with encephalitis have an early course similar to that of resistant rats, but 1-2 weeks later develop multifocal areas of replication and necrosis in the central nervous system in spite of high antibody titers. Viral genetic and molecular techniques are being used to understand these phenomena. Their relation to vaccination is under study, as is therapy. Terminated for management efficiency. Continued in W.U. 871 BC 148.

Publications: In Proc., Annu. Mtg. US Anim. Health Assoc., 1979; Lancet 1:886-887,1980; Virology 105:256-260, 1980; Am. J. Vet. Res. 41, in press 1980; In contrib. to Epidemiol. and Biostat., in press, 1980. (DAOG1537)

DD FORM 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

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M161102BS03)

Task No. 3M161102BS10 AP: Biology of Viral Agents of Potential BW Importance

Work Unit No. S10 AP 170: Rift Valley Fever Virus Infection: Genetic and
(S03 00 032) Cellular Aspects

Background:

Rift Valley fever (RVF) is a widespread African member of the family Bunyaviradae which poses a natural threat to military operations and which also possesses properties that make it feasible for use against us as a BW agent. In 1977, the virus was isolated in Egypt for the first time where it caused a major epidemic with an estimated million human cases over the succeeding 3 years. The widespread human disease and domestic animal wastage emphasized the devastating potential of this virus and raised the possibility of its continued spread into adjacent Middle Eastern countries or by viremic air travelers to distant sites. Although an experimental first-generation inactivated human vaccine has been developed by the US Army, the vaccine requires multiple injections to produce immunity and booster injections to maintain immunity. The vaccine is expensive to produce, is in relatively limited supply, and has not been standardized from lot to lot. We have been engaged in studies of RVF since January 1977 because of its global biomedical significance, its unique military importance, and the lack of definitive protective or control measures. Furthermore, RVF is the only member of the large taxonomic family Bunyaviridae whose pathogenesis is currently under study at USAMRIID. Our studies have been directed in the following areas: (a) understanding the pathogenesis of the severe forms of the disease in order to predict who would be at risk and to develop treatment modalities; (b) studying the properties of different RVF isolates and clones to understand the basis for their virulence as a probing study for the feasibility of an attenuated vaccine; (c) examining the molecular properties of RVF to provide definitive markers of the genetic basis for virulence, studying the evolution and epidemiology of the virus, and developing basic information on replication and structure of the virus to permit rational design of vaccines or antivirals for RVF and other Bunyaviradae; (d) assessing the relationship of RVF to fellow members of the proposed Phlebovirus genus both because of the intrinsic interest of other phleboviruses and to try to understand some of the apparently unique properties of RVF; and (e) attempting to understand the limitations of the currently used human vaccine, particularly optimization of dose schedules, inter-lot variations, duration of immunity, and determinants of host response.

Progress:

Pathogenesis (Anderson): We had previously shown that most fatal RVF infections of laboratory animals are due either to extensive hepatic necrosis occurring during the first week of infection or to late encephalitis. We had identified inbred rat strains having these different responses. Since the rat strains were inbred, the differences in their responses might have been a consequence of some simple genetic difference. We have now completed extensive breeding experiments to test this hypothesis. We have been unable to define any genetic basis for encephalitis. However, resistance to fulminant hepatic infection is inherited as a simple Mendelian dominant (Table I). This should permit us to define the gene operating and thus identify a critical step in RVF pathogenesis.

TABLE I. RESISTANCE TO FULMINANT HEPATIC NECROSIS HAS MENDELIAN DOMINANT INHERITANCE[a]

RAT CROSS	N	% SURVIVORS	
		Observed	Expected
1. LEW	14	100	100
2. WF	20	5	0
3. LEW x WF	19	100	100
4. LEW x (LEW x WF)	15	80	100
5. WF x (LEW x WF)	25[b]	40	50
6. WF x (Survivors of #5)	41	46	50
7. WF x (Survivors of %6)[c]	22	45	50

[a]Female rats inoculated SC with 5×10^3 PFU ZH-501; survivors evaluated on day 7. Similar results obtained with male rats and with converse breeding experiments.

[b]Challenged with only 50 PFU

[c]Four backcrosses onto susceptible WF background.

The inbred rat model is of particular interest since it mimics the 3 major forms of human RVF in a single laboratory host; benign disease (Lewis or LEW rats), fulminant disease (Wistar Furth or WF rats), and encephalitis (MAXX) rats. For this reason we have undertaken an in-depth study of the pathogenesis of RVF in rats by studying the sequential spread of virus at intervals after infection (titers and staining of organs) and evaluation of histological lesions. In the WF rat (Table II) infection is fatal 2 - 3 days after SC inoculation of 5×10^5 PFU virus; the evident cause of death is complete hepatic necrosis. There is also pulmonary arteritis and minor lesions in the spleen and adrenal. Viremia begins early and is progressive to levels of 10^7 - 10^{10} PFU/ml. FA studies of the liver demonstrate single cell infection at 8 - 16 h developing into spreading foci which culminate in complete infection of the organ in moribund rats. Vascular endothelium and glomeruli are widely involved, extending the parallel of this model to the peripheral vascular damage and anuria seen clinically in human hemorrhagic fever. Spleen, lymph node, lung, adrenal and renal tubular cells are also infected.

The LEW rat has no obvious signs of disease; minimal hepatic necrosis is the only histological lesions detectable. Viremia reaches titers of 2.9 ± 0.5 $\log_{10}$ PFU/ml and cannot be detected after 56 h postinfection. Focal liver infection occurs but is not detected until 24 h by FA and never exceeds clusters of 5-10 cells in diameter. Lymphoid (spleen and lymph node) and adrenal organs are involved by 24 h. Virus titers are clearly declining by 4 days and are undetectable when sampled at 14 days. There is never evidence of brain infection. Thus, the infection in the LEW rat generally follows the same early pattern seen in the WF rat but restriction of virus growth is evident from the first day, suggesting that cellular susceptibility or interferon might be critical factors in early resistance.

TABLE II. VIRUS TITERS AFTER INFECTION OF INBRED RAT STRAINS (n = 3/GROUP) WITH 5×10^5 PFU RVF (ZH-501 STRAIN)

TISSUE	TIME (h)	MEAN LOG_{10} PFU/ml OR g ± SD		
		LEW	MAXX	WF
Serum	8	1.0 ± 0.6	0.7	3.0 ± 1.0
	16	1.0 ± 0.4	0.7	3.9 ± 0.8
	24	2.3 ± 0.7	1.7 ± 1.0	7.8 ± 0.4
	32	2.9 ± 0.5	0.7	7.7 ± 0.5
	56	0.7	0.7	-[b]
Liver	8	0.7	0.7	2.5 ± 1.8
	16	0.7	0.7	4.3 ± 0.5
	24	3.1 ± 0.1	1.2 ± 1.1	8.2 ± 0.2
	32	2.5 ± 0.8	0.7	8.3 ± 0.3
	56	1.7 ± 1.9	2.7 ± 2.0	-
	80	2.0 ± 1.3	1.0 ± 1.1	-
	104	0.7		
Spleen	8	0.7	0.7	1.3 ± 1.4
	16	0.7	0.7	3.0 ± 0.7
	24	1.3 ± 1.4	0.7	7.0 ± 0.4
	32	1.4 ± 0.7	0.7	7.5 ± 0.4
	56	2.6 ± 1.7	3.0 ± 2.7	-
	80	4.3 ± 0.7	4.3 ± 0.5	-
	104	3.2 ± 0.5	4.3 ± 0.4	-
	7 days	ND[a]	3.8 ± 0.3	-
	10 days	ND	1.8 ± 1.3	-
	14 days	0.7	0.7	-
	19 days	ND	1.0 ± 1.1	-

[a] Not done

[b] All animals dead

MAXX rats appear well during the first week after challenge but about half develop encephalitis during the following 2 weeks. The only significant histologic lesions are found in the brains of encephalitic rats: multiple foci of acute necrosis with polymorphonuclear infiltrate and perivascular cuffing. Viremia is low (1.7 ± 1.0 log_{10} PFU/ml) and evanescent. Organ virus titers and FA studies during the first few days resemble those of LEW rats, but virus appears later and perhaps evidences a more prominent replication in reticuloendothelial tissue. Rats dying with encephalitis have a very distinctive pattern of brain involvement: large foci of intense fluorescence (presumably corresponding to the focal necrotic histopathologic lesions) occur with scattered infected cells bordering, and the rest of the brain uninvolved. One puzzling fact is the brief duration of viremia, a finding that MAXX rats share with 3 other species in which late encephalitis is a common outcome of RVF infection: gerbils, lauchas and cotton rats. Two species that rarely develop encephalitis (rhesus monkeys and sheep) have a viremia pattern which more closely resembles that of the LEW rat. Another perplexing problem is the latency of the encephalitis. The late occurrence of encephalitis in the presence of high titered serum antibody suggests an immunopathologic mechanism, such as cytotoxic T cells to viral antigens, postinfectious encephalitis or induced autoimmune disease. However, the pathology

and virologic evidence suggest a direct viral cytopathic effect. Direct intracranial inoculation of small doses of virus leads to fatal encephalitis within 3 - 5 days. There are 2 ways to reconcile these facts: (a) virus reaches the meninges or brain during the primary viremia and is somehow maintained in a latent state until the onset of disease, or (b) virus travels to the nervous system after the primary viremia, evading serum neutralizing antibody by some mechanism, such as entering in the form of infected cells. The persistence of RVF in spleen cells of MAXX rats (and gerbils in data not shown here) is consistent with the latter hypothesis. Studies are underway to elucidate these possibilities.

We have also examined the response to inactivated RVF antigens. Neutralizing antibody appears at the same time and in similar titers in WF, LEW and MAXX rats suggesting that the different clinical forms of disease are not due to differences in the humoral immune response to RVF antigens. Pilot studies of immunosuppression of LEW rats with procarbazine or cyclophosphamide are also relevant. Antibody response to inactivated antigens is delayed and suppressed with the regimens used. However, the resistant LEW rats are not converted to the WF phenotype. There were, however, some late deaths with high virus doses. This suggests that immune mechanisms (such as antibody) may be important in the final eradication of virus infection.

To study the cellular basis of resistance of RVF, we have established cell lines from each rat strain. When MAXX, LEW and WF cell lines are infected with the ZH-501 strain of RVF, titers and cytopathic effect are similar. However, if careful titrations are performed, the yield from WF cells is 2- to 5-fold higher. Furthermore, plaques under agar are larger on WF cells. It seems unlikely that these differences are important in the intact rat, but they may be more significant if multiplied through several replication cycles in a race between virus multiplication and the immune response. Probing studies with interferon (IF) are under way, but relatively little is known about rat IF, so that standards and assays must be developed first. RVF has been shown to be as sensitive as vesicular stomatitis virus to mouse IF and murine RVF can be successfully treated with poly(ICLC), which induces IF among its many physiological effects. It is also of interest that cyclophosphamide does not affect interferon production in mouse models (1, 2).

Virus Genetics. We have previously noted the selective avirulence of non-Egyptian RVF isolates for WF rats; several laboratories have achieved a degree of attenuation of RVF for mice by laboratory manipulations (reviewed in 3). We have now begun a study of the properties of randomly selected clones of ZH-501. First, we devised and validated techniques to clone in diploid cells suitable for vaccine production, so that our results could be applied to that problem as well as to the analysis of the genetic heterogeneity of virulent RVF stocks. We tested 12 clones for virulence in mice by SC inoculation: 3 were clearly attenuated, 8 were virulent, and one gave conflicting results when multiple subclones were tested (Table III). One of the attenuated clones was tested in more detail. It resembled Lunyo virus, a naturally occurring mouse-avirulent isolate of RVF (Table IV). Now that we have established the phenomenon, the immediate questions are: How do these heterogeneous viruses interact to determine the virulence of our RVF virus preparations, or the natural inocula? Are all attenuated clones as virulent by IC inoculation as the single one tested? Are there multiple functional lesions in the attenuated viruses which could be accumulated in a single candidate vaccine virus which would therefore be "failsafe" in genetically heterogeneous hosts? How many distinct genetic lesions are there in the attenuated clones?

TABLE III. VIRULENCE OF CLONES OF ZH-501

		TESTS OF RECLONED PROGENY				
Number	Virulence	No. Tested	Virulent	Intermediate	Avirulent	Possibly Neurotropic
205507	Virulent	2	2	0	0	
205508	Attenuated	2	0	1	1	
25509	Virulent	1	1	1	0	
205510	Virulent	13	12	1	0	2
205511	Attenuated	1	0	0	1	
205512	Virulent	2	2	0	0	1
205513	Virulent	1	1	0	0	
205514	Attenuated	8	0	0	8	
205517	Virulent	2	2	0	0	
205518	Virulent	5	5	0	0	
205519	Intermediate	3	2	0	1	
20537	Virulent	1	1	0	0	

[a]507 ICR Swiss mice given 10^3 PFU SC or IP. Virulent:0-1 survivors, avirulent: 0.1 die. Possible neurotropic: kills only 0-2 mice before day 7, but by day 28 only 0-1 remain alive.

TABLE IV. PATHOGENECITY OF RVF VIRUS STRAINS FOR MICE

	Log_{10} LD_{50}/ml		
	Suckling	Adult	
STRAIN	IC	IC	SC
Entebbe	7.1	6.0	5.1
Lunyo	6.2	6.2	<1.0
ZH-501	8.9	8.1	6.3
203222 Clone	7.5	7.2	<1.2

Molecular studies (Erlick). In our hands RVF strains are indistinguishable by the most sensitive serologic techniques. Four isolates were subjected to detailed molecular analysis (Table V). The molecular size of the 3 major virion structural proteins and of the 3 RNA components did not differ among the isolates. Definite differences were detected when tryptic fingerprints of the proteins were compared, all 4 isolates could be distinguished by comparing nucleoprotein fingerprints. The T1 oligonucleotide RNA fingerprints proved to be the most sensitive means of differentiating strains. Multiple "spots" distinguished the 4 viruses although they were obviously related.

Oligonucleotide fingerprinting is a cumbersome procedure; preparation of RVF RNA species was technically difficult but some additional data were obtained. Isolated large and medium RNA segments of ZH-501 were distinct from one another, proving that the medium segment is not a subunit or cleavage product of the large piece but rather an independent species with its own unique coding capacity. Fingerprints from 2 Rhodesian and an Egyptian isolate were also successful. In spite of their geographic proximity, the Rhodesian and South African isolates had multiple differences. In contrast, the 2 Egyptian isolates were virtually identical, one from a fatal human hemorrhagic fever case acquired near Zagazig in the Nile delta in 1977 and the other from a cow in Upper Egypt (Asyut) in 1978. This strongly supports a single introduction of RVF into Egypt with subsequent spread. It also suggests that hemorrhagic fever strains are very closely related genetically to other strains. Unfortunately, no virus strains from Sudan or Kenya are available for comparison, but it is clear that RNA fingerprinting is a potentially powerful tool that could be used to analyze RVF isolates if covert introductions or BW attack were suspected. We hope to apply it to the "microepidemiology" of RVF using a library of Rhodesian isolates from Dr. Swanepoel and to the study of the genetic basis of the virulence of the clones discussed above.

Relatively little is known concerning the minor structural proteins of the nonstructural proteins of the Bunyaviradae. For example, a high MW band (100-150,000) is often observed when RVF or other viruses are analyzed by PAGE and this is often alleged to be the viral polymerase. We have isolated this band and by tryptic fingerprinting demonstrated that it is an aggregate or a precursor of the surface glycoproteins G_1 and G_2. Our RVF preparations usually also have a 40-50,000 MW band. This was initially assumed to be actin, a "sticky" protein that often copurifies with immunoprecipitates or viruses. However, examination of this

TABLE V. MOLECULAR COMPARISON OF 5 RVF VIRUS ISOLATES AND PUNTA TORO

VIRUS	NO. OF STRUCTRUAL PROTEINS	NO. OF RNA SEGMENTS[d]	TRYPTIC PEPTIDES[e]		RNA FINGERPRINTING PATTERN[g]
			Nucleocapsids	Composite Glycoproteins	
ZH-501	4,[a] 1[b]	3	-	-	Egyptian[h]
Entebbe	4, 1	3	1, 23	0, 0	South African[i]
SA-51	4, 1	3	1, 23	0, 0	South African
SA-75	4, 1	3	3, 21	0, 0	ND
Lunyo	ND[c], 1	ND	8, 16	ND	ND
Punta Toro	4, 1	3	21, 3	NR[f]	NR

[a]Glycoproteins of ZH-501, Entebbe, SA-51, SA-75 have MW of 1×10^5, 6.5×10^4, 5.6×10^4, and 4.3×10^4. Those of Punta Toro are 1×10^5, 6.5×10^4, 6.0×10^4 and 4.5×10^4.

[b]Non-glycosylated nucleocapsid protein (N) molecular weight is 2.5×10^4 for ZH-501, Entebbe, SA-51, SA-75, and Lunyo. Punta Toro N protein molecular weight is 2.0×10^4.

[c]Not done

[d]MW of RNA segments are 2.7×10^6 (L), 1.7×10^6 (M), and 0.6×10^6 (S).

[e]Analysis of tryptic peptide mapping. First number represents number of major unique peptides and the second is common peptides when compared to ZH-501.

[f]Not related.

[g]Composite (1, M. S) oligonucleotide RNA fingerprint analysis

[h]Very closely related to Egyptian strain isolated 1978 (Asyut, Egypt, bovine).

[i]South African strains are not related to Egyptian strains, but are related to Rhodesian strains (R-34, Salisbury, 1970, human and R-35 Sinioa, 1974, bovine). Degree of relatedness requires further analysis.

species by tryptic fingerprinting proved that it was unrelated to authentic actin. We still do not know if it is a cellular contaminant or a virion protein, but these studies clearly demonstrate the value of deploying the effort and resources to examine critically the molecules resolved by PAGE rather than relying on analogy and speculation to assign their identity.

We have also attempted to develop techniques for 2-dimensional analysis of virion polypeptides utilizing PAGE for molecular size discrimination followed by isoelectric focusing to resolve charge heterogeneity. To date we have been successful only with the RVF nucleoprotein, where we can resolve 3 charge species with the appropriate 25,000 MW. Desialation (although the nucleoprotein is thought not to be glycosylated) or carbamylation are possible explanations, but it should be borne in mind that 3 RNA species and 3 distinct nucleocapsids can be isolated from some Bunyaviradae. Further studies of the molecular basis of this phenomenon are in progress.

Phlebotomus fever virus relationships. RVF was recently shown by Dr. Robert Shope, YARU, to be related by HI test to the Phlebotomus fever group of viruses. We have confirmed this connection and shown that it also holds for the fluorescent and neutralizing antibody tests. These results combined with molecular analysis have been used by the taxonomic committee to propose a new genus, Phlebovirus, within the Bunyaviradae family. We have selected 9 Phleboviruses on the basis of serologic or geographic relatedness to compare to RVF. To date, there is no evidence that any of these viruses bear a close resemblance to RVF using pathogenicity for laboratory rodents, replication in suckling mouse liver or protection against RVF challenge as criteria. Convalescent sera do not neutralize RVF, with the exception of a low-level reaction with Arumowat virus. Agar gel diffusion analysis with hyperimmune ascitic fluids show cross-reactivity within the group but does not suggest segment interchange as the origin of any of the viruses.

Because of the serologic relation within the group, we reassessed diagnostic procedures for RVF. Intracranial inoculation of suckling mice or IP injection of weanling hamsters is a sensitive and rapid method of isolating virus. Liver homogenates from dead animals yield a line of identity with standard RVF antigens in overnight agar gel diffusion tests. Propagation in Vero cell culture is almost as sensitive, and identification of infected monolayers is specific if the appropriate conjugate is used.

Human vaccine. A cooperative trial of 3 lots of diploid vaccine with the Israeli Defense Forces (Dr. Jeremy D. Kark) has reached the stage of preliminary analysis. The vaccine was well tolerated except in subjects with history of allergy who had an increased incidence of reactions. The 2 lots compared to date clearly differ in potency. Antibody responses of the recipients resemble those obtained in US Army volunteers. We have also provided advice and reference serology for the USDA research effort at the PIADL, Plum Island, NY, as well as serological monitoring of many vaccinees at USAMRIID.

Studies at USAMRIID of the human immune response to the RVF vaccine and animal models for testing vaccine potency have been the primary responsibility of MAJ Meadors and are discussed in the Medical Division annual report.

Presentations:

1. Peters, C.J., G. Meadors, J. A. Reynolds, T. Slone, D. E. Jones, D. G. Harrington, and E. L. Stephen. Protection from Rift Valley fever virus infection. Presented, 28th Annu. Mtg. Am. Soc. Trop. Med. Hyg., Nov 1979, Tucson, AZ.

2. Peters, C. J. RVF and other sandfly fever virus infections. Presented Tropical Medicine Course, 8 Aug 1980, Walter Reed Army Institute of Research, Washington, DC.

3. Erlick, B. J. Molecular analysis of Rift Valley fever virus. Presented, 28th Annu. Mtg. Am. Soc. Trop. Med. Hyf., Nov 1979, Tucson, AZ.

Publications:

1. Yedloutschnig, R. J., A. H. Dardiri, J. S. Walker, C. J. Peters, and G. A. Eddy. 1979. Immune response of steers, goats and sheep to inactivated Rift Valley fever vaccine, pp. 253-260. In Proceedings 83rd Annual Meeting of the United States Animal Health Association.

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2. Haller, O., H. Arnheiter, and J. Lindenmann. Genetically determined resistance to infection by hepatotropic influenza A virus in mice: effect of immunosuppression. Infect. Immun. 13:844-854, 1976.

3. Peters, C. J. and G. W. Anderson, Jr. Pathogenesis of Rift Valley fever. In Contributions to Epidemiology and Biostatistics, S. Karger, Basel, in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OG2762	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
80 01 16	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	033
b. [illegible]				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Procede with Security Classification Code)

(U) Role of coated vesicles in receptor-mediated endocytosis of biological substances

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
80 01	80 09	DA			C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: NA d. AMOUNT:

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 80	0.7	64
FISCAL YEAR CURRENT 81	0	0

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Provide KWOC with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Endocytosis; (U) Coated vesicles; (U) Viruses

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

23 (U) Determine for coated vesicles the regulatory mechanisms for receptor localization, intracellular route, target destination, formation and disassembly. Investigate their role in mediating cellular virus infection. A better understanding of their function may improve the capability to intervene in a possible BW situation.

24 (U) Apply a variety of physical and biochemical techniques to purified coated vesicles. Antibody to these vesicles will be prepared by conventional means in order to perform dual localization studies so that one can visualize internalization of various molecules.

25 (U) 80 01 - 80 09 - Preliminary studies of the mode of entry of Pichinde virus into BHK-21 cells have been performed. Antibody against purified coated vesicles has been raised in rabbits and characterized by a novel immunoassay technique. Interactions of calmodulin with coated vesicles were studied further.

Publications: J. Cell Biol. 83:289a, 1979.

Terminated for management efficiency. Continued in W.U. S10 AP 198. (DAOG1526)

* Available to contractors upon originator's approval

DD FORM 1 MAR 80 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.

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M161102BS03)

Task No. 3M161102BS10 AG: Enhancement of Host Defense Against Agents of Potential BW Importance

Work Unit No. S10 AQ 179: Role of Coated Vesicles in Receptor-Mediated Endocytosis
(S03 00 033) of Biologicl Substances

Background:

Coated vesicles are found in almost all eukaryotic cells and participate in a variety of systems whose common feature is the requirement for targeted delivery of a molecule within a cell. They have been recognized as the organelles responsible for the receptor-mediated endocytosis of a number of hormones, growth factors, macromolecules, viruses and toxins (1). In particular, it has been demonstrated that certain enveloped viruses enter cells via coated vesicles under physiological conditions (2). Calmodulin is a ubiquitous Ca-binding protein that regulates a wide variety of Ca-dependent intracellular functions (3). It has been demonstrated that calmodulin interacts with coated vesicles, but the nature of this interaction is not yet understood (4,5). Since coated vesicles appear to play a key role in the internalization of bacterial toxins, hormones and viruses, it is important to determine if these agents share a common cellular delivery pathway and to elucidate the steps in this pathway. Investigation of the basic regulatory mechanisms of coated vesicle functions may reveal a primary site susceptible to modification or intervention by therapeutic agents.

Progress:

Preliminary studies of the mode of entry of Pichinde virus into cultured BHK-21 cells have been performed. The results were evaluated by electron microscopy of thin sections of infected cells. Although definitive results were not obtained from these early studies, the studies revealed important necessary modifications in the experimental design. It was found that BHK cells that have been maintained overly long in continuous culture express an enodgenous virus particle that interferes with the interpretation of the experimental virus infection. Thus, appropriate measures have been taken to characterize cells appropriate for these experiments.

Antibody to purified coated vesicles was raised in rabbits, and a new method was developed for evaluating the antibody titer. Since the method does not require a radiolabeled reagent or precipitating antibody, it may have general applicability as an immunoassay where other conventional methods fail or are untenable. The anti-coated vesicle antibody will be used in future studies to determine unambiguously whether viral particles and toxins utilize the coated vesicle pathway for entry into various cells.

A series of studies on the interactions of purified, solubilized coated vesicles with a calmodulin-affinity resin were extended. These studies demonstrated conclusively that the interaction of calmodulin with solubilized coated vesicle proteins was Ca-dependent. They also showed that calmodulin most likely interacted with a minor protein constituent of coated vesicles. These results will be pursued further.

The coated vesicle purification procedure was characterized with respect to contamination by subcellular organelles using enzyme assays specific for each organelle. These studies were performed in collaboration with Dr. Peter Canonico and showed that purified coated vesicles were free of any significant contamination by other cell membrane components.

Presentation:

Linden, C. D., T. F. Roth, and T. R. Dedman. The association of calmodulin with coated vesicles. Presented, Amm. Mtg. Am. Soc. Cell Biol., Toronto, Canada. 7 Nov 79 (J. Cell Biol. 83:289a, 1979).

Publications:

None

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4. Linden, C. D., J. G. Chafouleas, J. R. Dedman, A. R. Means, and T. F. Roth. 1980. Coated vesicles, calmodulin and receptor mediated transport. J. Supramol. Struct. 4(Suppl.):91.

5. Linden, C. D., J. R. Dedman, J. G. Chafouleas, A. R. Means and T. F. Roth. 1981. Interaction of calmodulin with brain coated vesicles. Proc. Natl. Acad. Sci. USA 78: in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C5024	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
80 07 23	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	034
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Mechanism of viral binding to its cell surface receptor and internalization

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
80 07	80 09	DA	

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: NA d. AMOUNT:

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR (DIRECT EFFORT) 80	0.3	11
FISCAL YEAR (CONTRACT) 81	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Barquist, R. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME:

Virology Division

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Fort Detrick, MD 21701

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

NAME: Huggins, J.W.

TELEPHONE: 301 663-7244

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Canonico, P.G.

NAME: Jahrling, P.B.

POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Alphavirus

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

23 (U) Characterize the involvement of host cell surface viral receptors in virus binding and internalization. Such knowledge is needed for developing antiviral drugs for diseases of military importance, including Junin, Lassa, and Machupo.

24 (U) Initially, establish the presence of a receptor and define it biochemically in murine cell lines. Radiolabels will be used on the model virus, Pichinde, to assist the study. Once established, studies will continue on internalization of virus. Potential antiviral drugs will be tested for ability to block binding and/or internalization.

25 (U) 80 07 - 80 09 - Literature survey completed and work initiated. Preliminary studies have established a working system for measuring virus attachment. Binding is specific and saturable.

Terminated for management efficiency. Continued in W.U. 871 BE 146.(DA0G3815)

*Available to contractors upon originator's approval

DD FORM 1498 MAR 88

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

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BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)
(3M162776A841)

Task No. 3M161102BS10 AP: Biology of Viral Agents of Potential BW Importance

Work Unit No. S10 AP 171: Mechanism of Viral Binding to its Cell Surface
(S03 00 034) Receptor and Internalization

Background:

The plasma membrane of the eukaryotic cell acts as a barrier to limit severely the movement of macromolecules between the environment and the cell interior. It also acts as the communication link between the cell and its environment, containing receptors for many substances which profoundly alter cellular metabolism and function. The cell surface acts as the site of interaction for hormones, growth control factors, differentiation signals, bacterial and plant toxins, chemotactic signals, modifiers of cell action and receptors for viruses.

Other properties such as locomotion, phagocytosis and pinocytosis intimately involve the cell surface complex. Many macromolecules must be moved across the plasma membrane to maintain cell function; this movement is only partially understood, especially for proteins and larger complexes, such as viruses and bacteria. Morphological studies have provided a general description of an endocytic mechanism(s) which causes the particle to be trapped in a vesicle at the plasma membrane and become internalized. Several different mechanisms appear to exist.

Viruses replicate and survive inside mammalian cells by partially or fully utilizing host cell machinery. The process of entry into cell, across the host cells plasma membrane is at best poorly understood. The entry of opsonized particles (bacteria, rickettsiae, viruses, and test particles such as red blood cells) is one of the better understood processes. Recognition and binding is mediated via cell surface receptors for the opsonins Fc and C_3. Particles however also enter cells which appear to lack opsonins, suggesting the involvement of other receptors as well. The resulting phagocytosis has been shown by Stossel to involve proteins of the cytoskeletal system.

Macromolecules also enter cells by binding to a specific receptor via a process known as "receptor-mediated" or absorptive endocytosis. Macromolecules taken up by absorptive endocytosis are concentrated at the cell surface by binding to their specific receptors.

When studied morphologically, absorptive endocytosis has been found to occur at specialized regions of the plasma membrane called coated pits. Recent work by Helenius et al. with Semliki Forest virus (SFV) has provided striking evidence that this alphavirus enters the cell by absorptive endocytosis.

Some classes of viruses (parainfluenza such as Sendai, Newcastle disease, SV5, measles virus, vaccinia, encephalitic Germiston virus, vesicular stomatitis virus, herpes viruses type 1 & 2, cytomegalovirus) enter the host cells by fusion of the viral envelope with the cell membrane. Viruses which enter cells by this

manner also, in general, are able to produce cell-cell fusion under appropriate conditions. Arena- and alphaviruses are not reported to produce cell-cell fusion, suggesting that fusion is not a likely mechanism for entry by these viruses. The mechanism of virus entry into a cell is important in understanding a basic cellular mechanism used by cells to internalize substances.

The binding of a virus to its cell surface receptor is a necessary step in the cycle of virus replication. The presence of a receptor on a cell is a good candidate for the explanation of selective growth in certain target tissues. An understanding of the cell receptor is important in understanding the virus replication cycle. Among the various steps in viral replication, most depend on biochemical pathways furnished by the host cell and used by the virus for its own replication. The early events of entry are almost certainly dependent on host cell functions normally utilized by the cell for other purposes. The normal function of the receptor must at this point be pure speculation. It probably is a macromolecule (most known cases are proteins) which normally undergoes internalization by a host-cell coded event, since even early virus-directed host cell changes seem to require entry of virus into the cell.

To understand the replication cycle, as a prelude to designing reagents to interfere with it, several processes must be understood. Entry of the virus into a cell is the first step leading to virus replication. The process can be divided into 3 general areas: binding to its receptor, movement to the internalization site and internalization.

Progress:

New work unit was approved 17 July 1981. Literature search and background work have been completed. Research was undertaken to establish a model system for studying virus attachment to cultured cells. Preliminary studies have shown the interaction of TC-83 strain of VEE with a cultured mouse macrophage-like cell line (BWJM) to be a usable system to study the initial attachment of a virus to its cell receptor. Work is underway to develop a binding assay and to determine its parameters.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OG 017S	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. ENTER HISTORY	8A. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 05	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	132
b. CONTRIBUTING				
c. [redacted]	STOG 80-7.2:2			

11. TITLE (Include Security Classification Code)

(U) Identification of pathogens of military importance using nucleic acid hybridization

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
79 07	CONT	DA	C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING: 80	1.0	104
FISCAL YEAR CURRENT: 81	1.0	97

19. RESPONSIBLE DOD ORGANIZATION

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PRINCIPAL INVESTIGATOR (Include Area Code if U.S. Autovon prefix)
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TELEPHONE: 301 663-7341
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME: POC:DA

21. GENERAL USE

Foreign Intelligence considered

22. KEYWORDS (Include Each with Security Classification Code) (U) Military Medicine; (U) BW defense; (U) Bacteria; (U) DNA homology; (U) Identification; (U) Legionnaires' disease bacteria

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

23 (U) Establish the technique for determining base sequence homology of DNA of bacterial pathogens. Create a reference band of individual DNA varieties from representative bacterial genera. Develop the capacity to isolate, grow and identify rare or unusual pathogens, which may have been altered antigenically or physiologically. Initially, determine homology of Legionella pneumophila and its 4 serotypes.

24 (U) Measure reassociation between DNA homology of test and reference strains of bacteria, using radiolabeled material, heated hydroxyapatite columns or heated centrifuge. Initally, determine the guanosine (G) + cytosine (C) percent in the DNA of an unknown and compare to know G+C values. On this basis, select known reference bacterial DNA most similar to the unknown. Label unknown (or known) bacterial DNA with P-35, initiate reassociation between known and unknown DNA and perform calculations.

25 (U) 79 -10 - 80 -09 - DNA-DNA hybridization techniques have been established for purposes of bacterial identification. Such techniques require a large bank of reference DNA from known organisms against which to test DNA from an organism to be identified. Our bank of DNA now exceeds 110 reference DNA and includes organisms of military importance such as Francisella tularensis, Yersinia pestis, Pseudomonas pseudomallei, Shigella spp. and Salmonella spp. DNA from L. pneumophila and Legionella-like organisms has also been isolated. Using these DNA and hybridization it was determined that the Pittsburgh Pneumonia Agent (PPA) and L. pneumophila are not related at the species level. These data were used to propose and publish the name Legionella pittsburgensis species nova for PPA. Cyclic regulatory nucleotides were found to alter colonial and cell morphology of L. pneumophila. Efforts to modify existing DNA hybridization techniques for purposes of routine identification using small quantities of cells have been made and are still in progress.

Publication: Abstr, Ann. Mtg, ASM-1980, p. 91; J. Infect. Dis. 141:727, 1980.

(Available to contractors upon originator's approval)

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BODY OF REPORT

Project No. 3A161101A91C: In-House Laboratory Independent Research (U)

Work Unit No. 91C LA 132: Identification of Pathogens of Military Importance Using Nucleic Acid Hybridization Techniques

Background:

The usefulness of DNA-DNA hybridization techniques for purposes of bacterial identification has been realized at this Institute during the past year. This is important in that with the advent of genetic engineering during recent years, the natural occurrence of spontaneous mutants and the discovery of new bacterial species, there is an everpresent potential for the emergence of organisms which are refractory to conventional identification procedures which are based primarily upon comparisons of traits of an unknown organism with those of known organisms. Therefore alterations in any trait, whether physiological, tinctorial or antigenic can introduce error and possibly result in misidentification. The advantage of using DNA homology determinations for identification is that this method is not influenced by such alterations in cellular properties.

Progress:

DNA-DNA hybridization techniques have been established at this Institute for bacterial identification. The number of reference DNA against which unknown DNA can be tested now exceed 110. These have been obtained from a wide range of organisms which include such bacteria of military importance as Francisella tularensis, Pseudomonas pseudomallei, Vibrio cholerae, Yersinia pseudotuberculosis, Yersinia pestis, and Shigella and Salmonella spp. DNA has also been isolated from most strains of Legionella pneumophila and the Legionella-like organisms (LLO) (i.e., WIGA, TEX-KL, Pittsburgh Pneumonia Agent, HEBA, and TATLOCK).

Techniques have also been established for both in vivo and in vitro radioactive labeling of DNA. In vitro labeling of Pittsburgh Pneumonia Agent (PPA) and TEX-KL DNA with tritiated thymidine triphosphate was accomplished using the Nick Translation kit from New England Nuclear, Boston, MA.

Using labeled and unlabeled DNA described above, numerous DNA-DNA hybridization studies have been performed which have confirmed the relatedness of the different L. pneumophila strains at the species level, which is in agreement with the work of Dr. D. J. Brenner at CDC, Atlanta, GA. Although not related at the species level our studies have shown a low level of homology (approximately 10%) between L. pneumophila and PPA. These data were utilized in a recent publication co-authored with this investigator in which the name Legionella pittsburgensis was proposed for PPA (1). The name Legionella micdadei was simultaneously proposed for PPA by Hebert et al. (2) and the final decision as to which name will be accepted has not been decided upon by the International Committee of Bacterial Systematics.

Other studies performed under this work unit have centered around determination of what roles if any cyclic regulatory nucleotides may play in regulation of various cellular parameters in L. pneumophila and the LLO. A presentation was given at the Annual American Society for Microbiology Meeting concerning which alterations in cellular and colonial morphology for L. pneumophila grown on media containing

various nucleotides. It was noted that cyclic GMP caused colonies ro become more xanthic and the cells to be more filamentous as opposed to cyclic AMP which produced more cyanic colonies and bacillary cellular forms.

No alterations in virulence, antigenicity or growth rate by these cyclic nucleotides have been detected in L. pneumophila to date. Experiments using electron microscopy are in progress with Dr. John White (Pathology Division) to determine possible control of flagellation by these cyclic nucleotides in L. pneumophila and LLO.

Presentation:

Ezzell, Jr., J. W., and J. D. Ristroph. Crowth stimulation of Legionella pneumophila by cyclic adenosine 3', 5' - monophosphate. Presented, 80th Ann. Mtg., ASM, May 1980, Miami, FL (Abstracts I41, p. 91).

Publication:

Pasculle, A. W., J. C. Feeley, R. J. Gibson, L. G. Cordes, R. L. Myerowitz, C. M. Patton, G. W. Gorman, C. L. Carmack, J. W. Ezzell, and J. N. Dowling. 1980. Pittsburgh Pneumonia Agent: direct isolation from human lung tissue. J. Infect. Dis. 141:727-732.

LITEPATURE CITED

1. Pasculle, A. W., J. C. Feeley, R. J. Gibson, L. G. Cordes, R. L. Myerowitz, C. M. Patton, G. W. Gorman, C. L. Carmack, J. W. Ezzell, and J. N. Dowling. 1980. Pittsburgh Pneumonia Agent: direct isolation from human lung tissue. J. Infect. Dis. 141:727-732.

2. Hebert, G. A., A. G. Steigerwalt and D. J. Brenner. 1980. Legionella micdadei species nova: classification of a third species of Legionella associated with human pneumonia. Cur. Microbiol. 3:255-257.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J5422	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. ORIGN INSTR'N	8A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 12 07	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	133
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code): (U) Detection and characterization of plasmids in pathogens of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
79 05	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRIOR: 80	1.0	89
FISCAL YEAR CURRENT: 81	1.0	53

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
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20. PERFORMING ORGANIZATION
NAME: Bacteriology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Knudson, G. B.
TELEPHONE: 301 663-7341
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Mikesell, P.
NAME: POC:DA

21. GENERAL USE
Foreign Intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Bacterial genetics; (U) Legionella-like organisms; (U) Legionnaires' disease

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

23 (U) Establish a technological base for genetic analysis of pathogenic bacteria, with emphasis on determinations of the presence and nature of plasmids. Awareness of the threat of recombinant organisms as BW offensive agents is of considerable military significance.

24 (U) Techniques of curing, agarose gel electrophoresis, density gradient centrifugation and electron microscopy will be used to establish a rapid procedure for detection and characterization of plasmids. The first organism used as a model will be E. coli with a known plasmid.

25 (U) 79 10 - 80 09 - Methods have been developed for the rapid detection, isolation and characterization of naturally occurring plasmids and genetically engineered recombinant DNA plasmids, which are biologically active in pathogens. Techniques have been established for the rapid detection of plasmids by the use of agarose gel electrophoresis and ethidium bromide staining. Methods of purifying plasmids by cesium chloride-ethidium bromide ultracentrifugation of cleared lysates have been established. Several methods for the elimination of plasmids from the host bacterial cells have been established with cell lines known to harbor plasmids with drug resistance markers.

These newly developed methods were applied to study genetics of Legionella pneumophila and Legionella-like organisms (LLO). All known serogroups of L. pneumophila were examined for the presence of extrachromosomal genetic elements. Plasmids were isolated and partially characterized from 2 strains, Atlanta-1 and Atlanta-2, Serogroup II. The genetics of LLO (OLDA, WIGA, TEK-KL, TATLOCK, HEBA), and 5 strains of Pittsburgh Pneumonia Agent were also studied. Plasmids were isolated from OLDA, which has recently been shown by DNA homology to be a strain of L. pneumophila, and from WIGA and TEK-KL, which will soon be classified as new species of Legionella.

* Available to contractors upon originator's approval

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BODY OF REPORT

Project No. 3A161101A91C: In-House Laboratory Independent Research (U)

Work Unit No. 91C LA 133: Detection and Characterization of Plasmids in Pathogens of Military Importance

Background:

Multiple drug resistance plasmids complicate the treatment of infectious diseases by rendering their hosts resistant to specific antimicrobial agents. This drug resistance can be conjugally transferred in vivo to other bacteria even across species lines. Plasmids have been shown to carry genes which code for toxins such as botulinum, diphtheria and Streptococcus erythrogenic toxins, and the Escherichia coli enterotoxin (1). Recombinant DNA technology has been used to splice foreign genes into a plasmid vector which can then be cloned in a bacterial host (2). In this way plasmids could be used to construct genetically engineered pathogens which produce venom or toxin proteins, are drug resistant, have altered surface proteins, or provoke new autoimmune diseases (3). This project is designed to establish the defensive capability of rapidly detecting, isolating, and characterizing naturally occurring and genetically engineered plasmids in pathogens of potential military importance. This research has also led to a better understanding of the genetics of novel pathogens.

Progress:

This research project began with the development of methods for the purification and visualization of plasmids in model species of bacteria which contain plasmids of known MW. These general methods were then applied to screen strains of Legionella pneumophila and Legionella-like organisms (LLO). They will subsequently be applied to other pathogens of military importance.

When conducting a molecular genetic analysis of a new pathogen, our approach has been to first apply several rapid screening procedures which effectively detect plasmids in a wide variety of bacterial species. One method which has proven to be particularly useful in this screening process involves lysing a small quantity of cells from a single colony directly in the well of an agarose gel, followed by electrophoresis and staining with ethidium bromide (EtBr). Once plasmids have been detected they can be isolated and purified for further genetic analysis. Purification is accomplished by preparing lysozyme-Triton X-generated, cleared lysates which are essentially free of chromosomal DNA, followed by $CsCl_2$-EtBr ultracentrifugation (4). Alternatively, plasmid DNA is purified by alkaline denaturation of the chromosomal DNA from lysozyme-SDS-generated lysates, followed by ethanol precipitation of the plasmid DNA.

Cases of legionellosis have now been reported from more than 40 states of the U.S. and from 14 countries. It has been estimated that there are approximately 26,000 undiagnosed cases of legionellosis in the U.S. each year (5). Our report of plasmids in 2 strains of L. pneumophila has significant clinical implications, since it is well known that R factors can arise through the acquisition of various transposons by indigenous cryptic plasmids. The presence of an R-plasmid coding for resistance to erythromycin would severely restrict present treatment of the disease.

There are about 800,000 cases of pneumonia in the United States each year for which no known viral or bacterial agent can be identified (5). It is becoming apparent that L. pneumophila and LLO are the etiologic agents in many of these identified plasmids in OLDA, WIGA, and TEX-KL. The MW of the plasmids were determined by least-squares regression analysis to range from 47-60 Mdal. Methods have also been developed for the elimination of plasmids from host cells. These methods are presently being applied in a study designed to establish the role that plasmids play in the pathogenicity of these novel microorganisms.

Publication:

Knudson, G. B. and P. Mikesell. 1980. A plasmid in Legionella pneumophila. Infect. Immun. 29:1092-1095.

LITERATURE CITED

1. Maas, W. K. 1977. Genetics of toxin production by bacteria, pp. 1-13. In Perspectives in Toxinology (A. W. Bernheimer, ed.), John Wiley and Sons, New York.

2. Cohen, S. N., A. C. Y. Chang, H. W. Boyer, and R. B. Helling. 1973. Construction of biologically functional bacterial plasmids in vitro. Proc. Nat. Acad. Sci. USA 70:3240-3244.

3. King, J. 1978. New diseases in new niches. Nature 276:4-7.

4. Guerry, P., D. J. LeBlanc, and S. Falkow. 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. 116:1064-1066.

5. Fraser, D. W. and J. E. McDade. 1979. Legionellosis. Scient. Am. 241 (4):82-99.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6421	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'S	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	K. COMPLETION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	137
b. CONTRIBUTING				
c. COPY/PASTE THIS/	STOC 70-7,2:2			

11. TITLE (Provide with Security Classification Code)

(U) Laboratory diagnosis of viral diseases of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
79 04	80 10	DA			C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 80	1.0	39
	CURRENT 81	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

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TELEPHONE: 301 663-7211

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Woodruff, N. H., Jr.

NAME: POC:DA

21. GENERAL USE

Foreign Intelligence considered

22. KEYWORDS (Provide EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Virus diseases; (U) Serology; (U) Rapid diagnosis

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

23 (U) Develop a capability for rapid diagnosis of viral diseases for a clinical laboratory. There is a need to have methodology for early and accurate identification of virus infections for use by the military, especially for those of BW importance.

24 (U) Apply the enzyme-linked immunosorbent assay (ELISA) to a variety of viral disease specimens, comparing them to other routine, but slower methods.

25 (U) 79 10 - 80 09 - Forty-one (13%) viruses were isolated from 310 clinical specimens using only 2 types of cell culture. The majority of these were enteroviruses (Coxsackie B) and were associated with mild febrile illnesses with occasional skin rashes in children. Rapid diagnosis was made possible in many cases by electron microscopic (EM) examination of infected cell cultures which showed early cytopathic changes. Viral particles were demonstrated by EM 4 h after infecting cell cultures with prototype ECHO type 1, influenza A1, and VEE-TC83. Some of the parameters of indirect ELISA for detecting antibodies to VEE-TC83 were determined. A new Gilford processor/reader spectrophotometer for use in enzyme immunoassay (EIA-50) is being evaluated. This machine is semi-automated and could save technical time. Initial data indicate that EIA-50 is less sensitive than plaque-reduction neutralization and the conventional ELISA.

ILIR objectives have been met. The technology will be transferred to other investigations.

*Available to contractors upon originator's approval

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BODY OF REPORT

Project No. 3A161101A91C: Medical Defense Against Biological Agents (U)

Work Unit No. 91C-00-137: Laboratory Diagnosis of Viral Diseases of Military Importance

Background:

Many viral infections are not recognized because infected individuals are either asymptomatic or do not present the typical picture of the disease. Although a majority of these are mild and self-limited (e.g., rhino- and enterovirus infections), some can be relatively serious and so incapacitating that prompt medical treatment (e.g., hepatitis A and B, influenza, etc.) is required to reduce morbidity. Appropriate therapeutic measures can best be implemented if the identity of the causative virus is known.

Laboratory diagnosis depends on isolation (or demonstration) and eventual identification of the involved virus and also on serology (1). In many instances, it is difficult to recover a virus because of the lack of a proper in vivo or in vitro isolation system. Therefore, it becomes imperative to demonstrate serological responses of individuals in order to provide clinically useful information. The recently described enzyme-linked immunospecific assay (ELISA) is both sensitive and easy to perform in field situations. This is, therefore, a welcome development to many laboratories interested in rapid diagnosis. More sensitive modifications of ELISA have been described, but these are more complicated and time consuming (2,3).

Progress:

Three hundred ten clinical specimens, mostly throat swabs, that came through the clinical laboratory for bacterial cultures were processed for virus isolation using primary monkey kidney (MK) and human lung fibroblast (WI-38) cell cultures. Forty-one viruses (13%) were isolated and identified as follows: herpes simplex, 6; adenovirus, 2; parainfluenza type 3, 2; and picornavirus, 31 (Coxsackie B2, 22; Coxsackie B3, 4; Coxsackie B5, 2; and to be typed, 3). Majority of the picornaviruses were isolated during the summer months (Jun-Sep 79) and were associated with mild febrile illnesses with occasional skin rashes in children.

Routine virus isolation and identification in cell cultures takes several days to weeks to complete. To provide rapid diagnosis, very early cytopathic effects (CPE) were sought by examining inoculated cell cultures 1-2 times daily (except weekends). In 15 positive specimens, early CPE (1+, or 0-25% involvement of monolayer) were detected from 1-4 days (average, 2.5 days). In order to confirm the viral nature of the CPE, harvested cell cultures were negatively stained with phosphotungstic acid (after ultracentrifugation to concentrate virus) and examined with the electron microscope (EM). Eleven were identified as enterovirus, 3 as herpes, and 1 negative (CPE was due to toxicity). Thus, in these 14 specimens, diagnosis was made within 4 days. In another group of 9 specimens which were harvested at 3-7 days with advanced CPE (early CPE occurred during weekends), EM revealed viral particles in all. The rest of the specimens were not examined by EM.

A more rapid way of diagnosing viral infection is by direct EM examination of specimens without the benefit of amplification in cell cultures. Ten specimens which were positive for viruses (2 parainfluenza type 3, 1 adeno, 2 herpes, and 5 entero) were thus examined by EM, but viral particles were detected in only 2 (herpes-positive) instances. So, even if direct EM is more rapid, it is less sensitive, requiring at least 10^5 viral particles/ml (4). Immunoelectron microscopy increases the sensitivity of morphologic examination, but specific antisera, which may not be readily available, are required in the procedure (5).

Viral particles may be detectable in infected cell cultures prior to the appearance of CPE. This was proven by the inoculation of ECHO 1 (10^6/ml $TCID_{50}$) and influenza A1 (10^6/ml $TCID_{50}$) onto primary MK cells and VEE virus or VEE-TC83 (10^8/ml $TCID_{50}$) onto Vero cells. As early as 4 h following infection, a few viral particles were detected by EM. It should be noted, though, that these viruses had been adapted to the cell culture system; it may require a longer time for mild viruses in clinical specimens to replicate in cell culture.

Preliminary data for the detection of antibody to VEE-TC83 by indirect ELISA is promising. Of 24 sera tested by both ELISA and PRNT, there was agreement in 15; in 4, titers were higher with ELISA and in 2, titers were higher with PRNT. ELISA was positive in 3 preimmunization samples which were negative by PRNT, suggesting that this test may be detecting nonspecific antibodies.

Some of the parameters of ELISA have been determined. Microtiter plates coated with VEE-TC83 antigen were found to be stable up to 2 months when stored at 4°C, but only up to 30 days when stored frozen at -20°C. The incubation time following addition of the sera being tested and alkaline phosphatase conjugate may be shortened to 1 h each if kept at 37°C instead of room temperature. Likewise, after addition of substrate, incubation may be reduced to 30 min and readings of results by the naked eye may be comparable to those determined by the spectrophotometer.

The new Gilford processor/recorder spectrophotometer for ELISA (EIA-50) has the promise of substantial savings in technician's time because it is semi-automated. However, problems were encountered earlier due to inconsistent readings. By increasing the washing procedure to 10 times instead of 4, and by using 4°C bovine serum albumin as diluent for the conjugate, values obtained are becoming consistent. However, titers are lower compared to PRNT results. It may be necessary to compare the EIA-50 with other tests, such as complement fixation, hemagglutination-inhibition, and immunofluorescent antibody technique.

Publications:

None

LITERATURE CITED

1. Gardner, P.S. 1976. Rapid virus diagnosis. Lab-Lore 7:425-429.

2. Berg, R.A., R.H. Yolken, S.I. Rennard, R. Dolin, B.R. Murphy, and S.E. Straus. 1980. New enzyme immunoassays for measurement of influenza A/Victoria/3/75 virus in nasal washes. Lancet 1:851-853.

3. Yolken, R.H., and P.J. Stopa. 1979. Enzyme-linked fluorescence assay: ultrasensitive solid-phase assay for detection of human rotavirus. J. Clin. Microbiol. 10:317-321.

4. Lee, F.K., M.P. Macris, and A.J. Nahmias. Mar 1980. Electron microscopy for diagnosing viral infection. Lab. Management 18(3):35-39.

5. Milne, R.G., and E. Luison. 1977. Rapid immune electron microscopy of virus preparations. Meth. Virol. VI:265-281.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6428	80 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. GRP/SUBGRP	8B. SPECIFIC DATA-COM/FACTOR ACCESS	9. LEVEL OF SUM
79 12 07	H. TERMINATION	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	141
b. CONTRIBUTING				
c. [illegible]	STOG 70-7,2:2			

11. TITLE: (U) Rapid detection of immune complexes in infectious disease of unique military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
76 10	79 12	DA	C. In-house

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 80	0.2	6
	CURRENT 81	0	0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
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TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic participation)
NAME: Hedlund, K. W.
TELEPHONE: 301 663-7341
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign Intelligence considered

POC:DA

22. KEYWORDS: (U) Military medicine; (U) BW defense; (U) Antibody (U) Immune complexes; (U) Isotachoporesis; (U) Immunoglobulins; (U) Early detection

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

23 (U) Detect rapidly the presence of infectious agents or their component parts as well as antibodies directed against them in sera. This would significantly aid in the early diagnosis of infectious agents of military medical interest and BW importance.

24 (U) Immune complexes formed by the addition of well-characterized infectious bacterial or viral antigens and the antibodies directed against them can be readily defined by their migration in an electrical field by isotachophoresis of ion species of the same sign. The selective addition or removal of one specific reagent will elucidate the presence or absence of the other.

25 (U) 79 09 - 79 12 - We have demonstrated that soluble immune complexes could be identified by analytical isotachophoresis. The individual subclasses of human IgG could also be determined. The feasibility of studying the functional nature of human IgG subclasses by analytical isotachophoresis was also addressed. Preparative isotachophoresis has been used to isolate a new Legionella pneumophila toxin. Having met its objectives this work unit was terminated 7 Dec 1979.

Publications: J. Chromatography 162:76, 1979; J. Immunol. Meth. 25:43, 1979; In Electrophoresis-1979, pp. 765-773, 1980.

*Available to contractors upon originator's approval

DD FORM 1 MAR 80 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1, 1 MAR 69 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A161101A91C: In-House Laboratory Independent Research (U)

Work Unit No. 91C LA 141: Rapid Detection of Immune Complexes in Infectious Diseases of Unique Military Importance

Background:

The basic principle of isotachophoresis has been previously described (1, 2). In isotachophoresis the sample which is a mixture of anionic and cationic species is introduced between a leading electrolyte and terminating electrolyte. In the analysis of anionic species, the leading buffer is chosen so that its effective mobility is higher than all other anionic species, whereas the terminating anionic species is chosen with a mobility lower than those of all other anionic species. When an electric current is passed through such a system in the first stage, all ionic species migrate with a velocity determined by the pH, ionic strength, mobility and the potential gradient. After this stage in which the anionic species of the sample are separated according to differences in effective mobilities, a "steady state" is reached when all zones migrate with a velocity equal to that of the leading anionic species. Each zone will contain only one anionic species.

In the past, isotachophoresis has been used to separate inorganic ions, strong and weak acids, and their salts. More recently, in the early 1970s, the method was applied to the study of complex protein mixtures.

We have studied for the first time the nature of well-characterized antibody-antigen interactions. In addition, because IgG antibody subclass characterization gives clues to the biological and functional "usefulness and appropriateness" of an antibody response, we have studied human IgG subclasses as well.

Progress:

The ability of isotachophoresis to separate and identify both immunoglobulins and bacterial components like the Legionella toxin were successfully demonstrated and are now in standard use. Therefore the work unit which was directed at showing the feasibility of application has now moved to operational use. This work unit was therefore discontinued December 7, 1979.

Presentation:

Hedlund, K. W. Feasibility of studying the functional nature of human IgG subclass responses by means of analytical isotachophoresis. Presented, 2nd Conference on Electrophoresis, Munich, Germany, 15-17 Oct 1979.

Publication:

Hedlund, K. W., R. Wistar, Jr. and D. Nichelson. 1980. Feasibility of studying the functional nature of human IgG subclass responses by means of analytical isotachophoresis, pp. 765-773. In Electrophoresis '79 (B. J. Radola, ed.). Walter de Gruyter & Company, Berlin.

LITERATURE CITED

1. Arlinger, L. 1974. Analytical isotachophoresis--principles of separation and detection. Protides Biol. Fluids, Colloq. 22:661-667.

2. Everaerts, F. M., M. Geurts, F. E. P. Mikkers, and Th. P. E. M. Verheggen. 1976. Analytical isotachophoresis. J. Chromatogr. 119:129-155.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6424	80 09 15	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. GRP'N INSTR'N	9A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 10 01	K. COMPLETED	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	144
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 70-7.2:2			

11. TITLE (Include with security classification code)

(U) Mechanisms and determinants of microbial pathogenicity

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
77 10	80 09	DA			C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING		
	80	0.5	95
	CURRENT		
	81	0	0

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Little, J. S.

NAME: POC:DA

21. GENERAL USE

Foreign Intelligence considered

22. KEYWORDS (Provide KWICS with security classification code) (U) Military medicine; (U) BW defense; (U) Microbial pathogenicity; (U) Macrophages; (U) Pathogenic determinants; (U) Francisella tularensis

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

23 (U) Characterize pathogenic mechanisms and determinants of virulent microorganisms of military importance. Employ these data to establish biochemical criteria for selection, development and evaluation of vaccine candidates and for design, synthesis and testing of antimicrobial drugs.

24 (U) In vitro culture procedures are employed to evaluate the interaction and fate of pathogenic microorganisms with tissue cells. Fundamental information is obtained from tissue fractionation, enzyme analyses and cellular physiology studies.

25 (U) 79 10 - 80 09 - The treatment of macrophages with drugs which act as weak bases, such as chloroquine, caused a rise in the pH of the lysosomes of macrophages. This rise in intralysosomal pH resulted in survival of the avirulent strain of Francisella tularensis in both normal and immune macrophages. It was also shown that the capacity of immune macrophages to kill the virulent strain of F. tularensis is negated by chloroquine treatment. Thus a consequence of macrophage activation by way of immunization is the acidification of macrophage lysosomes below pH 4.5. The build-up of acid in the lysosomes of macrophages provides for a less favorable environment for survival of microorganisms and leads to their more rapid denaturation and death. A developing principle derived from these studies is that attenuation of intracellular parasites, such as F. tularensis which reside within the phagolysosome system of macrophages, is related to the pH optima of the pathogen for macromolecular synthesis and growth. Attenuation of such pathogens may be achieved by selection of strains for optimal growth at pH greater than that of the lysosomal compartment where they become sequestered. ILIR objectives were met; the work unit is terminated. Its important and far-reaching results, however, will be applied to many on-going infectious disease studies.

*Available in connection with author's approval

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

BODY OF REPORT

Project No. 3A161101A91C: In-House Laboratory Independent Research (U)

Work Unit No. 91C LA 144: Mechanisms and Determinants of Microbial Pathogenicity

Background:

Although the secretory functions of macrophages have received considerable attention for their role in the physiology and regulation of host responses in chronic inflammation, the destruction of invading microorganisms remains the primary function of macrophages in healthy hosts. Their activation must be viewed primarily as a mechanism to limit more efficiently the replication and dissemination of invading microorganisms. Important consequences of macrophage activation, therefore, include enhanced chemotactic and phagocytic capacity, engagement of metabolic pathways to meet increased energy demands and production of microbicial products. These adaptations are generally sufficient to limit the invasion of most microorganisms. However, a number of pathogens have developed the ability to survive and grow in macrophages. They have, in fact, learned to "coax" the macrophage to protect, nourish and disseminate them to distant sites within the host (1).

Insight into mechanisms of macrophage microbicidal functions can be gained by learning the ways virulent microorganisms have developed to permit their survival and replication within macrophages. It is with such information that molecular processes controlling macrophage activation can be uncovered, and agents for the nonspecific activation of macrophages can be developed.

Progress:

Certain microorganisms, such as Mycobacterium lepraemurium, Francisella tularensis, Listeria monocytogenes and Salmonella typhimurium, do not prevent phagolysosome function; rather they appear fully capable of resisting degradation by lysosomal enzymes and can grow and replicate within the acid environment of the phagolysosome. One organism belonging to this group which has been recently studied is F. tularensis. Both the virulent SCHU S4 strain and the attenuated live vaccine strain, LVS, when coated with specific antibodies are ingested and sequestered within phagolysosomes of rat peritoneal macrophages in culture (2). In this environment, the virulent strain survives and grows, but the attenuated strain is killed. Both strains are equally resistant to activated oxygen metabolites generated by hypoxanthine/xanthine oxidase system. The virulent strain, however, appears better adapted for survival in the acid environment of the lysosome than LVS. SCHU S4 can synthesize protein and RNA optimally at pH 4.5, the expected pH of phagolysosomes, but a more alkaline environment is required for optimal synthesis of macromolecules by LVS. Experiments were conducted, therefore, to clarify the relationship between intralysosomal pH of macrophages and F. tularensis survival in normal, immune and nonspecifically activated macrophages. In these studies, sodium caseinate-induced rat peritoneal exudates were collected and macrophages attached on plastic tissue culture flask in an appropriate medium. Macrophages were then infected with the live vaccine strain (LVS) of F. tularensis and incubated for 18 hr in the presence of a weak base. Diffusion of the base into the lysosomal compartment of the macrophage resulted in accumulation of the base in lysosomes and partial neutralization of the acid intralysosomal environment. Initially, NH_4Cl was used as the weak base at a concentration of 10 mM, but this was found to be bactericidal. Hence,

chloroquine (5 µg) was used in subsequent experiments. This concentration of drug raised the pH of a macrophage lysosome from 4.8 to 6.2 (3).

In constrast to control cultures, chloroquine-treated macrophages were unable to kill LVS. In fact, the bacteria proliferated so as to increase their intracellular concentration 5 to 10-fold within 18 hr (Table I). These data are consistent with the hypothesis that shifting the intralysosomal pH toward neutrality enhances the survival of intracellular LVS.

TABLE I. FATE OF OPSONIZED F. TULARENSIS AFTER IN VITRO INFECTION OF RAT PERITONEAL MACROPHAGES.

STATUS OF MACROPHAGE DONOR	CHLOROQUINE ADDED TO MEDIUM (5 µm)	STRAIN	NO. BACTERIA/MACROPHAGE	
			0 hr	18 hr
Normal	-	LVS	6.0	0.6
Normal	-	SCHU S4	11.9	71.3
Immune	-	SCHU S4	18.4	2.3
Endotoxin	-	SCHU S4	14.1	0.3
Normal	+	LVS	5.0	25.0
Immune	+	SCHU S4	7.1	67.8
Endotoxin	+	SCHU S4	7.6	26.4

Macrophages from immunized rats, in contrast to those from nonimmune rats, ingest and kill the virulent SCHU S4 (S4) strain. Since S4 does not survive at pH below 4.5, it was proposed that immunization might confer enhanced microbicidal capacity to macrophages by causing a decrease in the lysosomal pH of immune macrophages. To test this hypothesis, macrophage cultures were prepared from rats immunized with LVS 3-4 weeks prior to induction of peritoneal exudates. The phagocytes were infected with LVS 3-4 weeks prior to induction of peritoneal exudates. The phagocytes were infected with opsonized S4 then incubated for 18 hr in the presence of 5 µM chloroquine. In this model, the drug increases the lysosomal pH, which is then expected to permit survival and replication of S4 within the immune macrophage. As shown in the accompanying table, S4 survive and replicate only in chloroquine-treated immune macrophages.

Killing of S4 also occurs in endotoxin-activated macrophages. Presumably, endotoxin activation also results in lowering of lysosomal pH which would be reversed by chloroquine treatment. Experimental data confirm that the enhanced microbicidal capacity of endotoxin-activated macrophages is abrogated by treatment with a weak base (Table I). These data are consistent with the hypothesis that lysosomal pH is the principle cellular mechanism for the killing of F. tularensis.

Two principles are implied by the results. First, a consequence of macrophage activation, whether through the interaction of these cells with exogenous activating agent or specific immune products of lymphocytes may be the acidification of lysosomes below pH 4.5. Hyperacidification of lysosomes would provide for a less favorable environment for survival of microorganisms and lead to their more rapid denaturation and death. Hyperacidification of macrophage phagolysosomes could result from the activation of the putative lysosomal proton pump (4). Measurement of intralysosomal pH may be useful for the screening and identification of agents which are nonspecific activators of macrophages. The second implication of these studies is that the attenuation of intracellular parasites, such as F. tularensis which reside within the phagolysosome system of macrophages is related to the pH optima of the pathogen for macromolecular synthesis and growth. Attenuation of such pathogens may be achieved by selection of strains for optimal growth at pH greater than that of the lysosomal compartment where they become sequestered.

The work unit is completed; ILIR objectives have been met.

Presentations:

1. Canonico, P. G. Interaction of Francisella tularensis with rat peritoneal macrophages. Presented, 80th Ann. Mtg., ASM, May 1980, Miami, FL (Abstracts, D30, p. 43).

2. Little, J. S., R. A. Kishimoto, and P. G. Canonico. The intracellular fate of Coxiella burnetii in guinea pig peritoneal macrophages. Presented, 80th Ann. Mtg., ASM, May 1980, Miami, FL (Abstracts, D11, p. 39).

Publications:

1. Little, J. S., R. A. Kishimoto and P. G. Canonico. 1980. In vitro studies on the interaction of rickettsia and macrophages: effect of ultraviolet light on Coxiella burnetii inactivation and macrophage enzymes. Infect. Immun. 27:837-841.

2. Canonico, P. G., J. S. Little, M. C. Powanda, K. Bostian and W. R. Beisel. 1980. Elevated glycosyltransferase activities in infected or traumatized hosts: a nonspecific response to inflammation. Infect. Immun. 29:114-118.

LITERATURE CITED

1. Mims, C. A. 1976. The pathogenesis of Infectious Disease, Academic Press, New York.

2. Canonico, P. G. 1980. Interaction of Francisella tularensis with rat peritoneal macrophages. Abstracts of the Ann. Mtg., ASM, D30, p. 43.

3. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci., USA, 75:3327-3331.

4. Schneider, D. L. 1979. The acidification of rat liver lysosomes in vitro: a role for the membranous ATPase as a proton pump. Biochem. Biophys. Res. Commun. 87:559-565.

APPENDIX A
VOLUNTEER STUDIES

PROTOCOL TITLE AND NO. (No Volunteers[a])	COMMENT AND RESULTS
Proposal for the Clinical Evaluation of a Two Dose Schedule of Inactivated Rocky Mountain Spotted Fever Vaccine, Undiluted. (IND 862) Addendum: To Assess Booster Dose Efficacy and Safety Protocol 80-1 (2 MRVS - 5 Volunteers)	Six volunteers (there were 1 control subject) previously vaccinated with inactivated Rocky Mountain Spotted Fever Vaccine were boostered with a 0.5 ml dose of undilute RMSF vaccine (IND 862). All 6 complained of local pain. One case was moderately severe with 6 x 6 cm area of induration. Two subjects noted erythema and one person noted transient temperature elevation to 99.6°F. A large booster in the immunofluorescent antibody titers was noted. Microagglutination titers remained essentially unchanged.
Immunization with Live Attenuated Dengue Virus Vaccine Study No. 3 Response to Varied Doses of Den-2 (PR 154/5-1) Vaccine in Adult Volunteers with Prior Yellow Fever Immunization Protocol 80-2 (1 MRVS - 15 Volunteers)	Sixteen subjects previously immunized with Yellow Fever vaccine were divided into four groups. Three groups of volunteers received 0.5 ml, SC of vaccine in varying dilutions. One volunteer received placebo. Viremia was demonstrated in two subjects on days 10 and 14 respectively. Each had received 10^{-1} dilution of vaccine ($4.3 \times 10.^{3}$ pfu) and were unique in the study group in that they had previously received only one YF vaccination within 4 months of this study. Only one of the volunteers experience a fever of > 38C contributable to Den-2 vaccination. Five of 14 sero negative recipients sero converted including those who were viremic. Thirty days post vaccination antibody titers were consitent with those seen after

	infection. Antibody response appeared to be sustained with 5 seroconverted subjects maintaining HI and neutralizing antibody titers 6 months post immunization. Four subjects had neutralizing antibody titers greater than 1:100. There were no local reactions; 3 of 16 subjects reported febrile illness, one directly attributable to vaccine; and 2 of 5 seroconverters had leukopenia.
Immunization with Live Attenuated Dengue Virus Vaccine Study No. 5. Response to Administration of DCN-2 (PR-159/21) Adult Volunteers by an Intradermal Route. Protocol 80-3 (8-MRVS)	Two of six vaccinated volunteers had antibody response measured by virus neturalization. Subject one developed titer of 1:480, subject two developed titer of 1:25. Subject one had a platelet factor 3 levels above the normal range on days 7, 11 14, and 21 with normal levels on days 0 and 60. This subject was the only one with high antibody titer response to vaccination. Conclusions are limited by the fact that only two subjects converted and only one had a antibody titer.
Addendum to FY76-1, Initial Clinical Evaluation of Rocky Mountain Spotted Fever Vaccine, Formation-Inactivated Sheila Smith Strain, Chick Embryo Cell Origin, Lot 1, For Safety and Immunogenicity. Protocol 80-4 (4 Volunteers)	Four volunteers selected on previously elevated micro-agglutination (MA) or indirect fluorescent antibody (IFA) titers to RMSF. Each subject inoculated subcutaneously with 0.5 ml of 1:10 dilution either Lots 2 or 3. There were no local or systematic reactions. There were no changes in MA titers at either 7 or 28 days. One suoject showed a 4-fold titer rise by IFA. Lots 2 and 3 appear safe to use in immune subjects.
Evaluation of Immunologic Response to Booster Administration of Botulinum Toxoid Adsorbed Monovalent (B) Lot 91. Protocol 80-5 (5 volunteers)	Five volunteers already in the USAMRIID botulism immune plasma program and who had previously been immunized with 8-12 boosters of pentavalent botulism toxoid were boostered with

pentavalent toxoid. Twelve months later these subjects received a booster of pentavalent toxoid and, in the opposite arm, a 0.5 ml dose of MDPH monovalent B toxoid. Volunteers reported much less immediate pain with MDPH monovalent B toxoid. Local reactions were no greater in frequency than with the pentavalent series. Three subjects who had moderate reactions had received the monovalent B toxoid. Increase in B titer compared to that elicited from pentavalent toxoid alone was not statiscally significant when measured in mouse neutralization test.

Addendum to FY79-3: Evaluation of Immunologic Response to Booster Administration of Botulinum Toxoid, Adsorbed, Pentavalent (ABCDE)

Protocol 80-6 (27 volunteers)

Volunteers previously immunized with botulinum toxoid were boostered concurrently with botulinum toxoid adsorbed penta- (ABCDE), and an initial dose of botulinum toxoid adsorbed monovalent (B) Lot 91. To create a pool of donors for a plasmapheresis program 24 volunteers completed the 16 week course of biweekly plasmapheresis. Plasma was collected as single donor unit material.

APPENDIX B

PUBLICATIONS OF U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FISCAL YEAR 1980

1. Alluisi, E. A., W. R. Beisel, B. B. Morgan, Jr., and L. S. Caldwell. 1980. Effects of sandfly fever on isometric muscular strength, endurance, and recovery. J. Motor Behavior 12:1-11.

2. Anderson, A. O., and J. A. Reynolds. 1979. Adjuvant effects of the lipid amine CP-20,961 on lymphoid cell traffic and antiviral immunity. J. Reticuloendothel. Soc. 26(Suppl.):667-680.

3. Anderson, A. O., and N. D. Anderson. 1981. Structure and physiology of lymphatic tissues. In The Cell Biology of Immunity and Inflammation (J. J. Oppenheim, D. A. Rosenstreich, and M. Potter, eds). Elsevier/North Holland, New York, in press.

4. Anderson, A. O., N. D. Anderson, and J. D. White. 1981. Lymphocyte locomotion, lymphatic tissues and lymphocyte circulation in the rat. In Animal Models of Immunological Processes (J. B. Hay, ed.). Academic Press, New York, in press.

5. Anderson, Jr., G. W., and J. V. Osterman. 1979. Host defenses in experimental rickettsialpox: genetics of natural resistance to infection. Infect. Immun. 28:132-136.

6. Anderson, N. D., and A. O. Anderson. 1980. Lymphocytes, pp. 155-197. In Fundamentals of Clinical Hematology (J. F. Spivak, ed.). Harper & Row, Hagerstown, MD.

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APPENDIX C

CONTRACTS, GRANTS, MIPRs AND PURCHASE ORDERS IN EFFECT

FISCAL YEAR 1980

NO.	TITLE, INVESTIGATOR, INSTITUTION
DAMD17-78-C-8035	Mass Spectrophotometric Rapid Diagnosis of Infectious Diseases. M. Anbar, State University of New York, Buffalo
DAMD-17-80-C-1054	Mechanisms of Protective Immunogenicity of Microbial Significance. M. S. Ascher, University of California College of Medicine
DAMD17-78-C-8017	Genetic and Molecular Studies of the Phlebotomus Fever Group of Viruses. D. L. Bishop, University of Alabama
DAMD17-79-C-9108	Analysis of Flavivirus Replication. M. Brinton, Wistar Institute
DADA17-77-C-1035	Rapid Diagnosis of Arbovirus and Arenavirus Infection by Immunofluorescence. J. Casals, Yale University
DAMD17-78-C-8042	Togavirus - Specific Cellular Immune Effector Mechanisms. G. A. Cole, School of Hygiene and Public Health, Johns Hopkins University
DAMD17-80-C-0100	Study of Toxic and Antigenic Structures of Botulinum Neurotoxins. B. Das Gupta, University of Wisconsin Madison
DAMD17-79-C-9024	Lassa Fever Immune Plasma. J. D. Frame, Columbia University
DAMD17-79-G-9494	Isolation of the Etiologic Agent of Scandinavian Epidemic (Endemic) Nephropathy from Human Patients (and from Wild Rodents) as Presumptive Strain in a Vaccine against Korean Hemorrhagic Fever. G. Friman, Uppsala University Hospital, Uppsala, Sweden
DAMD17-77-C-7043	Development of Psoralen Photoinactivated Alphavirus and Arenavirus Vaccines. C. V. Hanson, California Department of Health
DADA17-73-C-3090	Studies on the Antigenic Composition of *Coxiella burnetii*. D. J. Hinrichs, Washington State University
MIPR-2025	Preparation and Characterization of Mouse and Human Monoclonal Antibodies to Botulinum Toxins. K. W. Hunter, Uniformed Services University of the Health Sciences
DAMD17-79-C-9032	Regulation of Staphylococcal Enterotoxin Biosynthesis. J. J. Iandolo, Kansas State University

DAMD17-80-C-0091 In Vitro Selection of an Attenuated Variant of Sindbis Virus: Investigation of the Molecular Basis for Attenuation. R. E. Johnston, North Carolina State University

DAMD17-79-G-9468 Korean Hemorrhagic Fever. H. W. Lee, Korea University Medical College, Seoul

DAMD17-77-C-7034 Resident Research Asscciateship Program (Postdoctoral and Senior Postdoctoral) with the Walter Reed Army Institute of Research. H. W. Lucien, National Academy of Sciences

DAMD17-79-C-9046 The Synthesis and Study of New Ribavirin Derivatives and Related Nucleoside Azole Carboxamides as Agents Active against RNA Viruses. R. K. Robins, Brigham Young University

DAMD17-77-C-7023 Role of Cellular Components of Mosquito Cells in Viral Replication and Transmission. R. H. Schloemer, Indiana University School of Medicine

DAMD17-78-C-8018 Development of Special Biological Products. A. Shelokov, Salk Institute

DADA17-72-C-2170 World Reference Center for Arboviruses. R. E. Shope, Yale University

DA49-193-MD-2694 Biochemical Changes in Tissues during Infectious Illness - Bioenergetics of Infection and Exercise. R. L. Squibb, Rutgers - State University

DAMD17-79-C-9053 Serological Screening Test for any Botulinum Toxin Type. H. Sugiyama, University of Wisconsin Madison

DAMD17-80-C-0099 Genetic and Physiological Control of Protective Antigen by Bacillus anthracis. C. B. Thorne, University of Massachusetts

DAMD17-79-D-0006 Preparation of Hyperimmune Botulinum Toxin. S. Ware, Pine Bluff Biological Products

DAMD17-80-G-9472 Investigation and Management of Ebola Virus Infection in Non-Human Primates. A. J. Zuckerman, London School of Hygiene and Tropical Medicine, England

GLOSSARY

ADCC	Antibody dependent cell mediated cytotoxicity
ADP	automatic data processing
AHF	Argentine hemorrhagic fever
BHF	Bolivian hemorrhagic fever
BUN	blood urea nitrogen
CBC	complete blood count
CEC	chick embryo cell (culture)
CF	complement fixation
CHO	Chinese hamster ovary
CL	chemiluminescence
CPE	cytopathic effect
CPK	creatinine phosphokinase
DEN	Dengue virus
EBO	Ebola
ED_{50}	median effective dose(s)
EEE	Eastern equine encephalitis(virus)
EF	edema factor
EM	election microscope
EP	endogenous pyrogen
FA	fatty acid(s)
GH	growth hormone
GOT	glutamic-oxalacetic transiminase
HA	hemagglutinins, hemagglutination
HAI	hemagglutinating inhibition
HAZ	hazara
HI	hemagglutination inhibition

ID	intradermal (ly)
ID_{50}	median infectious dose (s)
$IPLD_{50}$	infectious intraperitoneal lethal dose (s)
IM	intramuscular (ly)
IN	intranasal
IP	intraperitoneal (ly)
IV	intravenous (ly)
JE	Japanese encephalitis
JUNV	Junin virus
KHF	Korean hemorrhagic fever
LAC	LaCrosse virus
LCFA	long chain fatty acids
LAS	Lassa fever
LD	median lethal dose(s)
LCM	lymphocytic choriomeningitis
MA	microagglutination, microagglutinin
MAC	Machupo virus
MMD	mass median diameter
$MLPLD_{50}$	median infectious intraperitoneal lethal dose(s)
mRNA	messenger RNA
MTD	mean time to death
NIH	National Institutes of Health
ORO	oropouche
PA	protective antigen
PEC	peritoneal exudate cells
PFU	plague forming unit (s)
PGMK	African green monkey kidney

PIC	Pichinde virus
PMN	polymorphonuclear luekocytes
PR_{50} or PR_{80}	50% or 80% plaque reduction
RBC	red blood cells
RES	reticuloendothelial system
RIA	radioimmunoassay
RMSF	Rocky Mountain spotted fever
rRNA	ribosomal RNA
RVF	Rift Valley fever
SC	subcutaneously
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B
SEC	staphylococcal enterotoxin C
SF	Semiki forest virus
SF-N	Sandfly fever - Naples
SF-S	Sandfly fever - Sudian
SGPT	serum glutamic pyruvic
SIN	Sindbis virus
SP	small plaque
UV	ultraviolet
VEE	Venezuelan equine encephalomyelitis (virus)
WBC	white blood count
WEE	Western equine encephalities (virus)
WRAIR	Walter Reed Army Institute of Research
YF	Yellow fever

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