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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 1978 is presented.

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FOREWORD

This FY 1978 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 Facilites 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.

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PROGRAM OVERVIEW

Research programs of the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) are targeted toward: (a) infectious illnesses which pose special problems to our military forces; (b) medical defense against biological warfare (BW); and safe study of infectious, highly dangerous microorganisms in our unique and special containment facilities.

During FY 1978, the research programs followed the revised priorities established during FY 77, which emphasized studies on some of the most virulent and pathogenic microorganisms known. Based upon agreements reached with the Center for Disease Control (CDC) and the new priorities, USAMRIID initiated work this year with Lassa fever (LAS) and Congo-Crimean hemorrhagic fever (C-CHF) viruses, the bacterial agent causing Legionnaires' disease and the anthrax bacillus. Laboratory containment suite modifications were begun to permit the safe, simultaneous handling of all of these organisms, as well as with the start of FY 79, the generally lethal Ebola and Marburg viruses.

USAMRIID and U.S. Navy researchers became involved in an unexpected 1977-1978 outbreak of Rift Valley fever (RVF) which occurred for the first time in history throughout Egypt. Because there were thousands of cases, with hemorrhagic disease, encephalitis and blindness as complications, and the occurrence of many human deaths, additional emphasis was placed on in-house studies of RVF virus, diagnostic methods and viral chemotherapy. In addition, industrial production through contract was stepped up and given first priority to produce 300,000 doses of a new RVF vaccine. USAMRIID is the only known source of human vaccine against RVF. Since it is quite possible that RVF could spread into other middle eastern countries, the problem has potential military medical importance.

Work continued on other high priority organisms, including Machupo virus (the etiologic agent of Bolivian hemorrhagic fever, BHF), as well as Korean hemorrhagic fever (KHF) virus and botulism. These organisms all possess significant BW potential, may be lethal for man and present important safety problems. USAMRIID is one of the few laboratories in the free world where such agents can be studied with minimum risk to laboratory personnel and no risk to the surrounding environment. The goal is to develop safe and effective vaccines or toxoids for these highly dangerous, but poorly understood, diseases. Work in pathogenesis and immunogenesis continued to support vaccine development.

Second-order priorities included studies on dengue-1 (DEN-1), Japanese B encephalitis (JE), Argentine hemorrhagic fever (AHF), Chikungunya (CHIK) and Venezuelan equine encephalomyelitis (VEE). Toxin studies continued with bacterial exo- and enterotoxins. New diagnostic capabilities were developed, as were new treatment methods for viruses, bacteria and their toxins. Rickettsial studies continued on exogenous tick-borne spotted, epidemic typhus and Q fevers. The organisms or toxins in this priority are also dangerous for man, possess significant BW potential and pose special safety problems at an intermediate order of magnitude. Third-order

priorities included studies on western and eastern equine encephalitis (WEE and EEE), melioidosis and tularemia.

Reactivation of the USAMRIID human studies program in FY 77 was followed this year by an increased number of human trials addressing both USAMRIID and Walter Reed Army Institute of Research (WRAIR) research goals. The new Medical Research Volunteer Subject (MRVS) project replaces the old "Project Whitecoat." Each specific human protocol must undergo extensive scientific, safety and ethical review within the Institute and at higher command levels before final approval is given. In other important progress regarding human studies this year, USAMRIID's clinical facilities were upgraded further for safely caring for patients with rare, highly contagious diseases and for supporting such care with diagnostic, microbiologic and clinical laboratory tests. In addition, an agreement was reached with CDC to accept their potentially contagious patients in transfer for diagnostic studies and treatment at USAMRIID. A similar agreement is being negotiated with Plum Island Animal Disease Center, U.S. Department of Agriculture, where studies with RVF will begin in early FY 79.

Specific statements on USAMRIID progress are included under a number of headings.

Clinical Studies. Clinical studies performed during FY 78 included those on new experimental vaccines in volunteers, long-term, phase-III tests of the existing experimental vaccines being used to protect laboratory personnel, collaborative work with WRAIR and the National Institutes of Health (NIH), and, finally, clinical care of persons admitted to our high-containment suite as patients suspected of having been exposed to a highly dangerous, infectious microorganism.

Major areas of clinical research included 2 vaccine trials in volunteers with an inactivated Rocky Mountain spotted fever (RMSF) vaccine and an initial trial in phase-I testing in volunteers of a live, attenuated dengue-2 (DEN-2) vaccine. The latter study was performed in collaboration with investigators from WRAIR, where the vaccine was developed. A major clinical study also involved the first systematic nontherapeutic evaluation in volunteers of human transfer factor; this was conducted in cooperation with investigators from the National Institute of Allergy and Infectiou Diseases.

The phase-III testing of a large number of experimental vaccines was continued in laboratory workers of USAMRIID and other collaborating institutions. These vaccines were administered primarily for the safety of "at-risk" laboratory workers and included the following vaccines: live, attenuated VEE (TC-83), inactivated EEE, WEE and phase II Q fever, live, attenuated tularemia (LVS), inactivated RVF and CHIK and polyvalent botulinum toxoid. Additional assessment studies performed on both TC-83 VEE vaccine and killed TC-83 vaccine (C-84) suggested that a combination of both vaccines should be used for future protection of personnel. Initial VEE vaccination will be performed with live TC-83; booster vaccinations with C-84 will be given to those individuals whose anti-VEE titers either do not reach, or fall below, values deemed to be protective. This procedure will improve the safety and efficacy of the procedure.

The unique USAMRIID isolation facility for hospitalization of patients was used on several occasions during FY 78. Two USAMRIID investigators were hospitalized following potential exposure to Machupo virus and both were given prophylactic treatment with hyperimmune anti-Machupo human plasma. In neither instance was there ever evidence of an actual infection. These unique facilities were also used for 2 laboratory workers transferred from CDC and a Peace Corps worker from Africa, who each had been suspected of being exposed to LAS virus. Again, illness did not develop.

Important new safety measures were taken during FY 78 to upgrade the abilities of USAMRIID to handle highly contagious patients. These efforts included acquisition of additional Trexler-type containment equipment designed to care for a hospitalized patient within a specially constructed, plastic isolator module containing its own independent air supply and filtration system. Orders were placed to acquire additional modules for hospitalized patients and modules for transport of contagious patients in aircraft or ambulances.

To further the safe care of highly infectious patients at USAMRIID, facilities were set aside and a training program established to permit clinical laboratory and microbiologic samples to be handled and assayed under the strictest forms of containment using pressurized plastic suits with filtered air supply. In addition, a building modification program was initiated to upgrade the unique ward facilities to permit care of patients by hospital personnel who will be dressed in similar protective suits.

Vaccine Development. The development of new vaccines constitutes a major requirement in the USAMRIID research mission; the major aspect of this program dealt with attempts to create new vaccines against important viruses, with emphasis on arenaviruses that produce highly lethal hemorrhagic fevers. The viral vaccine program also included new studies on RVF virus, more work to create an attenuated DEN-1 vaccine, and continued efforts to improve vaccines for the alphaviruses, including VEE, WEE, EEE and CHIK.

Attempts to develop a potent inactivated BHF vaccine in a certifiable substrate continued to be frustrated by low yields of virus antigen and difficulties in obtaining consistent virus inactivation. Alternative approaches to existing virus inactivation procedures were studied by contracts, in an effort to resolve this problem. On the other hand, in an exciting breakthrough, the attenuated strain of Junin virus (virulent strains of which cause AHF) was found to protect monkeys and laboratory rodents against both AHF and BHF infections. Since the attenuated Junin virus has already been used in 600 human recipients in Argentina, additional studies with it are planned. Another approach has been to examine the immunizing potential of subunit polypeptides obtained from arenaviruses. Both glyco- and nucleoprotein subunits of Pichinde and lethal Machupo viruses were obtained through fractionation and purification. When used as vaccines, these purified subunits protected animals against homologous virus challenge.

Similar studies were then initiated with RVF virus; again, appropriate growth, purification, concentration and radiolabeling techniques allowed for the identification of 3 major subunits, 2 of which were glycoproteins. These will now be tested for immunogenicity. The strains of RVF virus collected in 1977 and 1978 in Egypt, were compared with older isolates and shown to have comparable antigenic markers. This indicated that the newly produced vaccines should protect equally well against all strains of RVF virus.

Studies with selected clones of the DEN-1 virus were continued and additional tests were developed to identify virulence "markers" to permit selection of an avirulent virus subpopulation that could be used as a human vaccine. A major breakthrough in this regard was the development of a new method to detect low-titer viremia in monkeys. The absence of viremia in subhuman primates may eventually turn out to be the optimal "marker" available for selecting a vaccine candidate appropriate for human use.

In another fundamental breakthrough with respect to development of safe vaccines, it was found that hydroxylapatite column chromatography could be used to separate physically alphaviruses on the basis of differences in virion surface charge. This permitted large numbers of virus strains of EEE, VEE and WEE to be differentiated and separated from each other. This new capability may permit the selection of single, nonheterogeneous populations of a virus which can then be used for experimental vaccine development. This chromatrographic technique is being applied to another alphavirus, CHIK, and may work as well for RVF virus and perhaps even for the arenaviruses.

Initial studies commenced on development of vaccines against melioidosis and Legionnaries' disease.

Another important facet of the vaccine development program includes the need for highly standardized, carefully monitored laboratory animal data when experimental vaccines are initially tested. USAMRIID programs have been developed to do this. The requirement for extensive record-keeping and quality control animal measurement data is essential so that acceptable and complete data can eventually be submitted for approval to support the value of a new vaccine prior to its initial testing in man.

Vaccine Adjuvant Studies. A portion of vaccine development research has been devoted to the study of potential adjuvants that could improve the immunogenicity of marginally effective vaccines. Adjuvants selected for applied study emphasized those with a potential for being approved for use in man. These included two interferon-inducers, lysine-stabilized poly(I)·poly(C) [poly(ICLC)] and an analog of the drug tilorone-HCl. Other adjuvants included dialyzable leukocyte extracts, biodegradable oil-water emulsions and muramyl dipeptide.

In the studies on tilorone analogs or poly(ICLC), an adjuvant-vaccine combination significantly enhanced resistance in mice against all test virus infections when compared to control mice receiving vaccines without

adjuvant. Similarly, a lipid emulsion given with VEE vaccine was effective in both mice and monkeys on the basis of both enhanced resistance to virus challenge and development of higher serum neutralizing antibody titers.

In more basic studies, the experimental adjuvants were compared to the effects achieved with Freund's complete adjuvant, which, although it is not safe for use in man, has long acceptance as being the most potent product available for enhancing the immunogenicity of weak antigens in animals. In these comparisons, Freund's adjuvant was found to potentiate both cellular and humoral primary immune responses, whereas muramyl dipeptide in oil appeared to improve the primary cell-mediated immune responses, but potentiated only anamnestic humoral responses to inactivated VEE antigens.

Immunologic Responsiveness Studies. Another sizable portion of the in-house program involved studies to determine how immune responses can be measured and manipulated in order that efficacious safe vaccines can be developed. These studies included investigations into the relative efficacy of experimental vaccines administered via different routes, the responsiveness of cell-mediated immune mechanisms to vaccines or infection, the effects of "selective" and general immunosuppression (such as that produced by acute irradiation), the immunologic functions of macrophages and lymphocytes and the production of immune complexes in plasma.

Because BW defensive measures must consider the exposure of troops via an aerosol, research was conducted to determine optimal immunologic methods for generating protective immunity on mucosal surfaces throughout the respiratory tract. These included studies of aerosol immunization to tularemia via the lungs and airborne infections with JE in monkeys and mice, and Pseudomonas pseudomallei (melioidosis) infections in mice and hamsters. Mice surviving an initial infection with JE were solidly protected against rechallenge, but neither killed virus vaccines nor passive administration of immune serum protected them. Aerosol infections also produced lethal melioidosis in hamsters and squirrel monkeys. Initial studies suggested that some protection against respiratory melioidosis could be provided by killed cell vaccines.

To evaluate cell-mediated immunity, comparisons between leukocyte-adherence inhibition and macrophage-migration factor tests were followed during tularemia infection in mice. Other studies in mice identified, through Mishell-Dutton assays, the extent of participation of B- and T-lymphocytes and macrophages in response to vaccination with either live or killed tularemia vaccines. Methods were also 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, LVS. Protection against highly virulent tularemia organisms appeared to require both T- and B-lymphocyte activity. In other studies, the participation of cell-mediated immune mechanisms was examined in nude mice, because of their congenital lack of thymic functions. This approach was especially valuable in attempting to determine why some of the arenavirus infections were capable of producing delayed lethal encephalitis.

Studies using nude mice led to the conclusion that lethal encephalitis caused by Tacaribe virus was immune-mediated and dependent upon the presence of intact functioning T-lymphocyte mechanisms.

Methods were developed to study the production of immune complexes in the circulation following the exposure of immunized or nonimmunized animals to virulent microorganisms. This new approach uses isotachophoresis to identify the complexes through their patterns of migration in an electrical field.

Additional basic work was conducted to define the role of lymphocytes and macrophages in immune mechanisms. A series of novel studies defined for the first time the physical and chemical requirements for lymphocyte chemotaxis under in vitro conditions. Lymphocytes migrating between agarose and glass layers were attracted by gradients of chemotactic factors, which differed chemically from those known to attract polymorphonuclear leukocytes.

Because some anti-inflammatory agents may affect the induction of immunity through their cytoskeletal effects, 2 cytoskeletal probes (cytochalasin-A and colchicine) were used to define the role of cellular microfilaments and microtubules in regulating lymphocyte movement through lymph nodes and during cell cooperation actions and recirculation throughout the body. It was also shown that high endothelial cells of lymph node venules could remove antigen-receptor complexes from the surfaces of recirculating lymphocytes without damage to the lymphocytes. The "scrubbing" process was postulated to be of value in exposing new receptors which rendered the lymphocytes more responsive to subsequent antigenic stimulation.

Studies of macrophage function continued to use cells collected from the pulmonary alveoli of cynomolgus monkeys during respiratory infection. The IV administration of the drug, glucan, prior to an aerosol exposure to an infectious organism was found to exert a nonspecific stimulating effect on the monocyte-phagocyte system; this treatment materially reduced mortality during intracellular infections, such as tularemia in rats. Mechanisms were developed for studying the detailed function of macrophage organelles following bacterial phagocytosis. The mannose-rich capsule of tularemia organisms was found to be important in allowing the organisms to be destroyed within macrophages. However, these phagocytic cells were capable of taking up virulent tularemia organisms whether or not their mannose-rich capsule had been removed.

Still other immune responses were studied in animals whose immune system had been suppressed. Suppression studies associated with high doses of whole-body irradiation were limited during FY 78 because the USAMRIID high-voltage X-ray source could no longer be repaired; it is being replaced. In other suppression studies, cyclophosphamide treatment given prior to immunization of animals with various types of vaccines was used in an effort to abolish humoral antibody responses, while at the same time allowing cell-mediated immune functions to be preserved, or even potentiated if these were being repressed by blocking antibodies. When treated with cyclophosphamide, animals showed a loss of protection when challenged with VEE virus, unchanged protection against tularemia, but enhanced protection against Q fever and tick-borne spotted fever.

Pathogenesis Studies. Pathogenesis studies in animal infections are needed to test new vaccines, diagnostic techniques and therapeutic measures. During the year, USAMRIID attempted to create suitable model infections in laboratory animals for Legionnaires' disease, KHF, LAS, C-CHF, RVF and arenavirus infections less dangerous than LAS, i.e., Pichinde and Tacaribe. In searching for a model, especially for such a difficult disease to study as KHF, a large variety of common and rare laboratory animals were tested, including some (cotton rats, vesper mice and voles) which are not available commercially and must be bred in-house. Such studies also call for different subhuman primate species as well as inbred strains of the more common laboratory animals.

Emphasis has also continued on defining the unique pathogenic patterns of illness produced by aerosolized organisms, including bacterial broncho- and lobar pneumonias, and the study of viruses, such as JE, which appear capable of entering the central nervous system from the nasal mucosa via olfactory nerves traversing the cribriform plate.

Additional studies were conducted to define some of the physiologic and biochemical responses that accompany infectious diseases. These included body fluid and electrolyte shifts in yellow fever and the hepatorenal failure which may accompany various infections. This work has been strengthened by development of computerized techniques for collecting and recording data from many on-going simultaneous physiologic measurements. Biochemical studies included additional work to define the mechanisms used to provide metabolizable energy for the infected host and to characterize the role of the liver in producing the large variety of new "acute-phase reactant" serum glycoproteins and hepatic metallothioneins during a variety of different infections.

Diagnostic Studies. Diagnostic studies covered several different areas. A major new research thrust was initiated to establish and maintain immunologically based diagnostic capabilities for a wide variety of virus diseases of special importance to the USAMRIID medical defense program. These new approaches involved development of improved fluorescent antibody (FA) technology, radioimmunoassay (RIA) methods, enzyme-linked immunoabsorbent assay (ELISA), and chemiluminescent-immunoassay techniques, all of which are being compared with each other and to the more time-consuming microbiologic techniques, e.g., plaque neutralization tests, which can be employed to provide validation for each new method. Spot tests on microscope slides for FA identification of a large number of viruses were prepared, standardized and tested for safety. Additional slides to test for new viruses are currently being developed both in-house and under contract.

Unique new aspects of diagnostic work included confirmation of the reliability of the FA technique for diagnosis of KHF. Serum specimens obtained from nephroso-nephritis patients in 3 Scandinavian countries were positive with KHF antigen, providing important new evidence that the two diseases are linked, or identical. Tissue culture growth of KHF virus was achieved for the first time. There were also initial breakthroughs showing that RIA and ELISA had great usefulness for virological diagnosis. The ELISA has been applied to bacterial organisms and toxins; it appears to provide more rapid method for diagnosis of Legionnaires' disease.

A major accomplishment was the development of a fully contained P4-level diagnostic laboratory, previously described in Clinical Studies. This included the training of personnel necessary to permit all diagnostic microbiologic and clinical laboratory samples to be handled and processed in fully contained biological safety laboratories, with investigators and technicians working in total-body positive-pressure safety suits.

Additional approaches in the diagnostic area included further studies to determine if changes in chemiluminescence or the biochemical composition of white blood cells or platelets could be used as early indicators of the presence of an infectious disease. Further progress was made on computerizing the various diagnostic approaches of a biochemical nature to determine if patterns might emerge which would be of greater value than changes in single parameters. Finally, a new technique using isotachophoresis was developed to permit the isolation of immune antigen-antibody complexes from serum that might be of diagnostic value.

Therapy Studies. Therapy studies involved important new work on antiviral compounds against unique, highly dangerous viruses. Work continued on the use of aerosolized antibiotics in pulmonary infections and metabolic and physiologic approaches for providing supportive therapy. The latter need is especially important during overwhelming infections, including those complicated by disseminated intravascular coagulation (DIC) or hepatorenal failure.

A dramatic achievement was the discovery that ribavirin was effective in animal models for treatment of such dangerous infections as those caused by arenavirus hemorrhagic diseases, RVF and possibly yellow fever. Earlier work with poly(ICLC) continued and new studies were introduced to determine if antioxidant drugs had value in infections due to lipid-containing viruses; the major emphasis was placed on studying the therapeutic effectiveness of ribavirin. While ribavirin was previously shown to have prophylactic efficacy, the 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 in monkeys. Work is on-going to determine the localization of ribavirin within tissues and the molecular mechanism of its antiviral activity within cells. In addition, arrangements were made to obtain new analogs of it that might cross the blood-brain barrier.

Studies to extend knowledge in the areas of aerosol therapy with antibiotics indicate that pulmonary concentrations of drugs, such as kanamycin, can reach therapeutic levels without dangerous accumulation in the kidneys. Aerosol therapy was more effective against bronchopneumonia in animal models than against frank lobar consolidation.

Since antimicrobial agents are not available for all lethal infections, continued emphasis was placed on improving metabolic and physiologic support and correction of imbalances. It was possible to control many of the infection-induced abnormalities in amino acid, protein, carbohydrate, insulin, free fatty acid, and ketone metabolism during experimental infec-

tions by therapeutic administration of appropriate metabolic substrates. Studies are underway to control hepatorenal failure by using hemoperfusion techniques and correction of salt and water imbalances.

The catastrophic complication of DIC during a variety of bacterial, viral and parasitic infections was approached with a protease inhibitor, aprotinin, as a drug to influence the kinin system. Studies in monkeys were conducted to determine the pharmacokinetics of aprotinin during experimentally induced infection and to determine its effects on blood clotting mechanisms.

Bacterial Toxin Studies. Studies included those on botulinum neurotoxins, anthrax toxins, several staphylococcal enterotoxins, cholera and Shigella spp. enterotoxins, diphtheria exotoxin and Pseudomonas exotoxin A and exoenzyme S.

In a major new program, effort was initiated to produce an improved multivalent botulinum toxoid, with excellent progress being made. Representative, previously used strains of toxin-producing organisms were obtained. Initial efforts began with neurotoxin A and were highly successful, allowing for production of sufficient volumes of culture to permit extraction and purification by modern chemical techniques of milligram quantities of the neurotoxin. Modern technology was used to toxoid this into a safe and immunogenic vaccine. A program was initiated to collect many liters of high-titer human antibotulinum plasma from individuals who previously had been immunized repeatedly with polyvalent botulinum toxoid. Approval by the Army and the Food and Drug Administration was obtained to use this investigational human hyperimmune botulinum plasma for therapy of acute botulism if this should become necessary. In addition, arrangements were made under contract to convert large quantities of the plasma into hyperimmune botulinum immunoglobulin.

Studies on anthrax toxins and protective immunogens had been at a virtual standstill for at least a decade. During FY 78, USAMRIID entered this field in an attempt to produce a more effective immunogen which could be used in man. The currently available vaccine is a crude filtrate which requires 18 mon for the primary vaccination series. Anthrax organisms produce at least 3 poorly characterized exoproteins: protective antigen, edema factor and lethal factor. It is necessary to reestablish methodology to culture selected strains, in order to produce sufficient exoprotein to allow for the eventual differentiation, purification and toxoiding by modern techniques.

Much basic work was accomplished in defining the secondary and tertiary structures of staphylococcal enterotoxin B (SEB) and its component peptides, and in comparing them with comparable portions of SEA and SEC_1 enterotoxins so as to define which portions of the protein molecules were immunogenic and which caused toxicity. In addition, a capability for sequencing proteins was established. Initial steps were taken to ascertain the correct amino acid sequences of SE toxins, with emphasis on SEC_1 and the dermatolysins. In other basic work, the mechanism used by staphylococci to excrete their exoprotein toxins was shown to depend upon the fatty acid composition of external membranes. Evidence was also obtained

that a proteinase was required to release the toxin into the culture medium.

The mechanism of action of SEB was studied with respect to the exchange and loss of fluid across the intestinal mucosa of the rabbit. A potential breakthrough in therapy emerged, when it was shown that SEB could be taken up and bound by activated charcoal. Initial attempts were then made to utilize this concept in hemoperfusion studies which would allow toxin in the blood stream to be removed during extracorporeal circulation through a charcoal filter. Exploratory physiologic studies were also performed to determine if either cholera or Shigella enterotoxins were toxic following exposure by parenteral or aerosol route, rather than the usual gastrointestinal route.

Progress was made in defining the nature of Pseudomonas exotoxins. Additional studies for laboratory scale production and purification of Pseudomonas exotoxin A were made, with toxoiding mechanisms being developed; the resultant toxoid provided partial protection against Pseudomonas infection in burned mice; there was little protective efficacy in burned, infected rats. Attempts to purify another Pseudomonas exoprotein, exoenzyme S, yielded initial successes. Major advances were made in studying the specific toxic mechanism of action of Pseudomonas exotoxin and the closely related diphtheria exotoxin. In vitro studies in cultured cells employed radioactively labeled exotoxin molecules to demonstrate for the first time that specific cellular receptors exist for each of these exotoxins. The presence of a specific cellular receptor is necessary to allow the toxin to enter a susceptible cell. The demonstration that such receptors exist on susceptible cells has already been followed by identification of drugs which prevent the binding of toxins to cells, thus obviating toxin entry and lethal action. In closely related, equally novel work, both diphtheria and Pseudomonas exotoxins have been conjugated with either ferritin or colloidal gold in order to visualize these receptors on cells by electron microscopy.

Rickettsiology Research. Rickettsiology studies emphasized continuing attempts to improve or evaluate rickettsial vaccines. Other research involved pathogenesis studies by light and electron microscopy in order to elucidate the nature of the earliest stages of the vascular endothelial lesions which characterize many rickettsial diseases.

Q fever studies proceeded along 3 paths: additional data were gathered from monkeys to evaluate the relative efficacy of the inactivated phase I Q fever vaccine, prior to initial testing in man. These tests used a newly standardized cynomolgus monkey model in which the disease closely resembles the illness seen in man, including interstitial pneumonia and hematologic, physiologic and immunologic responses. Additional attempts were made to isolate purified components of Coxiella burnetii in hopes of identifying specific components that might be highly immunogenic and effective in preventing Q fever, without having the undesirable side-effects of the existing phase II vaccine. Soluble phase I antigens of C. burnetii were treated with various enzymes, including proteinase, lipase or lysozyme, with the last producing an immunogen which appeared to exhibit reduced reactogenicity. A third line, involved the

infection of athymic, nude and normal euthymic control mice with small-particle aerosols of C. burnetii. Euthymic mice recovered rapidly and cleared rickettsiae from peripheral blood and spleen within 14 days; immunodeficient nude mice showed continued presence of rickettsiae for at least 60 days. This finding adds to the evidence concerning the importance of cell-mediated immunity in host resistance to Q fever.

Additional studies of the new RMSF vaccine were conducted in volunteers (see Clinical Studies). In addition, studies in laboratory animals were continued to produce broadly protective vaccines against all varieties of rickettsial spotted fevers. Studies in guinea pigs showed that our RMSF vaccine prepared for human use was protective against virulent strains of Rickettsia rickettsii obtained from diverse geographical areas. Cross-protection studies were conducted in guinea pigs and mice relative to the immunologic similarities of Rickettsia: rickettsii, conorii, sibirica and akari. Although none of these species produces lethal illness in guinea pigs, they each induce cross-protection against the others, as evidenced by prevention of a febrile response. This type of cross-protection was afforded BALB/c mice infected with R. rickettsii, R. conorii or R. sibirica which subsequently resisted infection with normally lethal R. akari.

In a final aspect of immunization studies against spotted fevers, 2 additional approaches were used. Soluble immunogenic antigens derived from suspensions of whole rickettsiae were found to protect laboratory animals. Limited success was achieved in attempting to use a relatively avirulent organism, Rickettsia montana, as a live vaccine.

Since spotted fever rickettsiae produce vascular lesions, studies were conducted using light and electron microscopy to demonstrate the earliest changes in endothelial cells in infected tissue culture cells or in the vascular endothelial cells of arteries obtained from guinea pigs. As an alternative approach, a chick embryo model was used to study the earliest lesions in vessels of allantoic membranes. In each instance, the rickettsiae were always present. In the egg, vascular lesions appeared before the embryo was immunologically competent and before proteins of the complement and coagulation systems had developed. These data, in combination, suggest that vascular lesions can best be explained by direct effect of the rickettsiae. In one final area, the methods used successfully to produce a whole-organism spotted fever vaccine from tissue culture were extended to Rickettsia prowazekii to determine if an improved epidemic typhus vaccine could be prepared. This new vaccine was at least as efficacious as the presently available commercial product. Further tests are planned to compare these products in subhuman primates.

Summary

This brief review of the USAMRIID progress during FY 1978 illustrates the success of a multidisciplinary approach in fulfillment of the mission of the Institute.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6410	78 10 01	DD-DR&E/AR(A)36

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
78 04 21	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	62776A	3M162776A841	00	001
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)
(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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 78	0.5	160.0
FISCAL YEAR — CURRENT 79	1.0	353.7

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
NAME: Medical Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Anderson, J. H., Jr.
TELEPHONE: 301 663-7361
SOCIAL SECURITY ACCOUNT NUMBER

21. GENERAL USE
Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME:
NAME:

POC: DA

22. KEYWORDS (Precede EACH 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 (Furnish 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 and 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) 77 10 - 78 09 - Administration of vaccines to at-risk laboratory personnel continued, as did clinical and serological observation of laboratory workers, support personnel and professional staff. Vaccine trials were conducted with an inactivated Rocky Mountain spotted fever, an inactivated VEE, inactivated Rift Valley fever, and attenuated live virus dengue-2 vaccines. A major clinical study in the evaluation of transfer factor was begun in cooperation with NIH. Evaluation of prophylaxis utilizing hyperimmune plasma and/or globulin continued with admission to the special isolation unit of 2 persons exposed to Machupo virus and 2, to Lassa fever virus. Utilization of the unique isolation facilities for patients began with the admission of 2 laboratory workers from CDC and a Peace Corps worker from Africa.

Publication: J. Infect. Dis. 138:217-221, 1978.

*Available to contractors upon originator's approval

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 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 consolidation of the work unit (A841 00 021) entitled "Evaluation of Prophylaxis and Therapy of Infectious Diseases in Man" with this one. The combination of the 2 results in a comprehensive research effort to develop, test and utilize experimental vaccines, antimicrobial drugs, hyperimmune plasma and special medical isolation procedures in man for BW defense against infectious diseases of special military importance. The new Army program of Medical Research Volunteer Subjects (MRVS), which replaces the old Project Whitecoat Program, has made important clinical studies involving human volunteers available again at the Institute.

Progress:

A. Vaccines

Rocky Mountain Spotted Fever. As noted in last year's annual report a revised protocol (Med. Div. Protocol No. 77-2), "Proposal for the Clinical Evaluation of a Two Vaccination Schedule of Inactivated Rocky Mountain Spotted Fever Vaccine (IND 862) (Diluted 1:3)," was approved by the Clinical Studies Branch, National Institute of Allergy and Infectious Diseases, NIH, and funding was received enabling work to begin in late September 1977 for selection of volunteers. Ten (9 MRVS and 1 staff member) were picked and after giving fully informed consent, received a preliminary medical and serological examination. Each of the volunteers was in good health and serologically negative for RMSF. Each volunteer received 0.5 ml of the vaccine SC 28 days apart. There were no local nor systemic reactions and no untoward effects in any of the laboratory parameters followed. By fluorescent antibody techniques (FAT), 9 of the 10 individuals demonstrated an increase in titer. However, the magnitude of the titer was not equal to that seen in either the natural disease or in previously immunized individuals who had only a 1:10 dilution of vaccine, but were not antigenically naive. Microagglutination (MA) titers, however, revealed an increase in all 10 volunteers, although again the response was not comparable to those responses seen in naturally acquired disease; titers are presented in Table I.

TABLE I. PROJECT FY 77-2

IDENTIFICATION	SAMPLE DATE	RECIPROCAL TITER FA	MA
R.S.	9/30/77	8	2
	10/27/77	8	2
	11/23/77	64	8
	1/5/78	32	16
W.V.	10/27/77	8	2
	11/23/77	32	2
	1/5/78	32	16
M.C.	9/30/77	8	0
	10/27/77	8	4
	11/23/77	64	8
	1/5/78	256	8-16
J.F.	9/30/77	8	0
	10/27/77	8	4
	11/23/77	8	8
	1/5/78	8	16
T.C.	10/27/77	8	2
	11/23/77	32	4
	1/5/78	64	32
S.M.	9/30/77	8	2
	10/27/77	8	4-8
	11/23/77	8	8
	1/5/78	16	8
J.G.	10/27/77	8	4
	1/5/78	64	16
J.N.	10/27/77	8	4
	11/23/77	8	4
	1/5/78	32	8-16
R.M.	9/30/77	8	2
	10/27/77	8	2
	11/23/77	8	8
	1/5/78	8	16
D.K.	9/30/77	8	0
	10/27/77	8	2
	11/23/77	32	8
	1/5/78	64	16

Because of the immunogenic response elicited by the vaccine at a 1:3 dilution, a new protocol (Med. Div. Protocol No. 78-4), "Proposal for the Clinical Evaluation of a Two Dose Schedule of Inactivated Rocky Mountain Spotted Fever Vaccine, Undiluted (IND 862)," was written and submitted for approval to test the vaccine undiluted. This protocol was approved by the Division Chief's review, USAMRIID Human Use Committee review, the Scientific and Human Use Review Committee of the NIH and The Surgeon General's Office. Ten MRVS have been selected, the initial studies performed and the first immunization given. There were no systemic or local reactions with the first immunization. At this time there are no titers available.

Rift Valley Fever. Rift Valley Fever (RVF) (Med. Div. Protocol No. 78-1), "Evaluation of the Human Response to the Administration of Rift Valley Fever Vaccine, Formalin-Inactivated, Tissue Culture Origin, NDBR 103, Lots 1-6 (IND 365)". Because of increased demands for vaccine for use in the treatment of epidemics of RVF, the need arose to test those lots of vaccine (Lots 1-5) which had not been used previously in clinical trials. A protocol was designed in accordance with the previously approved RVF protocol submitted in 1969 to the Army Investigational Drug Review Board (AIDRB). This protocol was approved by the Division Chief review and USAMRIID Human Use Committee. Two informed members of the Institute staff received 0.1 ml of each of Lots 1-5 of RVF vaccine. When no ill effects were observed, a group of 5 informed volunteers from the professional staff of USAMRIID each received 0.5 ml of 2 lots on a schedule so that each lot was given to 2 individuals, but no individual received the same 2 lots. After 10 days with no ill effects noted, a group of 21 MRVS were selected, randomly placed in groups of 3 and each group assigned to one of the RVF vaccine Lots 1-5 and Lot 6, Runs 1 and 2. Each volunteer was begun on the standard immunization schedule of 3 injections of 1 ml each given on days 0, 10 and 28. Appropriate clinical and serological studies were carried out to determine the safety and immunologic response of Lots 1-5 compared to Lot 6 which has had extensive clinical use. Safety testing indicated the lots were identical with respect to reactogenicity. None of the volunteers experienced any local or systemic reaction.

In direct comparison testing utilizing the pre-immunization and day 35 sera, serologic results were comparable for all groups based on comparison with Lot 6-2, utilizing a PRN_{50}. Lots 1 and 2, as well as Lot 6, Run 1 gave PRN_{80} and PRN_{50} titers of $\geq$ 1:1280 by day 35, a response that was also seen with Lot 6-2. Lot 3, while giving a PRN_{50} response of $\geq$ 1:1280, gave a PRN_{80} response of only 1:640. Serologic testing on Lots 4 and 5 was invalidated due to cell contamination and is scheduled to be repeated.

Dengue Fever. (DEN-2), Med. Div. Protocol No. 78-2, "Evaluation of Human Response to the Administration of Dengue Virus Vaccine (Type 2), Live, Attenuated," (IND 1257), was initiated on 3 March 1978. This vaccine trial utilized a vaccine developed by a WRAIR group and was done in conjunction with them. In the initial part of the project, 2 fully informed staff volunteers from the WRAIR with a high degree of competence in virology received the vaccine. During the 14 days of inpatient care on the project ward there was no evidence of reaction to the vaccine. By day 12 of the study both volunteers had leukopenia with an increase in lymphocytes. In one, this was accompanied by fatigue, slight photophobia, but no other systemic signs. After a period of 5 days the WBC count returned to normal. Virus was recovered from the blood of the volunteers during this period of leukopenia and both volunteers developed significant antibody titers.

In the second part of the study 4 fully informed volunteers from the WRAIR professional staff, each with a high degree of competence in virology, received the vaccine. Each volunteer underwent a complete history and physical examination and base-line biomedical studies. Each volunteer had previously been screened for hepatitis-B surface antigen and PRN titers to DEN-1, -2, -3 and -4 virus types. Volunteers were confined on the clinical research unit for 3 days prior to immunization, at which time each volunteer received another physical examination and was questioned in detail about potential exposures prior to arrival on the research ward. Base-line biochemical and virological studies were repeated. Each of the volunteers received 0.5 ml of vaccine SC, which contained ~ 4×10^5 PFU of virus. Volunteers were followed for 10 days as inpatients and for another 11 days as outpatients. Each volunteer was questioned about symptoms of headache, myalgia, eye pain, fever, chills, nausea, vomiting, diarrhea, abdominal pain, epistaxis or melena. Volunteers were also examined for evidence of rash, petechiae, local and general adenopathy, abdominal tenderness, hepatomegaly, splenomegaly and conjunctival suffusion. Inoculation sites were examined for evidence of edema and erythema. One volunteer reported 48 hr of mild fatigue, but developed no other signs or symptoms, nor did he develop leukopenia. This one volunteer was found, in retrospect, to have had a probable dengue infection in the past. One volunteer had a mild headache for ~ 36 hr on days 9-10. Although this volunteer had short, early leukopenia, in addition to the expected decrease subsequently, his base-line white counts were lowest of the group. One volunteer developed headache, myalgia and a low-grade fever on day 12 in addition to an erythematous, macular rash which persisted for 5 days. Leukopenia appeared on day 14. The fourth volunteer developed signs and symptoms warranting readmission for observation. The volunteer developed fever of 102°F, headache, photophobia and fatigue. He was hospitalized for 3 days during which time he had one day of leukopenia. A full battery of bacterial cultures revealed no growth in blood or urine and normal flora in the throat

and stool. Viral isolations on HEK, HR-21 and RMK cells, and in eggs and mice were all negative. During the 3 days of hospitalization all signs and symptoms resolved except the fatigue. He did not develop a rash.

To summarize the project, including all 6 volunteers, the following information is given:

The most common symptom was a headache occurring in 4 of 6 volunteers. In one subject with a local reaction, the headache occurred on day 3, lasting only a few hours and may have been related to over-exercise. Three of the volunteers experienced headaches on days 10-13. In one of these 3, headache was an isolated finding, while the other 2 had both fever and other symptoms. Fatigue was the next most common complaint, although this occurred on days 12-15. The fatigue coincides with the return to work after 2 weeks of isolation and may be more physiologic than related to the vaccine. Mild photophobia was reported in 2 of the volunteers on days 11-13. Myalgia occurred in 1 volunteer on days 12-13, coinciding with fever and rash. Nausea was reported by 1 volunteer on day 4 and it may have been related to dietary indiscretion. Of the 6 volunteers only 1 had any reaction at the site of inoculation, 14-mm erythema on day 2 and 5-mm induration with 12-mm of erythema on day 3. By day 4 the induration was gone and by day 5 there was no evidence of inoculation site reaction. Five of the 6 volunteers developed leukopenia (defined as a total WBC count < 4,000); WBC dropped in each on about day 10 and began to increase on about day 16. In those individuals who developed fever, leukopenia appeared about 24 hr after the onset of fever; 3 volunteers developed fever, one with a temperature of 100°F at the time of his local reaction and also on days 11-15. One individual developed fever of 102°F which resulted in his being rehospitalized.

In summary, the first human DEN-2 vaccine trial has been completed; 3 individuals had either insignificant symptoms or none at all. One individual had mild transient signs and symptoms, and a local reaction; 2 volunteers developed signs and symptoms of systemic dengue infection not unlike mild, natural disease.

Venezuelan Equine Encephalomyelitis Vaccine, Inactivated. Med. Div. Protocol No. 77-1, Addendum-1, "Acceptability Study of Venezuelan Equine Encephalomyelitis Vaccine, Inactivated, Dried, MNLBR 109, LOT NO. C-84-1, IND 914," was outlined in the previous report. The original protocol evaluated the response of 15 individuals who received 0.5 ml of the inactivated vaccine; 7 of these received a booster of 0.5 ml 28 days after the initial immunization. The following table summarizes the neutralization titer data 2 weeks following booster immunization:

TABLE II. SUMMARY OF VEE TITERS

RECIPROCAL TITER	
Boosted (n=7)	Nonboosted (n=8)
640	<10
640	40
80	80
1280	10
320	20
160	<10
2560	40
	20

A review of these titers demonstrates significant serologic response to the vaccine, but not to the degree seen after natural disease or the live TC-83 vaccine. An additional Addendum-2 to 77-1 was written and submitted to gain approval to administer a 3rd immunization to 4 of the previously boosted and 4 of those not boosted. The additional immunization at 6 mon was sought to determine the potential to induce prolonged titers to VEE. The study was approved and 7 individuals (including one control) from the original project gave informed consent and took part in the project. Serologic response to this booster immunization is currently being evaluated.

B. Transfer Factor

A joint project between USAMRIID and NIAID, NIH, was begun with Med. Div. Protocol No. 78-3, "Transfer of Cell-Mediated Immunity to Microbial Antigens with Dialysable Leukocyte Extracts (Transfer Factor)." The study was approved, 6 MRVS were recruited and the project begun. Clinical evaluation and serologic responses are not yet available and will be presented in a subsequent report.

C. Prophylaxis

Bolivian Hemorrhagic Fever (BHF) Globulin (IND 1114). Two USAMRIID personnel were exposed to Machupo (MAC) virus, the etiologic agent of BHF, when a plastic vial leaked material containing the virus. These 2 workers were admitted to the isolation unit of Ward 200 for observation and therapy. BHF hyperimmune globulin was administered to both patients. Because of recurrence of a chronic, undiagnosed, pulmonary disease in one patient, no additional BHF globulin was administered. The second patient received 2 additional doses 5 days apart. The dosage selected, 0.25 ml/kg globulin for an initial dose and 0.125 ml/kg

for subsequent doses, was based upon experimental data in nonhuman primates, which demonstrated this dosage to be well above the minimal protective dose and below that at which the monkeys developed subsequent neurologic sequelae. After 17 days with no clinical appearance of symptoms and no laboratory evidence of infection, the patients were discharged. Subsequent follow-up has revealed no evidence of illness, or sequelae from the administration of the hyperimmune globulin.

Lassa Fever (LAS). Two researchers from CDC were admitted to the isolation facility at Ward 200 because of exposure to LAS virus. The 2 individuals had been sorting vials of viremic rodent sera when one of the partially thawed vials came apart, splattering blood on the chin and shirt of one investigator. Neither individual was wearing protective clothing or suitable respiratory protection. The work was not being done in a cabinet; it was the concensus that a significant exposure had occurred. The exposed individuals were flown to Hagerstown, MD from Atlanta, transferred by ambulance with protective clothing and reverse respirators to the USAMRIID special isolation unit. Both individuals presented with normal physical examinations and laboratory values. Both individuals were given LAS immune plasma, 2 U on admission and an additional unit 7 days later. Neither individual developed significant signs or symptoms during hospitalization, which extended to 21 days postexposure. Virologic evaluation revealed no evidence of LAS virus or replication in either individual, nor was there any evidence of autonomous antibody production. The individuals involved have been followed closely for a study of the rate of disappearance of antibody levels and this study has continued after their discharge from the isolation unit.

Med. Div. Protocol No. 77T-1, "Evaluation of WR 171,669 in the Treatment of Multi-Drug Resistant P. falciparum" is complete. All individuals were followed with no recurrence of malaria and no related medical problems.

Med. Div. Protocol No. 77T-3, "Rejuvenation, Preservation and Characterization of the African (Uganda I) Strain of Plasmodium falciparum" is essentially complete. No individual had recurrence of malaria. There were no complications in 3 individuals; the 4th developed mononucleosis with mild liver dysfunction. Evaluation of this problem is underway.

D. Summaries of Reports to AIDRB

VEE, Live, Attenuated, NDBR-102 (TC-83) (IND 142).

During the reporting period 26 immunizations were administered with complete clinical and serological data available on each. Two types of systemic reactions occurred: 4 individuals experienced mild

reactions 24-48 hr after immunization, characterized by mild headache, malaise and low grade fever in one individual. Two individuals experienced the more commonly seen systemic reaction at 6-8 days; these late reactions consisted of malaise, myalgia and chills with 1 of the 2 experiencing low grade fever. No local reactions were noted and no individuals were hospitalized. This systemic reaction rate of 23% is consistent with our previous experience as is the division of reactions into 2 distinct clinical syndromes. All individuals immunized were seronegative prior to immunization. Serologic data on the 26 individuals receiving primary immunization showed a conversion by HI titers in 24 (92%). Of the 2 individuals who were HI-negative following immunization, one had a positive PRN titer. The policy of doing PRN testing on HI-negative recipients, standard procedure at this time, further illustrates the relative value of the 2 tests.

Q Fever Vaccine (Henzerling strain) (WRAIR; IND 229).

During the latest reporting period 32 individuals received immunization with Q fever vaccine (Henzerling strain); 3 individuals experienced local reactions, 2 after the first injection and 1 after the second. The product is highly reactogenic and has led to an unacceptable incidence of local reactions, including sterile abscesses. For this reason the material is not considered adequate for maintaining booster immunizations in those people who have received a primary series. Work continues in the further investigation of the phase I vaccine at this Institute. Titers on those individuals immunized are not available because the CF test at USAMRIID has been discontinued.

EEE Vaccine, NDBR-104 (IND 266).

During the last reporting period 45 subjects completed primary immunizing series of 2 doses of the EEE vaccine (0.5 ml SC) separated by 28 days. There were no local or systemic reactions reported among these individuals. Serologic data is available on 40 individuals: 34 (85%) had detectable HI titers 1 mon after the 2nd injection; 6 remained HI-negative, although one had a positive PRN titer.

One-hundred forty-one subjects were administered 0.1 ml ID as a booster EEE immunization during the reporting period. Reactions were limited to local erythema appearing within 20 min in 33 individuals (23%) and delayed onset (24-48 hr) in 20 individuals (14%). Serologic data are available on 136 of the individuals: 117 were seropositive prior to the immunization by HI testing; 19 (14%) were seronegative, although 14 (74%) converted after the ID booster. The remaining 5 remained HI-negative, although 2 were positive by PRN test.

E. Isolation.

In addition to these clinical cases requiring isolation in the special isolation facilities of Ward 200, there were several individuals admitted in various degrees of isolation for observation following suspected illness or known exposure. The only other patient admitted to the special isolation facilities was a 24-year-old, white, male Peace Corps volunteer from Senegal, Africa, who after a 2-week history of fever, headache and malaise was flown to the United States where he was admitted to Walter Reed Army Medical Center. Following admission, he was transferred to USAMRIID and placed in the special isolation unit. Within the first 36 hr of admission, the lack of antibody response which would have been considered diagnostic of LAS in this individual was confirmed by laboratory investigation. In all individuals with LAS previously studied, there had always been an antibody response by day 12-15. The patient remained in the special isolation unit for another 36 hr, at which time there had been no evidence of any viral etiology for his disease. Since the patient had been afebrile and asymptomatic, he was transferred to the hospital ward with enteric isolation. The patient was subsequently discharged after observation; the exact etiology for his illness has not yet been confirmed.

Two individuals were hospitalized on Ward 200 on the open ward for observation following separate incidences of exposure to DEN-1 virus. The first individual admitted was a laboratory worker who inadvertently dropped a syringe of DEN-1 virus on the floor, causing ejection of liquid which reportedly splashed into the patient's eye. The patient was confined to Building 1425 to avoid the hazard of introducing virus into local mosquito populations. The second admission was an investigator who accidentally stuck his finger with a syringe needle he was using which contained DEN-1 virus. He was admitted for observation for 10 days. Blood studies identical to the experimental vaccine protocol were carried out in both subjects. Neither individual developed any evidence of clinical infection.

Publication:

Ascher, M. S., C. N. Oster, P. I. Harber, R. H. Kenyon, and C. E. Pedersen, Jr. 1978. Initial clinical evaluation of a new Rocky Mountain spotted fever vaccine of tissue culture origin. J. Infect. Dis. 138:217-221.

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. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 04 21	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
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11. TITLE (Precede with Security Classification Code)

(U) Enhancement of host resistance to facultative bacteria

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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	CURRENT 79	0	0

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POC: DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Cell-mediated immunity; (U) Francisella tularensis; (U) Salmonella typhimurium; (U) Syngeneic mice

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

23 (U) Define the role of humoral and cell-mediated immunity in facultative intracellular bacterial infections. This will lead to important information regarding treatment of and prophylaxis against BW agents.

24 (U) Using passive transfer of immune serum and/or lymphoid cells to syngeneic recipients with fully competent or selectively depressed immune mechanisms, determine murine resistance against F. tularensis challenge of graded virulence.

25 (U) 77 10 - 78 09 - Passive transfer of protection against lethal infection with fully virulent strains in the murine tularemia model can be effected by transfer of syngeneic immune splenocytes mixed with killed bacterial antigen. Splenocytes from unboostered donors are markedly inferior to those from recently boostered donors, indicating a requirement for highly activated populations. Although the immune lymphocyte dose is the most critical factor for protection, immune macrophages are also required. Studies on splenocyte depletion and on treatment with hyperimmune serum indicate the need for both T- and B-lymphocyte activity. Unlike protection against fully virulent strains, protection against strains of lesser virulence can be effected by sensitized T-cells alone or by immune serum. These studies have now terminated, due to higher priority efforts.

Publication: In The Prokaryotes, Springer-Verlag, Berlin, in press, 1978.

*Available to contractors upon originator's approval

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 002: Enhancement of Host Resistance to Facultative Bacteria

Background:

Our previous studies with murine tularemia (1, 2) described a model system wherein adoptive transfer of immune splenocytes ensures survival of nonimmune recipients against challenge with Francisella tularensis var. tularensis (SCHU S5R). Specific protection against these fully virulent strains appears to be antigen-dependent, requiring concomitant administration of syngeneic immune splenocytes and homologous killed bacteria (Ag). Treatment with either component alone may significantly delay dissemination and replication of SCHU S5R organisms, but this interference with bacterial growth results only in prolonged survival and does not reflect the capability of the host to recover from infection. Resistance to lethal challenge with virulent Eurasian strains (F. tularensis var. palaearctica), however can be evoked either with Ag or syngeneic immune serum (IMS).

Depletion of immune cell populations by treatment with various anti-leukocyte sera has failed to yield unequivocal evidence of the cell type(s) required for effective control of SCHU S5R infections. Data from the present studies indicate that a recently activated, fully competent immune complex consisting of T- and B-lymphocytes and probably immune macrophages is essential for protection against SCHU-type strains. The immune cell requirement for protection against Eurasian strains is less critical; the effective lymphocyte dose is 10-20% of that required for defense against the SCHU strains.

Progress:

Protection against F. tularensis var. tularensis. Unlike our previously reported findings, the present studies indicate that selected lots of IMS can substitute for the Ag supplement in adoptive transfer of protection by immune splenocytes to syngeneic nonimmune recipients. Splenocyte donor AKR/J mice were immunized SC with 10^3 organisms of the live vaccine strain (LVS) followed 28 days later by an IP booster of 10^6 LVS. Splenocytes (ISC_{10}) harvested 10 days postbooster were transferred IP to AKR/J recipients (donor:recipient ratio, 1:1) 20 hr before SC challenge with 25 SCHU S5R organisms (25 LD_{50}). Recipient and control groups were inoculated IP with Ag or IMS (titer, 1:640) at time of ISC_{10} transfer. Single factor treatment with ISC_{10}, IMS or Ag inhibited replication of challenge organisms in the spleen, prolonged survival time but failed to prevent fatal infection (Table I). The inhibitory effect was more pronounced in the IMS-control group ($P < 0.01$) than in Ag- or ISC_{10}-control groups ($P < 0.05$). Protection against lethality occurred only if Ag or IMS were accompanied by ISC_{10}.

TABLE I. RECOVERY OF BACTERIA FROM SPLEENS AND SURVIVAL CAPABILITY OF NONIMMUNE ISC_{10} RECIPIENT AKR/J MICE CHALLENGED WITH F. TULARENSIS SCHU S5R[a]

RECIPIENT TREATMENT			LOG_{10} F. TULARENSIS/SPLEEN ON DAY						SURVIVORS (6 mice/group)	
ISC_{10}	10^7 killed SCHU S4	IMS[b]	4	5	6	7	10	12	No.	Mean death time (day)
+	-	-	4.2	5.0	7.2	8.0			1	8.8
+	+	-	3.5	3.1*	5.4*	5.5*	2.6	2.4	4	10.0
[+	+][c]	-	4.3		5.3*		2.5	2.2	6*	
+	-	+	2.6	2.9*	5.1*	4.8*			4	14.0
-	+	-	5.5	5.8	8.3	8.2			0	7.7
-	-	+	3.4	4.5	5.5*	6.3*			0	9.8
-	-	-	7.6	8.0	8.4				0	5.8

[a] Immune splenocytes (ISC_{10}) transferred IP at a donor:recipient ratio of 1:1, 20 hr before challenge with 25 SCHU S5R organisms.

[b] Syngeneic hyperimmune antitularemia serum, titer 1:640; 0.25 ml IP/recipient at time of ISC_{10} transfer.

[c] ISC_{10} mixed with 10^7 killed SCHU S4 before transfer.

* $P < 0.01$, compared to killed antigen alone and untreated.

A series of subsequent experiments confirmed these findings and indicated that enhancement capability of IMS was reflected by its agglutinating antibody (Table II). High-titer sera (1:320-1:640) that effected enhancement were obtained at the height of secondary antibody response, i.e., 15 days after LVS-immunized mice were administered a 30-day IP booster injection with 10^6 LVS or 10^3-10^6 SCHU S4 organisms. Administration of a similar booster by the SC route failed to alter antibody titer and the 15-day post booster sera had no protective efficacy. Nonetheless, both high and low titer sera were equally effective for inhibiting dissemination of challenge organisms. The serum titer

probably serves merely as an index for the presence of protective activity; ISC_{10}-Ag recipients with no measurable circulating antibody at time of challenge with 25 LD_{50} survived as well as the ISC_{10}-IMS recipients that had high titer circulating agglutinins from passively transferred IMS. The only information, regarding the role of IMS, indicates that IMS is required for efficient phagocytosis of F. tularensis by AKR/J macrophages but that destruction of the bacteria occurs only in immune and not in nonimmune macrophages (H. P. Hawley, Bacteriology Division).

TABLE II. COMPARISON OF ENHANCEMENT ACTIVITY OF HOMOLOGOUS KILLED ANTIGEN WITH THAT OF LOW- OR HIGH-TITER SYNGENEIC IMMUNE SERA (IMS) ON THE SURVIVAL CAPABILITY OF NONIMMUNE ISC_{10} RECIPIENTS CHALLENGED WITH F. TULARENSIS SCHU S5R[a]

RECIPIENT TREATMENT				NO. OF TRIALS[c]	RECIPIENT PROTECTION ± SEM	
Donor ISC	10^7 Killed SCHU S4	IMS[b] 1:40	IMS[b] 1:320-1:640		Mean % Survival	Mean Death Time - Days
-	-	-	-	12	0 ± 0	5.9 ± 0.1
+	-	-	-	9	3 ± 1	8.1 ± 0.2
-	+	-	-	12	1 ± 1	7.2 ± 0.3
+	+	-	-	10	72 ± 7	10.1 ± 0.4
-	-	+	-	4	4 ± 4	8.8 ± 0.5
+	-	+	-	6	17 ± 7	9.2 ± 0.2
-	-	-	+	7	0 ± 0	9.5 ± 0.2
+	-	-	+	6	61 ± 6	11.2 ± 0.7

[a] Experimental conditions as described for Table I.

[b] Serum pools prepared from blood obtained 15 days after a 30-day booster dose of 10^3-10^6 SCHU S4 or 10^6 LVS administered SC (titer 1:40 - 1:80) or IP (titer 1:320 - 1:640).

[c] Groups of 6-10 mice/trial.

Various techniques were used to alter the relative concentrations of cellular elements prior to transfer of ISC suspensions or of splenocytes harvested 28 days after primary immunization (VSC). In freshly harvested suspensions the predominant cell population consisted of lymphocytes (94% ± 1%); direct immunofluorescent reactions indicated that 50-60% of ISC and VSC lymphocytes reacted with antithymocyte serum (ATS). The monocyte-macrophage fraction constituted 6 ± 1% of ISC or 2 ± 1% of VSC, while the granulocytic component was 0.8 ± 0.9% of ISC or 3 ± 1% of VSC. Virtually all the mononuclear cells showed direct immunofluorescent reactions with antileukocyte serum (ALS).

In vitro treatment with ATS, complement and DNAase removed 25-30% of the original lymphocyte population, i.e., 50-60% of ATS-reactive lymphocytes, had little effect on the monocyte-macrophage population, and reduced but did not eliminate the capability of ISC to transfer protection to syngeneic nonimmune recipients (Table III). In contrast, treatment with ALS effecting essentially the same overall reduction in lymphocytes and a 50-80% reduction in the monocyte-macrophage population destroyed the transfer capability of ISC. These data suggested that immune B-cells and/or immune monocyte-macrophages were also essential for protection.

TABLE III. EFFECT OF TREATMENT OF IMMUNE DONOR SPLENOCYTES (ISC) WITH RABBIT ANTIMURINE THYMOCYTE (ATS) OR ANTIMURINE LEUKOCYTE SERUM (ALS) ON THEIR CAPABILITY TO TRANSFER PROTECTION[a]

Treatment	ISC DONOR CELLS		NO. SURVIVORS/16
	Transfer Dose ($\times 10^7$)		
	Lymphocyte	Monocyte/macrophage	
Normal rabbit serum	8.2	1.4	10
ATS	6.2	1.4	6
ALS	6.0	0.7	1*

[a] ISC administered concomitant with 10^7 killed SCHU S4 organisms 20 hr before challenge with 25 SCHU S5R.

* $P < 0.01$, χ^2-Yates correction. Comparison with normal rabbit serum treatment group.

Removal of adherent cell populations (B-cells and macrophages) from VSC and ISC suspensions was effected by incubation on, or passage through, loosely packed glass wool. Approximately 90% of the B-lymphocytes (i.e., reactive with rabbit antimurine Ig) were removed by

slow passage through the column. Only 78 ± 4% of the monocyte-macrophage population adhered to glass wool but slow passage was as effective for their removal as a 1-hr attachment time. Recovery values for viable unattached T-lymphocytes decreased as incubation time increased; slow passage through the column allowed recovery of essentially all T-cells, whereas 30-min incubation resulted in approximately 25% loss and 60-min incubation, a 50% loss. In every instance, treatment of the unattached population with ATS caused lysis of 93-97% of the nonadherent cells.

At a donor:recipient ratio of 1:1, adoptive protection conferred by nonadherent ISC generally was markedly inferior to that of the original untreated population (Table IV). Even when the T-lymphocyte population was recovered with no appreciable loss (slow filtration), nonadherent ISC supplemented with Ag were significantly less protective than the original suspension. Somewhat better protection was achieved if IMS rather than Ag was used to supplement the nonadherent ISC transfer dose, suggesting that B-lymphocytes were an essential component of untreated suspensions (Table V). Experiments with VSC supported these findings. In a series of trials it was found that: (a) adoptive protection with untreated VSC suspensions was achieved only when relatively large numbers of splenocytes were transferred, (b) nonadherent VSC were nonprotective at a donor:recipient ratio of 1:1 but conferred partial protection as the ratio approached 2:1, and (c) an IMS supplement was as effective with VSC as with ISC suspensions.

TABLE IV. EFFECT OF REMOVAL OF ADHERENT LYMPHOCYTES ON THE CAPABILITY OF ISC TO TRANSFER PROTECTION TO NONIMMUNE RECIPIENTS [a]

PROTOCOL	NO. TRIALS	IMMUNE DONOR SPLENOCYTES			RECIPIENT SURVIVAL RATIO
		Treatment [b]	Mean Transfer Dose X 10^7 ± SEM		
			Lymphocyte	Monocyte/ Macrophage	
A	3	None	10.5 ± 1.2	1.1 ± 0.1	17/24
		Slow filtration	6.5 ± 1.0	0.3 ± 0.07	6/24*
B	3	None	10.9 ± 1.1	1.0 ± 0.3	21/28
		60-min attachment	3.6 ± 0.3	0.2 ± 0.11	1/28**

χ^2 analysis, Yates correction; comparison with corresponding value for untreated suspension. * $P < 0.05$, ** $P < 0.001$.

[a] ISC transferred IP (donor:recipient, 1:1) concomitant with 10^7 killed SCHU S4 organisms 20 hr before challenge with 25 SCHU S5R.

[b] ISC suspensions filtered slowly through, or incubated for 60 min on, glass wool prior to transfer.

TABLE V. PROTECTION CONFERRED BY TRANSFER OF IMMUNE SPLENOCYTES ON THEIR NONADHERENT CELLULAR COMPONENTS (GLASS FILTRATION) CONCOMITANT WITH 10^7 KILLED SCHU S4 (Ag) OR SYNGENEIC IMMUNE SERUM (IMS)

NO. TRIALS	DONOR SPLENOCYTES[a]					RECIPIENT SURVIVAL RATIO	
	Type	Treatment	Mean Transfer Dose ± SE			Ag Groups	IMS Groups
			D:R Ratio	Lymphocytes x 10^7	Monocyte-Macrophage x 10^7		
4	ISC	None	1.0 ± 0	10.2 ± 1.1	0.9 ± 0.2	24/34	9/24*
		Glass filtration	1.3 ± 0.2	6.2 ± 1.3	0.3 ± 0.09	4/44**	14/34*
3	VSC	None	1.0 ± 0	12.8 ± 0.8	0.8 ± 0.05	12/22	8/22
		Glass filtration	1.8 ± 0.1	11.2 ± 1.4	0.2 ± 0.03	6/28*	11/28

χ^2 analysis, Yates correction; compared to untreated splenocyte - Ag group. * $P < 0.05$, ** $P < 0.001$.

[a] Splenocytes harvested 28 days after primary immunization with LVS (VSC) or 10 days after a 28-day booster dose (ISC).

Spleens were harvested on day 6 and day 7-8 postchallenge from challenge-control mice and from recipients of IMS or Ag alone or in combination with untreated or filtered suspensions of ISC or VSC. Bacterial counts indicated that replication of F. tularensis was suppressed through day 6 equally well in single-factor recipients of IMS or untreated ISC or VSC and in protected or nonprotected multiple-factor recipients, i.e., 10^5-10^6/spleen as compared with 10^8/spleen in Ag or challenge-control groups. The F. tularensis count remained unchanged on day 7-8 in protected groups but was significantly higher ($P < 0.05$) in nonprotected groups. Delayed dissemination/replication would be compatible with an increased efficiency of phagocytosis due to the influence of IMS or products from activated immune B-cells. Continued partial suppression of replication in protected groups suggests interaction with immune donor macrophages, cells that are removed by filtration through glass wool.

Protection against F. tularensis var. palaearctica. Eurasian and North American water-borne strains of F. tularensis vary widely in virulence for man and lagomorphs but are as infectious for mice (LD_{50} = 1 organism) as the fully virulent F. tularensis var. tularensis. Differences in virulence, however, can be detected in studies with mice by observing the relative efficacy of various prophylactic procedures (Table VI). Passive administration of antibody was highly effective against SC and partially against IP challenge with a strain of lesser virulence (425) but was only partially protective against SC, and not at all against IP, challenge with a fully virulent Eurasian strain (503). A single injection of 10^9 killed organisms (Foshay antigen) administered 24 hr before SC challenge conferred specific protection against strain 425 but not against strain 503. More intensive immunization with this antigen, however, protected equally well against either strain 2 weeks after immunization. The immune response was inferior to that evoked by immunization with LVS as indicated by the inability of antigen-supplemented splenocytes from Foshay-protected donors to prevent lethal infection of nonimmune recipients. As was observed in studies with the SCHU S5R strain, VSC and ISC in the absence of Ag supplement were incapable of transferring protection. When supplemented with Ag, a high VSC transfer dose injected IP immediately before SC challenge protected against 100 LD_{50} of strain 425, partially against a similar dose of 503 and not against 25 LD_{50} of strain SCHU S5R; similar treatment with ISC was somewhat more effective against the SCHU challenge. In every instance requirements for defense appeared to be least critical for strain 425, somewhat greater for strain 503 and most stringent for strain SCHU S5R.

Titrations of the transfer dose for untreated VSC or ISC suspensions and for their nonadherent components indicated that immune B-lymphocytes and adherent monocyte-macrophages were not essential for protection against strain 503 (Table VII). The effective T-lymphocyte dose was 10-20% of that required for studies with SCHU S5R.

TABLE VI. PROTECTION CONFERRED BY IMMUNE SERUM, FOSHAY ANTIGEN OR IMMUNE SPLENOCYTES AGAINST CHALLENGE WITH F. TULARENSIS STRAINS BY REDUCED VIRULENCE (425, 503) AND AGAINST A FULLY VIRULENT STRAIN (SCHU S5R).

TREATMENT	TIME TO 0-HR CHALLENGE	CHALLENGE ROUTE	CHALLENGE STRAIN					
			425		503		SCHU S5R	
			LD_{50}	Survival Ratio	LD_{50}	Survival Ratio	LD_{50}	Survival Ratio
Immune serum (1:640)	-24 hr	SC	50	6/6	25	3/6	25	0/6
0.25 ml, IP	+ 4 hr	SC	50	6/6	25	1/6	-	-
Foshay antigen (killed SCHU S4)								
1 dose of 10^7 IP	- 20 hr	SC	100	11/12	100	0/12	25	0/12
	0	SC	100	2/12	100	0/12	25	0/12
3 doses of 10^9 SC	- 2 wk	SC	100	10/10	100	6/10	25	0/12
	- 2 wk	IP	100	10/10	100	0/10	-	-
Immune splencoytes[a]								
Foshay	- 20 hr	SC	-	-	25	4/10	-	-
	0 hr	SC	100	1/10	25	0/10	-	-
LVS-VSC	- 20 hr	SC	-	-	100	6/6	25	6/6
	0 hr	SC	100	6/6	100	3/6	25	0/6
LVS-ISC	- 20 hr	SC	-	-	100	6/6	25	6/6
	0 hr	SC	100	6/6	100	3/6	25	3/6

[a] Splenocytes inoculated IP concomitant with 10^7 killed SCHU S4.
Foshay: harvested 14 days after the last of 3 SC doses of 10^9 killed SCHU S4.
LVS-VSC: harvested 28 days after primary SC dose of 10^3 LVS.
LVS-ISC: harvested 10 days after a 28-day IP booster dose of 10^6 LVS.

TABLE VII. RELATIONSHIP OF ADOPTIVE TRANSFER DOSE OF IMMUNE SPLENOCYTE SUSPENSIONS OR THEIR NONADHERENT CELLULAR COMPONENTS (GLASS-FILTERED) TO THE PROTECTION CONFERRED AGAINST VIRULENT EURASIAN (503) AND NORTH AMERICAN (SCHU S5R) STRAINS OF F. TULARENSIS

NO. TRIALS	Type	Treatment	DONOR SPLENOCYTES[a]			RECIPIENT SURVIVAL RATIO AFTER CHALLENGE WITH:	
			Mean Transfer Dose				
			D:R Ratio	Lymphocyte x 10^7	Monocyte x 10^7	503 100 LD_{50}	SCHU S5R 25 LD_{50}
4	VSC	None	1.5	14	1.0	6/6	12/12
			1.0	9	0.6	16/16	4/16
			0.75	5	0.4	5/6	
			0.5	5	0.3	5/6	
		Glass-filtered	2.0	15	0.3	5/6	4/6
			1.5	10	0.25	18/18	4/18
			1.25	7	0.2	8/12	0/6
			1.0	5	0.15	9/10	1/10
3	ISC	None	1.0	11	1.0	12/12	9/12
			0.75	7	0.6	12/12	4/14
			0.5	5	0.4	6/6	
			0.25	2	0.2	6/6	
			0.1	1	0.1	2/6	
		Glass-filtered	2.0	?	0.7		8/8
			1.3	7	0.4	11/12	4/14
			0.8	5	0.3	11/12	3/6
			0.5	2	0.2	4/6	
			0.25	1	0.1	6/6	

[a] Splenocytes harvested 28 days after primary immunization with 10^3 LVS (VSC) or 10 days after a 28-day booster dose of 10^6 LVS (ISC). A portion of each suspension was passed slowly through glass wool. The splenocyte dose with 10^7 killed SCHU S4 was inoculated IP with recipients 20 hr before SC challenge. (D/R ratio, donor: recipient ratio).

Overall, the data indicate that specific antibody with nonimmune macrophages protects against a F. tularensis strain of relatively low virulence (strain 425), and nonimmune macrophages in conjunction with low numbers of specifically activated immune T-lymphocytes can protect against a virulent Eurasian strain (strain 503). In contrast, activated immune T- and B-lymphocytes and macrophages are required for defense against the fully virulent North American strains (SCHU S5R). Damage to immune T-lymphocytes (ATS treatment) or loss of adherent immune cells (glass filtration) causes a marked reduction in defensive capability. Specific antibody can substitute for adherent immune B-cells but cannot restore full competence to a nonadherent splenocyte population. A marked increase in the donor:recipient ratio is required for nonadherent immune cells to achieve an effect equal to that of the original immune population. The critical component lacking in suspensions of nonadherent cells has not been identified, but the effectiveness of stimulation with specific Ag suggests interaction with a residuum of immune macrophages. The efficiency of ISC as compared with VSC suspensions, the 20-hr interval between transfer and challenge, and the enhancement by specific antigen point to a need for a primed immune population to control the rapid replication of fully virulent organisms. The significant suppression in bacterial count effected by specific antibody may reflect a balance between replication and destruction of infecting organisms whose processing could result in production of primer material.

This project is now terminated. Further clarification of the host defense mechanism must await development of satisfactory techniques for adoptive transfer of immune macrophages and information regarding their processing of organisms and the interaction of the resultant products with other immune cells.

Publication:

Eigelsbach, H. T., and V. G. McGann. 1978. The genus Francisella, In The Prokaryotes (Editors: M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, H. G. Schlegel) Springer-Verlag, Berlin, in press.

LITERATURE CITED

1. Eigelsbach, H. T., D. H. Hunter, W. A. Janssen, H. G. Dangerfield, and S. G. Rabinowitz. 1975. Murine model for study of cell-mediated immunity: protection against death from fully virulent Francisella tularensis infection. Infect. Immun. 12: 999-1005.

2. U.S. Army Medical Research Institute of Infectious Diseases. September 1977. Annual Progress Report, FY 1977, pp. 9-18. Fort Detrick, MD.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6419	78 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
77 10 01	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	003
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(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	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	175.0
FISCAL YEAR CURRENT 79	1.0	142.9

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) Respiratory pathogens; (U) Airborne infections; (U) Mycoplasma pneumoniae; (U) Animal models

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

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) Immune responses (humoral, local, cell-mediated) are studied in Dunning-Fisher rats vaccinated by aerosol and parenteral routes with attenuated live vaccine strain (LVS) F. tularensis and subsequently challenged with aerosols of virulent SCHU-S4, F. tularensis.

25 (U) 77 10 - 78 09 - Inbred rats were vaccinated with F. tularensis, LVS by SPA, IN IP, IM or SC route. Transient bacteremia and LVS concentrations in tissues were dependent on vaccination route. A systemic infection without significant pathology occurred by day 3 which cleared in 15-28 days. Humoral agglutinins appeared by 7 days, highest in the IP group. Serum IgM levels were significantly elevated in the 3 vaccinated groups tested. After aerosol challenge with virulent organisms, SCHU S4, all nonvaccinated control rats became ill with 94% mortality; no vaccinated animals became ill or died. Although pulmonary infection was not prevented by any method of vaccination, proliferation of SCHU S4 in lungs was considerably suppressed with no histopathology.

*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

* U.S. GPO: 1974-540-843/8691

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 003: Mechanisms of Immunoprophylaxis Against Aerosol-Disseminated Respiratory Diseases

Background:

Francisella tularensis has long been considered a potential respiratory pathogen in BW against our military forces (1). This organism has also been well-characterized on the basis of aerosol stability and virulence (2). Protection against airborne tularemia ideally would be via immunoprophylaxis; much work has been reported on the protective effect of parenteral vaccination against this disease. Much more information is required, however, to define optimal methods of vaccination to induce the most effective immune defense mechanisms of the host, particularly against aerosol challenge. One approach in recent years has been to deposit antigen locally in the respiratory tract by intranasal (IN) instillation to stimulate local immunity, e.g., local secretory antibodies or cell-mediated immunity (CMI). Unfortunately, there is a paucity of data on how effective these "immune responses" may be in protecting against subsequent aerosol challenge. The research reported here describes initial experiments to study the relationships between host immune responses and protection against subsequent infections.

Progress:

Experiments were completed to determine the immunoresponsiveness of inbred Fischer 344 rats to attenuated and virulent strains of F. tularensis. The attenuated live vaccine strain (LVS) was administered by various routes; the vaccinated animals subsequently were challenged with aerosols of the fully virulent SCHU S4 strain. Early reference experiments showed this rat to be reproducibly susceptible to aerosols of SCHU S4. Over 90% of the rats died in 5-12 days after aerosol exposure to 5 logs. Another important aspect of these experiments compared the distribution and persistence of LVS in selected tissues of the animals administered the antigen by different methods. For aerogenic vaccination, rats were exposed to small particle aerosols (SPA) of LVS, thus depositing the antigen predominantly in the lung. Other rats, lightly anesthetized with Halothane, were vaccinated with 500 µl of LVS given by either IP, IM or SC inoculation or in a 20-µl volume administered by IN instillation (which is totally retained in the upper respiratory tract.) This was determined in earlier studies using carbon black as a tracer system.

Over all groups, the mean vaccination dose per animal was 5.7 log colony forming units (CFU) of LVS (range 4.5-5.6)

The frequency of infection and concentrations of LVS in selected tissues of the treated rats were measured from days 1-42 after vaccination. Rechallenge of these rats occurred on day 42. The data in Table I depicts the LVS recoveries up to assay day 14 only. LVS was recovered only rarely between 15 and 28 days. Data for control animals sham-vaccinated either IN or with aerosols of sterile broth were all negative and are not shown.

Relatively high lung concentrations (5.0 logs CFU) of LVS were obtained from days 1-14 from 100% of the SPA animals. LVS titers peaked to over 7 logs on day 3. At day 28, all lungs were cleared of LVS and remained so through day 42 when the animals were rechallenged with aerosols of SCHU S4. Most of the IP vaccinated animals (89%) also had LVS in their lungs at day 1 but at lower concentrations than in the SPA group. Frequency of infection in the IP rats diminished through 14 days (89 to 27%). LVS was not isolated from the lungs of the IN, IM, and SC rats at day 1, but 30-50% were positive on day 3, 60-100% on days 7-10. LVS concentrations did not exceed 3 logs. Lungs of all assayed animals were examined for histopathology, and the reaction of the lungs to LVS infection was minimal. Some mild lung lesions attributable to LVS were observed only for the SPA animals on day 7 when over 7 logs of LVS organisms were present.

While >6 logs of LVS were present in the spleen of 89% of the IP animals on day 1, only about 3 logs of LVS were measured at this time in the spleen of the IM rats and in 0-1 of the rats in the SPA, IN and SC groups. From days 3-10, most of the vaccinated animals had solid infections of the spleen but frequency of infection was diminishing by day 14, and all spleens were cleared of LVS by day 28. Again, the benign nature of the infection was demonstrated with the observation of only mild splenitis in some of the IP animals on day 7. Consistently higher levels of LVS were observed for this group than for the other groups.

In general, the frequency and concentration of LVS recovered from a pool of the deep cervical and mediastinal lymph nodes were similar from days 3-14 among the SPA, IN and IP groups and from day 7 on among the IM and SC rats. Of interest here was the evidence that the antigen administered in a 20-µl volume IN was not aspirated into the lung, but was transported via the draining

TABLE I. DISTRIBUTION OF LVS IN RATS VACCINATED BY VARIOUS ROUTES

VACCINATION ROUTE (log_{10} dose)	TISSUE[b]	NO. RATS POSITIVE/NO. RATS ASSAYED[a] BY DAYS				
		1	3	7	10	14
SPA (4.5)	Lung	9/ 9 (5.2)	9/ 9 (7.5)	14/14 (6.7)	9/ 9 (5.7)	14/14 (4.7)
	Spleen	1/ 9 (4.3)	7/ 7 (4.8)	14/14 (3.9)	9/ 9 (3.1)	7/14 (2.6)
	Lymph nodes	2/10 (3.0)	9/10 (3.4)	9/ 9 (3.9)	10/10 (3.2)	11/11 (3.0)
	BAW	3/ 3 (2.7)	3/ 3(>4.0)	6/ 6 (3.9)	3/ 3 (2.9)	3/ 6 (1.9)
IN (5.6)	Lung	0/10	3/10 (2.5)	11/15 (3.0)	2/12 (2.1)	2/14 (2.8)
	Spleen	0/10	9/10 (4.9)	15/15 (4.3)	6/ 9 (3.0)	11/14 (2.5)
	Lymph nodes	2/11 (2.7)	11/11 (4.9)	10/10 (4.0)	6/10 (3.4)	11/12 (3.5)
	BAW	0/ 3	0/ 3	0/ 6	1/ 3 (1.5)	0/ 6
IP (5.4)	Lung	8/ 9 (4.7)	7/ 8 (4.2)	8/12 (3.7)	3/ 5 (2.9)	3/11 (2.4)
	Spleen	8/ 9 (6.6)	8/ 8 (7.0)	12/12 (4.3)	5/ 5 (3.3)	1/11 (2.3)
	Lymph nodes	3/10 (3.2)	7/10 (3.6)	5/ 7 (3.5)	5/ 6 (3.7)	4/ 9 (2.8)
	BAW	0/ 3	1/ 3 (1.5)	2/ 5 (1.5)	1/ 3 (1.0)	0/ 6
IM (5.1)	Lung	0/ 2	1/ 2 (2.8)	1/ 2 (2.9)	1/ 2 (1.7)	1/ 7 (1.7)
	Spleen	2/ 2 (2.7)	2/ 2 (5.8)	2/ 2 (3.8)	2/ 2 (3.3)	2/ 2 (2.4)
	Lymph nodes	0/ 2	0/ 2	2/ 2 (4.2)	2/ 2 (3.3)	2/ 2 (2.0)
	BAW	0/ 2	0/ 2	0/ 2	0/ 2	0/ 2
SC (5.1)	Lung	0/ 2	1/ 2 (2.3)	2/ 2 (3.2)	2/ 2 (2.2)	1/ 2 (1.7)
	Spleen	0/ 2	2/ 2 (5.1)	2/ 2 (4.2)	2/ 2 (3.7)	2/ 2 (3.0)
	Lymph nodes	0/ 2	0/ 2	2/ 2 (3.5)	1/ 2 (3.9)	0/ 2
	BAW	0/ 2	0/ 2	0/ 2	0/ 2	0/ 2

[a] Numbers in parentheses are log_{10} mean estimates of LVS/tissue of positive animals.

[b] BAW = bronchoalveolar wash fluid.

regional lymph nodes through the lymphatics and eventually to the lungs. On day 3, all of the IN vaccinated animals had lymph nodes and lungs positive for LVS. Again, as with the other tissues, the lymph nodes for all vaccinated groups were cleared by 28 days.

Serum and bronchoalveolar wash (BAW) fluids were sampled for LVS. Since transient LVS bacteremia was observed in only some animals of the vaccinated groups, except for the IP group, the serum LVS data is not shown in Table I. LVS was recovered from the serum of 100% of the IP animals from days 1-3, indicating hematogenous spread of the organism. For all groups, however, concentrations of LVS did not exceed 2 logs CFU/ml of blood. The frequency of LVS in BAW fluids was dependent on method of vaccination. LVS was present in BAW fluid of all SPA animals through day 14 with peak concentrations of >4 logs on day 3. LVS was isolated from the BAW of 33% of the IP group days 3-10 to indicate extensive systemic infection. In contrast, LVS was not recovered from BAW of SC, IM and IN animals, except for one of the IN groups on day 10. These findings suggest lymphatic transport of the organism when administered by these 3 routes.

LVS agglutinin titers measured for the serum and the BAW fluids are presented in Table II. Regardless of method of vaccination serum antibody titers were first evident by day 7 with titers peaking by day 10 for all groups. Highest titers (1:2030) were obtained for the IP group and were lowest for the SC group (1:320). By day 42, titers for all groups decreased to 1:80-1:240. BAW agglutinins were first detected at day 7 among the SPA and IP groups, with low titers of ~ 1:20 on day 10 among IN animals (1:10). Agglutinins in BAW were no longer measurable by day 14. No LVS agglutinins were detected at any time in BAW fluids for the IM or SC group. The role of the BAW agglutinins is not known at this time.

Immunoglobulin (Ig) levels were measured for the SPA, IN and IP vaccinated groups using the Mancini radial immunodiffusion assay procedures (data for the IM and SC groups are pending). Serum IgM levels were significantly increased ($P < 0.05$) in the SP and IP groups at 7 and 10 days compared to controls. At 14 days, IgM concentrations of all 3 vaccinated groups tested significantly higher than sham-vaccinated controls. By day 28, IgM levels were down to baseline values. IgM was detected also in the BAW fluids of 50% of SPA rats and in 7% of the IP rats tested on day 7. None of the IN rats showed IgM in BAW. IgM in BAW fluids have not been observed previously; their significance on host immunity remains to be determined. In either serum or BAW fluids, no increases over baseline levels were observed for IgA or IgG.

TABLE II. SERUM AND BAW AGGLUTININ TITERS IN LVS-VACCINATED RATS

ROUTE OF VACCINATION	FLUID	NO. RATS POSITIVE/NO. RATS ASSAYED[a] BY DAYS 1	3	7	10	14	42
SPA	Serum	0/ 7	0/ 9	14/14(352)	9/9(1478)	14/14(628)	6/6(126)
	BAW	0/ 3	0/ 3	4/ 6(24)	2/3(20)	0/ 6	0/6
IN	Serum	0/10	0/10	11/15(278)	7/9(452)	20/20(666)	4/5(226)
	BAW	0/ 3	0/ 3	0/ 6	1/3(10)	0/ 6	0/5
IP	Serum	0/ 7	0/ 7	12/12(1574)	5/5(2030)	11/11(878)	5/5(240)
	BAW	0/ 3	0/ 3	1/ 3(20)	0/3	0/ 6	0/5
IM	Serum	0/ 2	0/ 2	2/ 2(905)	2/2(905)	2/ 2(452)	2/2(80)
	BAW	0/ 2	0/ 2	0/ 2	0/2	0/ 2	0/2
SC	Serum	0/ 2	0/ 2	2/ 2(80)	2/2(320)	2/ 2(226)	2/2(80)
	BAW	0/ 2	0/ 2	0/ 2	0/2	0/ 2	0/2

[a]Reciprocal geometric mean agglutinin titer of positive animals in parentheses

Sham-vaccinated control rats and 5 groups of rats vaccinated by different methods were challenged with 5.3 $\log_{10}$ CFU of virulent F. tularensis, SCHU S4 aerosols at 42 days. On days 2, 7 and 14 after aerosol challenge, selected numbers of rats from each group were killed with a lethal injection of phenobarbital and assayed (Table III).

All control rats appeared ill by 3 days after aerosol challenge with a 94% mortality occurring between days 6 and 14 for the animals not killed to obtain response data. Even though high serum antibody titers were present (1:1076), concentrations of 10^7 SCHU S4 were present in the lungs of the control rats at 7 days and animals were dying of the infection. This lack of correlation between serum antibody titers and organism concentrations in the tissues has often been reported (3). Cellular immunity is considered generally to represent the primary mediator of protection against tularemia (4).

Although no vaccinated animals died or even appeared ill by 90 days after aerosol challenge, pulmonary infection was not prevented in any immunized rats. However, peak levels of SCHU S4 organisms in the tissues of the vaccinated animals were at least 3 logs lower than those in controls.

TABLE III. RESPONSE OF LVS-VACCINATED RATS TO AEROSOL CHALLENGE WITH F. TULARENSIS (SCHU-4)

ROUTE OF VACCINATION	DAY	NO. RATS POSITIVE/NO. RATS ASSAYED[a]					AGGLUTININ TITER[b]	
		lung	spleen	nodes	serum	BAW	serum	BAW
Broth Controls	2	7/7(6.1)	3/7(5.5)	4/7(4.7)	2/7(2.4)	7/7(5.4)	0/7	0/7
	7	4/4(7.2)	4/4(4.9)	4/4(>4.0)	4/4(3.0)	4/4(>4.0)	4/4(1076)	2/4(80)
	14	1/1(5.6)	1/1(3.6)	1/2(2.6)	0/1	1/1(1.8)	1/1(1280)	1/1(20)
SPA	2	5/6(4.4)	0/6	2/6(1.9)	0/6	3/6(2.5)	6/6(112)	0/6
	7	7/7(3.9)	1/7(3.0)	5/7(3.1)	0/7	5/7(1.9)	6/6(160)	0/6
	14	5/6(3.0)	0/6	5/6(3.1)	0/6	0/6	6/6(128)	0/6
IN	2	5/6(4.6)	0/6	2/6(1.9)	0/6	2/6(2.4)	5/5(180)	0/6
	7	6/6(3.9)	1/6(2.7)	5/7(3.1)	0/7	6/6(4.1)	6/6(285)	0/6
	14	4/4(3.5)	0/4	5/6(3.1)	0/6	0/4	3/4(254)	0/6
IP	2	5/6(5.1)	0/6	1/4(2.0)	0/6	4/6(1.9)	6/6(268)	0/6
	7	6/6(3.3)	1/6(3.4)	3/6(2.8)	2/6	2/6(2.4)	6/6(252)	0/6
	14	6/6(3.4)	0/6	5/6(3.1)	0/6	3/6(2.3)	6/6(285)	0/6
IM	2	2/2(7.0)	2/2(2.2)	2/2(>4.0)	0/2	2/2(2.7)	2/2(80)	0/2
	7	2/2(4.8)	1/2(2.2)	2/2(>4.0)	0/2	2/2(3.1)	2/2(160)	0/2
	14	2/2(2.7)	0/2	2/2(3.9)	0/2	0/2		
SC	2	2/2(6.3)	1/2(2.5)	2/2(>4.0)	0/2	2/2(>4.0)	2/2(80)	1/2(20)
	7	2/2(5.5)	2/2(2.7)	2/2(>4.0)	0/2	2/2(3.7)	2/2(640)	2/2(20)
	14	0/2	0/2	2/2(4.1)	0/2	0/2	2/2(452)	1/2(10)

[a] Numbers in parentheses are $\log_{10}$ mean SCHU S4 concentrations/tissue of positive animals.

[b] Reciprocal geometric mean agglutinin titer of positive animals shown in parentheses.

All vaccinated animals were protected against pulmonary pathology. No lung lesions attributable to challenge were observed in any vaccinated group. In contrast, all control animals showed pyogranulomatous lesions in the lungs.

Infection of lymph nodes after aerosol challenge followed a pattern similar to that of the SCHU S4-infected lung. This was expected, as these regional lymph nodes drain and filter out organisms from the upper and lower respiratory tract.

Infection of the spleens was prevented among SPA and IP vaccinated groups through 14 days, but not among the IM and SC rats. However, the levels of SCHU S4 were considerably lower in the latter 2 groups than were seen in the spleens of control animals. Tularemic lesions were observed only in spleens of controls. SCHU S4 organisms were cleared from spleens of all vaccinated animals by day 14, but not from controls. The lack of spleen infections among vaccinated animals indicated that hematogenous spread of the challenge organism and, therefore, a systemic infection was effectively prevented by LVS vaccination. In support of this contention is the fact that none of the vaccinated animals had a bacteremia except for 2 unexplained IP animals on day 7. All the challenged control rats had 3 logs of circulating organisms on day 7.

Although SCHU S4 were recovered from the BAW fluids of all challenged animals at day 2, their levels were considerably lower in vaccinated groups than in controls. Except for some of the IP group, SCHU S4 was cleared from all vaccinated animals by day 14, suggesting the involvement of some local immunity. Note that for animals vaccinated with aerosols of LVS, LVS were not eliminated from the BAW fluids even 14 days after vaccination (Table I).

Also of interest in these studies of the immune response of the rat to F. tularensis infection is the lack of an anamnestic humoral antibody response in vaccinated animals following aerosol challenge. No increase in serum titers of the vaccinated animals was observed over the prechallenge titers measured on day 42. This was in contrast to the high serum agglutinin titers obtained for the challenged control animals in which >90% of these animals succumbed to challenge. This again raises the question as to the role of the humoral antibody in F. tularensis infections. However, with the rat model system, comparative studies can be pursued with immune, nonimmune, immunosuppressed and perhaps even immunopotentiated animals to further evaluate the relative importance of humoral, local and cell-mediated immunity to respiratory tularemia specifically and respiratory diseases in general.

Publications:

None

LITERATURE CITED

1. Sawyer, W. D., and P. W. Summers (ed). 1963. Defense against biological warfare--A symposium. Milit. Med. 128:81-146.

2. Sawyer, W. D., J. V. Jemski, A. L. Hogge, Jr., H. T. Eigelsbach, E. K. Wolfe, H. G. Dangerfield, W. S. Gochenour, Jr., and D. Crozier. 1966. Effect of aerosol age on the infectivity of airborne Pasteurella tularensis for Macaca mulatta and man. J. Bacteriol. 91:2180-2184.

3. Thorpe, B. D., and S. Marcus. 1965. Phagocytosis and intracellular fate of Pasteurella tularensis. III. In vivo studies with passively transferred cells and sera. J. Immunol. 94:578-585.

4. Kostiala, A. A. I., D. D. McGregor, and P. S. Logie. 1975. Tularaemia in the rat. I. The cellular basis of host resistance to infection. Immunology 28:855-869.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0E6412	78 10 01	DD-DRE-1498

3. CATEGORY	4. KIND OF SUMMARY	5. SUMMARY SECURITY	6. WORK SECURITY	7. REGRADING	8A. DISTRIBUTION INSTRUCTION	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUMMARY
78 04 21	T. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
62776A	3M162776A841	00	004

(U) Properties and clinical therapy application studies of transfer factor

12. SCIENTIFIC AREAS: 003100 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START (YR MO)	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 08	78 10	DA	C. In-house

17. CONTRACT/GRANT: a. NUMBER: NA; b. TYPE; c. EXPIRATION; d. AMOUNT; e. NO. OF AWARDS; f. CUM. AMT.

18. RESOURCES ESTIMATE	A. PROFESSIONAL MAN YRS	B. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	70.0
FISCAL YEAR CURRENT 79	0	0

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21. GENERAL USE
Foreign intelligence considered

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POC: DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Cell-mediated immunity; (U) Infectious diseases; (U) Lymphocyte transformation; (U) Transfer factor

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

23 (U) Study cell-mediated immunity (CMI) by lymphocyte transformation to microbial antigens in man and experimental animals in model infectious diseases. Assess the role of transfer factor (TF) as an immunostimulant or a specific prophylactic in infections of military medical significance and potential BW threat.

24 (U) Measure CMI in tularemia, Venezuelan equine encepalitis, Q fever and other infections. Administer TF to humans and experimental animals and measure effects on CMI and skin reactivity to model microbial marker antigens. Purify and characterize the active component(s) in TF responsible for biologic activity.

25 (U) 77 10 - 78 09 - An altered state of immune responsiveness to microbial antigens is brought about by cyclophosphamide treatment prior to immunization, resulting in abolition of humoral antibody responses along with preservation or potentiation of delayed type hypersensitivity (DTH) and cellular immune reactivity. Immunized animals with such altered reactivity show loss of protection upon challenge with VEE virus, potentiation of protection when challenged with Q fever or Rocky Mountain spotted fever, and continued susceptibility to tularemia.

In a double blind controlled trial dialysable transfer factor administration to normal volunteers did not induce DTH or CMI to tularemia antigen.

The investigator is leaving the Army. The work unit is terminated. Further work on transfer factor will be conducted under other work units.

Publications: Infect. Immun. 18:318-323, 1977; J. Infect. Dis. 138:217-221, 1978; Clin. Immunol. Immunopathol. 12: in press, 1979.

*Available from cognizant or sponsoring agency only.

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 004: Properties and Clinical Therapy Application Studies of Transfer Factor

Background:

An important role has been assigned to cell-mediated immune (CMI) responses as a determinant of protective resistance in certain infectious diseases and cancer. Recent advances in assays have made it possible to assess the CMI responses of humans and experimental animals to microbial vaccines alone or in combination with host specific and nonspecific immunomodulators. This project addresses the role of CMI in certain animal model infections which employ infectious challenges as end-points. In addition, studies in man test the effects of dialyzable transfer factor (TF) on delayed-type hypersensitivity (DTH) reactions to microbial antigens.

Progress:

Studies on the significance of alterations of CMI in a mouse tularemia model have led to several interesting conclusions. Administration of killed vaccine in combination with any one of several potent adjuvants results in the development of DTH reactivity equivalent to that seen in convalescent recipients of live, attenuated tularemia vaccine. The former animals, however, are fully susceptible to virulent challenge whereas the latter are completely protected. One hypothesis for the lack of efficacy of killed vaccine in protecting mice is that suppressor cells which are generated in the course of immunization interfere with the full expression of CMI responses (1). We have used the immunosuppressive drug, cyclophosphamide (CY), to ablate selectively this suppressor cell activity and find only a slight increase in resistance of mice so prepared. We conclude that suppressor activity, although present, has very little biological significance in limiting the efficacy of killed vaccine in tularemia and that other factors such as nonspecific macrophage activation are probably more important determinants of active resistance than specific CMI in this model.

The selective nature of the immunosuppression produced by CY is such that animals pretreated with one large dose of CY and immunized develop vigorous DTH and CMI reactivity in the absence of both suppressor B cells and circulating antibody activity for a period of time. Using this technique we have now conclusively shown that the protective factor in immunization with VEE virus vaccine is antibody. This is based on studies in mice and guinea pigs in which animals with intense DTH and CMI are fully susceptible to infection when the antibody component of the immune response has been ablated by CY. This is the first direct demonstration of the role of antibody in this infection employing the tool of selective immunosuppression by CY and settles a confusing issue (2, 3).

The same general procedures have been employed in assessing the protective component of the immune responses to two rickettsial vaccines derived from Rickettsia rickettsii (cause of RMSF) and Coxiella burnetii (cause of Q fever). In contrast to the findings with VEE, the protection afforded by inactivated rickettsial vaccines is resistant to, and sometimes potentiated by, CY pretreatment. The animals at time of challenge have intact CMI reactions but no circulating antibody detectable, as in the case of VEE. This interesting model will allow us to test candidate immunopotentiators under conditions where potentiation of humoral antibody responses can be eliminated. The importance of CMI in rickettsial infections is not surprising but these studies again give some of the first direct evidence for it without having to resort to cumbersome adoptive cell transfer techniques.

Studies on the human immune response to microbial vaccines have included long-term follow-up of prior recipients of VEE and RMSF vaccine and at present are being conducted in support of a high-dose trial of RMSF vaccine.

A study on the effects of TF on the immune responses of humans has been conducted this past year. To test adequately the unanswered question regarding the mode of action of TF, that of specificity or nonspecificity, we have employed two unrelated antigenic markers, keyhole limpet hemocyanin (KLH) (4) and tularemia. TF donors were bled prior to and after immunization, one with KLH and 2 with live tularemia vaccine. These procedures resulted therefore in 6 different TF preparations which were then tested in 7 recipients each. There were no untoward reactions to TF or changes in clinical laboratory studies detected. To date there has been no activity detected in the TF recipients which would be manifest as conversion of DTH skin reactivity or in vitro lymphocyte reactivity from negative to positive. The dosage of skin tests to assess DTH after TF is being altered in an additional group of recipients. This is the first time that a double-blind controlled trial of TF in normal volunteers with defined mouse antigenic markers has ever been done.

The principal investigator is departing to assume a position at the University of California. The studies in this work unit will be continued by other means.

Presentations:

1. Archer, M. F., R. Edelman, C. N. Oster, H. E. Ramsburg, and G. J. Tilden. Further immunologic evaluation of inactivated Venezuelan equine encephalomyelitis (VEE) vaccine in man. Presented, 26th Annual Meeting, American Society of Tropical Medicine and Hygiene, Boston, MA, 9-11 Nov 1977.

2. Ascher, M. S. Modulation of cell-mediated immunity to microbial vaccines. Presented, Johns Hopkins University Immunology Council, Baltimore, MD, 8 Mar 1978.

Publications:

1. Ascher, M. S., D. Parker, and J. L. Turk. 1977. Modulation of delayed-type hypersensitivity and cellular immunity to microbial vaccines: effects of cyclophosphamide on the immune response to tularemia vaccine. Infect. Immun. 18:318-323.

2. Andron, L. A., and M. S. Ascher. 1978. Transfer factor in vitro: chromatography of components that enhance antigen-induced lymphocyte proliferation. Clin. Immunol. Immunopathol. 9:157-165.

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1. Ascher, M. S., D. Parker, and J. L. Turk. 1977. Modulation of delayed-type hypersensitivity and cellular immunity to microbial vaccines: effects of cyclophosphamide on the immune response to tularemia vaccine. Infec. Immun. 18:318-323.

2. Rabinowitz, S. G., and W. H. Adler. 1973. Host defenses during primary Venezuelan equine encephalomyelitis virus infection in mice. I. Passive transfer of protection with immune serum and immune cells. J. Immunol. 110:1345-1353.

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4. Burger, D. R., A. A. Vandenbark, P. Finke, and R. M. Vetto. 1977. De novo appearance of KLH transfer factor following immunization. Cell. Immunol. 29:410-413.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6420	78 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
78 04 21	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	62776A	3M162776A841	00	006
b. CONTRIBUTING				
c. ~~XXXXXX~~/ STOG 78-7.2.1, 3, 6				

11 TITLE (Precede with Security Classification Code)

(U) Immunochemical studies with the nonindigenous tick-borne rickettsiae

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	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		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	0.7	100.0
	CURRENT 79	0.5	280.4

19 RESPONSIBLE DOD ORGANIZATION

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21 GENERAL USE

Foreign intelligence considered

POC:DA

22 KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Immunology; (U) Vaccines; (U) Tick-borne rickettsiae; (U) Laboratory animals; (U) Biochemistry

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

23 (U) Develop vaccines of low reactogenicity for immunoprophylaxis against specific rickettsial diseases of military importance. Demonstrate the potential of components of tick-borne rickettsiae to protect against disease caused by members of this group. This program will encompass studies vital for military defense against the biological warfare potential of tick-borne rickettsiae.

24 (U) Study pathogenesis of the tick-borne typhus group rickettsiae and define suitable animal models for the evaluation of vaccine efficacy. Produce tissue-culture grown rickettsiae; isolate, purify and define various immunogenic components for the production of vaccines. Study cell-mediated and humoral immune responses to vaccines prepared from inactivated or subunit vaccines in suitable animal models of disease.

25 (U) 77 10 - 78 09 - Studies in guinea pigs showed that our CEC-grown RMSF vaccine protects against strains of R. rickettsii of diverse geographical origin. The immunological responses of guinea pigs to R. conorii infection were monitored and shown to be similar to those reported for R. rickettsii. Studies showed that spotted fever "group" protection can be elicited after infection with any one spotted fever group rickettsia, or by inactivated rickettsiae if sufficient numbers are used. Results of studies in BALB/c mice as a model of immunity to the spotted fever group are presented. Experimental R. conorii and R. sibirica infections in various subhuman primate species were examined. Studies on the genetic basis for susceptibility to R. akari in mice are reported.

Publications: Infect. Immun. 18:840-846, 1977; N. Engl. J. Med. 297:859-863, 1977; J. Clin. Microbiol. 8:102-104, 1978; Arch. Intern. Med. 138:735-738, 1978; Nutr. Rep. Int. 18:57-68, 1978; Infect. Immun. 22: in press, 1978.

*Available to contractors upon originator's approval

DD FORM 1498 PREVIOUS EDITIONS ARE OBSOLETE

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 006: Immunochemical Studies With the Nonindigenous Tick-Borne Rickettsiae

Background:

Included among the tick-borne rickettsial diseases are Rocky Mountain spotted fever (RMSF), the tick-borne rickettsiosis of the eastern hemisphere, and rickettsial pox (1). RMSF, caused by Rickettsia rickettsii, found in the United States, is the most severe of these diseases. Rickettsia conorii, the etiological agent of fievre boutonneuse, South African tick bite fever and Indian and Kenyan tick typhus, causes a severe nonfatal infection, and is prevalent throughout parts of Europe, Africa and the Mediterranean basin. North Asian tick-borne typhus of Russia and Mongolia (Rickettsia sibirica) also causes a severe nonfatal infection. Queensland tick typhus (Rickettsia australis) is a moderately severe tick-borne infection found in Australia, and rickettsialpox (Rickettsia akari) is a relatively benign mite-borne infection found in the United States and Russia. Due to the possibility of potential military traffic in selected geographical areas, R. rickettsii, R. conorii, R. sibirica should be considered of military concern. Neither R. australis, due to its geographical isolation, nor R. akari, due to low virulence and incidence, is of military significance.

A chick embryo cell culture-grown vaccine against R. rickettsii was prepared (2), and is under test in man. A prototype vaccine prepared from R. rickettsii and R. conorii was evaluated in guinea pigs and found to offer protection against the militarily significant spotted fever members. In concert with vaccine preparation, evaluation of immune responses to vaccination and infection is under investigation. Such studies in mice are presently focused on R. akari infection, which is lethal without "toxic action" (3).

Elucidating the genetic basis for varying degrees of natural resistance to infectious diseases is an area of increasing interest. Such resistance is important not only when animal models are developed to test the presumptive pathogenesis of a disease in man, but as a key to why an animal is susceptible to a particular disease. There appears to be a relationship between susceptibility of mice to a lethal rickettsial challenge and their genetic background. Studies have been initiated in mice using R. akari to explore such a genetic basis for susceptibility.

Progress:

A study was performed to determine whether our chick embryo cell (CEC)-grown RMSF vaccine protects against R. rickettsii of diverse

geographical origin. Guinea pigs were vaccinated and challenged 30 days later with yolk sac-grown rickettsiae. All strains of R. rickettsii had been isolated from human cases of RMSF. Protection was determined as a function of decrease in areas under fever curve. Results (Table I) indicate that our vaccine protects against all tested strains.

TABLE I. PROTECTION FROM STRAINS OF R. RICKETTSII OF DIVERSE GEOGRAPHICAL ORIGIN AS A FUNCTION OF IMMUNIZATION WITH LOT I CEC-GROWN RMSF VACCINE.

CHALLENGE STRAIN	GEOGRAPHICAL ORIGIN	PROTECTION	
		VACCINATED	NON-VACCINATED CONTROLS
Hansen	Montana	+[a]	-
Cape Cod	Massachusetts	+	-
Gladys Hill	Virginia	+	-
Swann	Montana	+	-
Sheila Smith[b]	Montana	+	-

a. + = protection, - = no protection
b. Strain used for vaccine preparation

Since R. conorii has military significance due to its wide geographical distribution, immune responses to infection in guinea pigs were examined and are summarized in Table II. There was no detectable rickettsemia found at any time after infection. Guinea pigs showed only marginal signs of illness (2 or 3 days of temperature 39.9-40.5°C) and slight scrotal swelling. The same guinea pigs were used for rickettsemia and all immunological determinations except macrophage migration inhibition (MMI). Similarly to those previously reported using R. rickettsii, microagglutinating (MA) and indirect fluorescence antibody (IFA) titers first appeared by about 3 days, reached a peak at 2-3 weeks, and fell off gradually through day 90, the last time tested. Lymphocyte transformation (LT) and MMI were used as measures of cell mediated immunity (CMI).

TABLE II. IMMUNE RESPONSE OF GUINEA PIGS TO R. CONORII INFECTION.

TIME AFTER INFECTION (days)	RECIPROCAL TITER MA (N=4)	IFA (N=4)	LT (N-4)	MICROPHAGE MIGRATION INHIBITION[b] (N=3)
-1	<4	<8	<2.0	<10
3	3	4		
5	18	96		
7	28	112	3.5	<10
10	80	256		
14	128	448	4.3	74
18	160	512		
21	128	768	8.4	72
31	136	544	9.3	
52	128	192		
90	96	288	3.4	<10

a. Stimulation index = $\frac{\text{cpm with antigen}}{\text{cpm no antigen}}$

b. % migration = $\frac{\text{No. units migration in presence of antigen}}{\text{No. units migration with no antigen}} \times 100.$

As found previously with R. rickettsii, LT was detected by 1 week, reached a peak at 2-4 weeks, and fell off thereafter. However, magnitude of LT response was not nearly as great with R. conorii as found with R. rickettsii. This may be a function of severity of disease, or magnitude of antigenic mass presented during disease. With RMSF in guinea pigs, circulating rickettsemia of 10^2 - 10^3 organisms/ml blood are routinely found. Measureable MMI appeared at 2 and 3 weeks with R. conorii infection, and disappeared thereafter. This, too, is similar to that found with R. rickettsii infection, but inhibition was greater in response to RMSF than to R. conorii infection.

A study was performed to determine the extent of heterologous protection to challenge after infection with each of the most militarily important spotted fever group rickettsiae. Results are shown in Table III. It appears that there is considerable, but not absolute spotted fever "group" protection after infection with any one member. R. rickettsii appears to be the most severe challenge, and complete protection was afforded only by homologous vaccine or by its subgroup member, R. sibirica, vaccine.

TABLE III. PROTECTION[a] OF GUINEA PIGS AGAINST HETEROLOGOUS CHALLENGE WITH SPOTTED FEVER GROUP RICKETTSIAE

CHALLENGE RICKETTSIA	PROTECTION[b] OF CONVALESCENT GUINEA PIGS TO RICKETTSIA					
	akari	australis	conorii	rickettsii	sibirica	none
akari	+	+	+	+	±	0
australis	+	+	+	+	±	0
conorii	+	+	+	±	+	0
rickettsii	±	±	±	+	+	0
sibirica	+	+	+	+	+	0

a. Protection measured as absence of fever after challenge.
b. + = protection, ± = partial protection, 0 = no protection

A second study was undertaken to determine if substantial heterologous protection could be stimulated by inactivated rickettsiae as was shown to occur after infection with live organisms. Guinea pigs were immunized with various concentrations of formalin-inactivated rickettsiae as presented in Table IV and challenged 4 weeks later with $\sim 10^6$ yolk sac-grown R. rickettsii. Immunization with concentrated (10^9) rickettsiae from any of the 5 species offered protection against R. rickettsii. This indicates that, as with live infection, group protection can be attained by inactivated rickettsiae as long as sufficient antigenic mass is employed.

TABLE IV. EFFECT OF CONCENTRATION[a] OF INACTIVATED SPOTTED FEVER GROUP RICKETTSIAE ON PROTECTION AGAINST R. RICKETTSII

Immunizing Rickettsiae	Protection[b] Afforded by Various Concentrations Concentration of Rickettsiae[1]			
akari	10^9	10^7	10^5	10^3
australis	±	0	0	0
conorii	+	±	±	0
rickettsii	+	+	±	±
sibirica	+	+	±	0

a. Concentration determined by direct count with acridine orange dye.
b. Protection is defined as absences of significant fever after challenge. + = full protection; ± = partial protection; 0 = no protection.

Studies in BALB/c mice as a model of immunity to the spotted fever group of rickettsiae were conducted. Mice were inoculated with various concentrations of viable spotted fever rickettsiae and challenged at 21 days with 10^4 PFU R. akari. Results are shown in Table V. It appears that "infection" with R. conorii, R. sibirica, R. rickettsii, or R. australis protects against R. akari, and from the PFU necessary to confer immunity, rickettsial replication must occur.

TABLE V. EFFECT OF PRIOR IMMUNIZATION WITH VARYING DOSES OF VIABLE SPOTTED FEVER RICKETTSIAE ON SUBSEQUENT CHALLENGE WITH R. AKARI (10^4 PFU)

IMMUNIZING RICKETTSIAE	LOG_{10}PFU/MOUSE	NO. SURVIVORS/5
conorii	6	5
	5	5
	4	4
	3	4
	2	3
	1	3
	0	0
sibirica	5	ND
	4	3
	3	4
	2	4
	1	3
	0	0
australis	6	ND
	5	5
	4	5
	3	5
	2	5
	1	5
	0	1
rickettsii	6	4
	5	4
	4	4
	3	4
	2	3
	1	0
None	-	0

Since mice with prior exposure to any spotted fever group rickettsiae were subsequently refractory to R. akari challenge, passive protection against R. akari infection with mouse or guinea pig spotted fever group immune serum was tested. Results (Table VI) show that passive protection can be transferred by only R. akari or R. australis immune serum. Species source of immune serum does not seem critical. It has been difficult to attain mouse R. australis antiserum of reasonable titer, which may explain why mouse antiserum does not passively protect as is seen with guinea pig antiserum.

TABLE VI. PASSIVE PROTECTION AGAINST R. AKARI INFECTION IN MICE BY SPOTTED FEVER GROUP IMMUNE SERA.

IMMUNE SERUM TO RICKETTSIA	NO. SURVIVORS/TOTAL AFTER IMMUNE SERUM[a]	
	Guinea Pig	Mouse
akari	3/4	4/4
australis	4/4	0/3
conorii	0/4	0/2
rickettsii	0/4	0/2
sibirica	0/4	0/2
None	0/6	

a. Prepared in guinea pigs or mice

Since protection against R. akari could be conferred with only R. akari and possibly R. australis antiserum, an experiment was performed to attempt spleen cell transfer of immunity. Mice were infected with 10^4 PFU of each spotted fever member. $5x10^7$ viable spleen cells were transferred 18 hr before challenge with R. akari. Results are shown in Table VII. It is seen that immunity is transferred by spleen cells from R. akari-convalescent mice, but not from spleen cells from any other convalescent mice.

The next experiment was performed to determine whether spotted fever group immune spleen cells plus homologous mouse immune serum would protect against R. akari challenge. Mice were injected IP with 0.7 ml of immune serum (MA titer $\geq$1:32) and ½ hr later were injected with $5x10^7$ homologous immune spleen cells. At 20 hr all mice were challenged with 10^4 PFU R. akari. Results (Table VIII) show that no protection was transferred by

TABLE VII. EFFECT OF SPOTTED FEVER IMMUNE SPLEEN CELL TRANSFER ON SUBSEQUENT CHALLENGE WITH R. AKARI

SPLEEN CELLS FROM MICE CONVALESCENT[a] FROM RICKETTSIA	DAYS POSTINFECTION OF SPLEEN CELL TRANSFER	NO. SURVIVORS AFTER R. AKARI CHALLENGE
akari	4	4/5
	7	3/3
	14	4/5
	21	5/5
	28	5/5
	53	3/3
australis	4	0/5
	7	0/3
	14	1/3
	21	2/5
	28	0/5
	53	0/3
conorii	4	0/5
	7	0/4
	14	0/3
	21	0/5
	28	0/3
	53	0/3
sibirica	4	0/5
	7	0/3
	14	0/3
	21	1/5
	28	1/2
	53	0/2
rickettsii	4	0/5
	7	0/4
	14	0/3
	21	0/5
	28	0/5
	53	0/4

a. Donor mice were rescued with 2 IM doses of oxtetracycline on days 2 and 3. Experiments ascertained that protection in recipient mice was not due to transferred antibiotic.

immune cells plus antiserum. Experiments are planned to determine if higher titered antiserum or lymphocytes from a source other than spleen will transfer immunity.

Efforts were made to determine whether protection by R. akari immune spleen cells was due to passive transfer of antibody. Immune spleen cells were harvested, washed, and divided into 2 aliquots. One aliquot was transferred to recipient mice, and the other was frozen and thawed 3 times, and then transferred. All whole cell recipients lived and all treated cell recipients died. Further studies are necessary to determine whether transfer of immunity is due to T cells or to B cells producing antibody in the recipients.

A study was performed to determine whether spleens from mice immunized with killed R. akari could transfer immunity to syngeneic recipients. Mice were immunized with $\sim 10^8$ killed rickettsiae; spleens were removed and transferred on day 14. After challenge, recipient mice survived while nonrecipient controls died.

TABLE VIII. EFFECT OF SPOTTED FEVER IMMUNE SPLEEN CELLS PLUS IMMUNE SERUM ON PASSIVE PROTECTION AGAINST R. AKARI

SPLEEN CELLS PLUS ANTISERUM FROM MICE CONVALESCENT FROM RICKETTSIA	NO. SURVIVORS/TOTAL
akari	4/4
australis	0/3
conorii	0/4
rickettsii	0/3
sibirica	0/4
None	0/4

Studies on the effects of antibiotic treatment of R. akari infection in mice and of oral versus IM administration were completed. Results are shown in Table IX. Using 10^4 PFU for infection, it appears to be cruicial that treatment be initiated before day 5, but little difference was evident between chloramphenicol or tetracycline treatment and IM or oral routes. Further studies are planned to determine the relationship of development of immunity to challenge and the ability to transfer this immunity with spleen cells.

TABLE IX. EFFECT OF CHLORAMPHENICOL AND TETRACYCLINE ON R. AKARI INFECTION IN MICE

DAY OF TREATMENT	NO. SURVIVORS/4			
	ORAL		IM	
	TETRACYCLINE	CHLORAMPHENICOL	TETRACYCLINE	CHLORAMPHENICOL
0	4	4	4	4
3	4	4	4	3
4	3	4	4	2
5	0	0	0	4
6	0	0	0	1
7	0	0	0	1
None				

Studies of R. conorii and R. sibirica infection in subhuman primates were completed and results are summarized in Tables X and XI. The rhesus monkey (Macaca mulatta) appears to be the best model for R. sibirica infection. Rhesus monkeys infected with 10^7 PFU showed overt signs of clinical illness, anorexia, depression and fever. Slight leukocytosis due to a neutrophilia was noted early in the illness; increases in serum alkaline phosphatase values were noted in the convalescent stage. Antibody titers were pronounced by day 21. The best model for R. conorii infection appears to be cynomolgus monkeys (Macaca fascicularis). When infected with 10^7 PFU, anorexia, depression and fever were noted.

TABLE X. R. CONORII INFECTION IN SUBHUMAN PRIMATES

SPECIES	NO.	DOSE (LOG_{10} PFU)	NO. WITH FEVER (%)	NO. WITH ESCHAR (%)	NO. WITH ANOREXIA, DEPRESSION
M. mulatta (rhesus)	4	7	4 (100)	0	4
M. fascicularis (cynomolgus)	5	7	5 (100)	2 (30)	5
	2	3	0	0	0
M. nemestrina (pigtail)	4	5	1 (25)	1 (25)	
Saimiri sciureus (squirrel)	3	7	1 (33)	2 (66)	1
	3	3	2 (66)	1 (33)	2
Cebus apella (capuchin)	4	5	4 (100)	1 (25)	0
Callithrix jaccus (marmoset)	4	5	4 (100)	3 (75)	2

TABLE XI. R. SIBIRICA INFECTION IN SUBHUMAN PRIMATES

SPECIES	NO.	DOSE (LOG_{10} PFU)	NO. WITH FEVER (%)	NO. WITH ESCHAR (%)	NO. WITH ANOREXIA, DEPRESSION
M. mulatta	4	7	4 (100)	1 (25)	4
M. fascicularis	2	7	1 (50)	0	1
M. nemestrina	4	5	2 (50)	2 (50)	0
S. sciureus	3	7	3 (100)	1 (33)	2
C. apella	3	4	2 (66)	2 (66)	0
C. jaccus	4	5	1 (25)	3 (75)	2

Studies were undertaken to determine susceptibility of different inbred, outbred, and inbred hybrid strains of mice to R. akari. Since initial studies showed the Kaplan strain was considerably more virulent in BALB/cJ mice than the Hartford strain, the Kaplan strain was used for this study. The $MILPD_{50}$ has been tentatively determined for 13 inbred, 3 inbred hybrids, and 4 outbred strains as shown in Table XII.

TABLE XII. SUSCEPTIBILITY OF MICE TO KAPLAN STRAIN R. AKARI ($MIPLD_{50}$)

INBRED	$MIPLD_{50}$ (PFU)	INBRED HYBRID	$MIPLD_{50}$ (PFU)	OUTBRED	$MIPLD_{50}$ (PFU)
BALB/cJ	2,000	C3D2F	11,250	ICR Dub	>26,000
BALB/cDub	6,578	$CB6F_1$	20,148	ICR WRAIR	>78,000
C3H/HeJ	<3	$AKD2F_1$	24,563	Swiss MBA	4,430
C3H/HeDub	17,342			CAW/CFI	111
C57L/J	29,000				
CBA	24,563				
DBA.2J	2,586				
SWR/J	8,120				
AKR/J	2,900				
C57BL/6J	166				
SJL/J	80				
A/HeJ	<3				

Presentations:

1. Kenyon, R. H. Current status of CEC-grown Rocky Mountain Spotted Fever Vaccine. Presented at N.I.A.I.D. Special Conference on Rocky Mountain spotted fever, Dec. 1977, Bethesda, MD.

Publications:

1. Kenyon, R.G., M.S. Ascher, R.A. Kishimoto, and C.E. Pedersen, Jr. 1977. In vitro guinea pig leukocyte reactions to Rickettsia rickettsii. Infect. Immun. 18:840-846.

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1. Woodward, T.E., and E.B. Jackson. 1965. Spotted fever rickettsiae. pp. 1095-1129. In Viral and Rickettsial Infections of Man, 4th ed. (F. L. Horsfall, Jr. and I. Tamm, eds.). J.B. Lippincott Co., Philadelphia.

2. Kenyon, R.H., and C.E. Pedersen, Jr. 1975. Preparation of Rocky Mountain spotted fever vaccine suitable for human immunization. J. Clin. Microbiol. 1:500-503.

3. Bell, E. J., and E. G. Pickens. 1953. A toxic substance associated with the rickettsias of the spotted fever group. J. Immunol. 70:461-472.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6419	78 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
78 04 21	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO. [illegible]	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	007
b. CONTRIBUTING				
c. //////////	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code) (U) Immunoprophylactic role of macrophages in airborne, rickettsial 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
75 07	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	145.0
FISCAL YEAR CURRENT 79	1.0	192.2

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 (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Respiratory diseases; (U) Macrophages; (U) Coxiella burnetii; (U) Phagocytosis; (U) Animal models

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

23 (U) Determine the role of macrophages as a host defense mechanism against infectious disease and to investigate means of enhancing host defenses by stimulation of the macrophage system or modification of cellular and humoral immune responses. This research is essential for developing approaches to successful immunoprophylaxis against this recognized BW threat and the treatment of casualties in BW operations.

24 (U) Study the ability of peritoneal macrophages from normal and immune guinea pigs to phagocytize and destroy Coxiella burnetii in vitro; then conduct similar studies with alveolar macrophages. To study the role of cellular and humoral immunity in improving host response against respiratory disease.

25 (U) 77 10 - 78 09 - Athymic nude mice and their normal euthymic litter-mates were infected by SPA of C. burnetii. Euthymic animals cleared rickettsiae from peripheral circulation and spleen within 14 days, whereas, organisms were isolated from spleen and blood of athymic mice through day 60. A cynomolgus monkey model was developed for Q fever. Infection in monkeys resembles Q fever in man including clinical illness, hematological and physiological changes, antibody production, and interstitial pneumonia. Studies to evaluate a new killed particulate, phase I Q fever vaccine are in progress. Transformation of peripheral blood lymphocytes from cynomolgus monkeys infected with C. burnetii was suppressed at 14-28 days. In contrast, production of specific humoral antibodies was not diminished during the acute and early convalescent stages of infection.

Publications: Infect. Immun. 19:194-198, 22: in press, 1978
Proc. Soc. Exp. Biol. Med. 158:626-630,1978
J. Infect. Dis., in press, 1979.

*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 MAY 69, FOR ARMY USE, ARE OBSOLETE. * U.S. GPO 1974-540-843/8691

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 007: Immunoprophylactic Role of Macrophages in Airborne, Rickettsial Respiratory Diseases

Background:

The continuing objectives of the research under this work unit were to: (a) assess further the relative importance of cellular and humoral immune systems in experimental Q fever infections utilizing the congenitally athymic nude (nu/nu) mice and their phenotypically normal (nu/+) euthymic litter-mades and (b) develop a subhuman primate model for Q fever infection with the ultimate goal of testing the efficacy of Q fever vaccines.

Progress:

Outbred Swiss (nu/nu) athymic mice and their phenotypically normal euthymic (nu/+) litter-mates were exposed to 10^4 MIPID$_{50}$ of phase I Henzerling strain of Coxiella burnetii presented in small-aerosol particles (SPA) as previously described (1).

No clinical signs of illness (fever, weight loss, lethargy, coryza) or mortality were observed in any of the athymic or euthymic mice following exposure to C. burnetii. Spleen weights began to increase on day 14 in both types of mice (Table I). However, spleen weights of euthymic mice declined to normal levels by day 21, whereas, spleen weights of athymic mice continue to increase: by day 30 spleens were ∿11 times greater, and on day 50, ∿5 times greater than controls.

TABLE I. SPLEEN WEIGHTS AND IMPRESSION SMEARS OF ATHYMIC AND EUTHYMIC MICE (n = 3-5) FOLLOWING CHALLENGE WITH C. BURNETII

DAYS AFTER EXPOSURE	SPLEEN WEIGHT (gm)		IMPRESSION SMEARS	
	Athymic	Euthymic	Athymic	Euthymic
1	0.1	0.1	-	-
3	0.1	0.1	-	-
5	0.2	0.2	-	-
7	0.2	0.2	+	+
9	0.3	0.3	+	+
14	0.5	0.4	+	+
21	0.6	0.1	+	-
30	1.1	0.1	+	-
60	0.5	0.1	+	-

Spleens from both strains of mice contained rickettsia between 7 and 14 days as shown by the impression smears stained with Giménez stain (Table II). Rickettsiae were no longer detected in euthymic animals on day 21, but populations of organisms that were too numerous to count were observed in each field of view in athymic mice for 60 days at which time the experiment was terminated. Titration of infected spleens in yolk sacs of embryonated eggs indicated that the spleens of athymic mice contained approximately 10^5-10^6 rickettsiae at 30 and 60 days, whereas, no rickettsiae were recovered from euthymic animals at these times.

Rickettsiae were isolated from blood of both athymic and euthymic mice on day 4, but were no longer detected in euthymic mice by 21 days (Table II). In contrast, rickettsemia persisted in athymic mice through day 60.

Antibody to phase I and II C. burnetii developed at the same rate in both strains of mice (Table II). Antibody against phase II antigen was initially detected on day 14 and persisted at elevated titers through day 60. Antibody to phase I antigen was demonstrable by 21 days and persisted at high levels. Uninfected control athymic and euthymic mice did not develop antibodies to either antigen.

Mild to moderate multifocal to diffuse interstitial pneumonia was noted between 7 and 14 days in both strains of mice. Severity of lesions and the temporal course of the infections were similar in both strains up to 21 days. Lung lesions were resolved by day 30 in euthymic mice. However, minimal interstitial pneumonia was still present in 5 athymic mice on day 60. At no time were lesions observed in any euthymic mice.

Moderate multifocal and acute hepatitis was noted on day 7 with moderate, multifocal, necrotizing hepatitis between 9 and 14 days in both strains of mice. The liver of euthymic mice was essentially normal by 60 days, whereas moderate necrotizing hepatitis with severe hepatocellular necrosis was still noted in athymic animals.

Lung, liver, spleen and heart of control athymic and euthymic mice were essentially normal at all sampling times.

Table II. ONSET AND DURATION OF RICKETTSEMIA AND INDIRECT FLUORESCENT ANTIBODY (IFA) TITERS OF ATHYMIC AND EUTHYMIC MICE (n = 3-5) FOLLOWING CHALLENGE OF C. BURNETII

DAYS AFTER EXPOSURE	RICKETTSEMIA[a]		(IFA TITER (LOG_{10} TITER) ATHYMIC		EUTHYMIC	
	Athymic	Euthymic	Phase I	Phase II	Phase I	Phase II
1	–	–	0	0	0	0
4	+	+	0	0	0	0
5	+	+	0	0	0	0
7	+	+	0	0	0	0
9	+	+	0	0	0	0
14	+	+	0	1.5	0	1.5
21	+	-	1.0	2.0	1.0	2.0
30	+	-	1.0	3.0	1.0	3.0
60	+	-	2.0	3.0	2.0	3.0

In a separate effort, studies were initiated to establish a well-defined model for Q fever infection in subhuman primates with the ultimate objective of evaluating a prototype vaccine. Ten conditioned cynomolgus monkeys (Macaca fascicularis) of both sexes, weighing 2.0-3.5 kg were exposed by SPA to 10^5 $MIPID_{50}$ C. burnetii, phase I (2); 2 of these were used for histopathologic examination; four monkeys were exposed to heart infusion broth as controls.

All monkeys exposed to C. burnetii developed clinical signs of illness (fever, increased respiratory rate, cough, anorexia, and depression) between days 5 and 12. All monkeys were rickettsemic between 3 and 13 days; with increases in serum alkaline phosphatase, SGOT, and total bilirubin. There was radiological evidence of pneumonia in all monkeys by day 9; there was microscopic evidence of interstitial pneumonia and subacute hepatitis in the 2 necropsied monkeys. Antibodies to phase I and II C. burnetii were detectable in all infected monkeys (Table III).

Table III. HUMORAL ANTIBODY RESPONSES OF CYNOMOLGUS MONKEYS (n=6) FOLLOWING EXPOSURE TO 10^5 C. BURNETII PRESENTED AS SPA

	GEOM. MEAN RECIPROCAL ANTIBODY TITER					
	IFA		MA		CF	
DAY	Phase I	Phase II	Phase I	Phase II	Phase I	Phase II
-7	0	0	0	0	0	0
7	0	306[a]	0	7	0	0.5
14	48	2487	19	733	0	55
21	351	7169	470	1070	0	309
28	240	6827	496	1070	6	263
35	405	5461	664	1387	8	389

To date, 16 cynomolgus monkeys have been immunized with the NDBR-105, Lot 4, killed, particulate, phase I Q fever vaccine (3). The immunization schedule was the same as that recommended for humans: skil test (0.02 µg/0.1 ml) and day 0, vaccinated with 0.5 ml (30 µg) on day 7, and skin test at 28 days after vaccination.

Eight of the monkeys were challenged in July (1 yr post-vaccination) and the other 8 in August (6 mon postvaccination) with 10^5 $MIPID_{50}$ of the phase I Henzerling strain of C. burnetii administered in SAP to test the protective efficacy of the vaccine. Results are pending.

During development of the subhuman primate model for Q fever infection, we noted a suppression of lymphocyte blastogenesis in the acute period. Other investigators have reported a similar suppression of lymphocyte blastogenesis in acute bacterial and viral infections. A study was undertaken to examine the cellular and humoral immune responses following infection of cynomolgus monkeys with C. burnetii. The functional activity of T-lymphocytes was measured by in vitro proliferative responses to phytohemagglutinin (PHA) and specific antigens; B-cell activity was measured by the production of specific antibodies.

Six cynomolgus monkeys were exposed by SPA to 10^5 $MIPID_{50}$ of the phase I Henzerling strain of C. burnetii using the procedure described by Berendt (2). Peripheral blood lymphocytes were separated using the technique of Böyum (4); in vitro lymphocyte transformation (LT) assay was performed as previously described (5).

Preliminary data indicated that lymphocytes from normal, uninfected monkeys cultured as a 1:10 dilution of the PHA had stimulation indices (SI) between 22 to 52. The SI of lymphocytes following culture in PHA or phase I or II C. burnetii is shown in Table IV. Between 14 and 28 days there was a significant reduction in LT by PHA. Lymphocytes became responsive to PHA again on day 35 with a concomitant increase in the SI when cultured with specific antigen.

TABLE IV. LYMPHOCYTE STIMULATION INDEX OF MONKEYS (n = 6) AFTER CHALLENGE WITH C. BURNETII

AFTER EXPOSURE	SI (±SE)		
	PHASE I	PHASE II	PHA
0	0.9 ± 0.1	1.0 ± 0.1	23 ± 8
7	1.0 ± 0.1	1.0 ± 0.1	44 ±14
14	1.6 ± 0.2	2.0 ± 0.2	4 ± 1
21	0.9 ± 0.1	1.0 ± 0.2	4 ± 4
28	1.1 ± 0.2	1.0 ± 0.2	6 ± 4
35	3.8 ± 1.0	6.7 ± 1.5	66 ±25

Serum antibody titers as measured by the IFA, MA and CF tests are presented above in Table III. Antibody to phase II C. burnetii was initially detected 7 days postexposure and persisted at high levels throughout the experimental period. Specific antibodies detected by the IFA test were consistently higher at every sampling time than the MA or CF results. Phase I antibody was detected on day 14 by the IFA and MA tests, but not detected until day 28 by the CF test.

Presentation:

Gonder, J. C., M. D. Kastello, and R. A. Kishimoto. Cynomolgus monkey model for experimental Q fever. Presented, American Society for Microbiology, Las Vegas, NV, 14-19 May 1978 (Abstracts of the Meeting - 1978, p. 16).

Publications:

1. Kishimoto, R. A., J. W. Johnson, R. H. Kenyon, M. S. Ascher, E. W. Larson, and C. E. Pedersen, Jr. 1978. Cell-mediated immune responses of guinea pigs to an inactivated phase I Coxiella burnetii vaccine. Infect. Immun. 19:194-198.

2. Powanda, M. C., S. V. Machotka, and R. A. Kishimoto. 1978. Metabolic sequelae of respiratory Q fever in the guinea pig. Proc. Soc. Exp. Biol. Med. 158:626-630.

LITERATURE CITED

1. 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.

2. Berendt, R. F. 1974. Simian model for the evaluation of immunity to influenza. Infect. Immun. 9:101-105.

3. Spicer, D. S., and A. N. DeSanctis. 1976. Preparation of phase I Q fever antigen suitable for vaccine use. Appl. Microbiol. 32:85-88.

4. *Böyum, A. 1968. Isolation of mononuclear cells and* granulocytes from human blood. Scand. J. Clin. Lab. Invest. (Suppl. 97) 21:1-90.

5. U.S. Army Medical Research Institute of Infectious Diseases. 1 Oct 1977. Annual Progress Report, FY 1978, pp. 63-70. USAMRIID, Fort Detrick, MD.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6415	78 07 11	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
77 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	008
b. CONTRIBUTING				
c. //////////	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(U) Effect of ionizing radiation on progression and immune defenses against infectious diseases of potential BW importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 014100 Radiobiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 07	78 07	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: 78	0.75	120.0
FISCAL YEAR CURRENT: 79	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) Laboratory animals; (U) Military medicine; (U) BW defense; (U) Radiation; (U) Vaccine; (U) Immune suppression; (U) Encephalomyelitis, equine (VEE)

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

23 (U) Radiation exposure from nuclear attack may be detrimental to immune and physiologic processes of military and civilian personnel possibly exposed to a simultaneous BW attack. This work unit investigates interrelationships between acute or chronic irradiation and immune or disease processes in animal models, so that limitations of protective vaccines or disease in irradiated personnel may be more effectively managed in a nuclear warfare environment.

24 (U) Acute or protracted whole-body irradiation is delivered to selected animal species before, simultaneously with, or after infection. Clinical and immune responses are observed and measured serially.

25 (U) 77 10 - 78 07 - The x-radiation source is inoperable and will be removed. A linear accelerator is under consideration to replace it and in lieu of a cobalt-60 device for high dose radiation. Evaluation of the existing shielding has shown that it is satisfactory for a 4 millielectron volt accelerator. Costs are being determined for the new equipment and any construction necessary.

No active research is underway at this time so the work unit is terminated. When plans are finalized for purchase and installation of a radiation source, a new research plan will be prepared.

Publication: Current Chemotherapy, p. 317-319, American Society for Microbiology, Washington, 1978.

*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 1498B 1 MAR 68 FOR ARMY USE ARE OBSOLETE. * U.S. GPO 1974-540-843/8691

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BODY OF REPORT

Project No. 3A762776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 008: Effect of Ionizing Radiation on Progression and Immune Defenses Against Infectious Diseases of Potential BW Importance

Background:

Radiation-induced immunosuppression has been shown to affect the recovery of animals from experimental virus infections (1). Much evidence points to increased susceptibility of hosts that are subjected to immunosuppressive treatments (1,2). Previous studies in monkeys given the live, attenuated VEE virus vaccine strain (TC-83) have shown delayed and prolonged viremia and elevated serum virus titers in acutely irradiated, vaccinated monkeys. Is the humoral immune response of animals given TC-83 and exposed to chronic low-dose rate, total-body irradiation affected in a similar manner?

Dengue viruses cause significant morbidity in U. S. military personnel during operations in tropical countries. Efforts to achieve dengue immunoprophylaxis capabilities have been slowed by lack of an animal disease model. Halstead and co-workers (USAMRDC Contract No. DADA 17-73-C3083) have found that the chemically immunosuppressed monkey may be a potential model for dengue infection. Because of great variability of chemical immunosuppressants in effecting changes in hematologic components, we proposed that irradiation-immunosuppressed monkeys may be a more reproducible means to achieve a dengue-infected host.

Several studies have been conducted to evaluate the interferon inducer, lysine-stabilized polyriboinosinic polyribocytidylic acid [poly(ICLC)] as an antiviral compound and an adjuvant. Recently, it has been observed that many interferon inducers are also radioprotective agents. It was hypothesized that if interferon-inducing agents are radioprotective, then poly(ICLC) may also have radioprotective properties useful in military medicine.

Progress:

The x-radiation source is inoperable and will be removed. A linear accelerator is under consideration to replace it and in lieu of a cobalt^{60} device for high dose radiation. Evaluation of the existing shielding has shown that it is satisfactory for a 4 millielectron volt accelerator. Costs are being determined for the new equipment and any construction necessary.

No active research is underway at this time so the work unit is terminated. When plans are finalized for purchase and installation of a radiation source, a new research plan will be prepared.

Publication:

Hilmas, D. E., E. L. Stephen, R. O. Spertzel, and H. B. Levy. 1978. Use of poly(ICLC) for the prophylaxis and treatment of Venezuelan equine encephalomyelitis virus infection in nonhuman primates, pp. 317-319. In Current Chemotherapy, Vol. 1, (W. Siegenthaler and R. Luthy, eds), American Society for Microbiology, Washington.

LITERATURE CITED

1. Nathanson, N., and G. A. Cole. 1971. Immunosuppression: a means to assess the role of the immune response in acute virus infections. Fed. Proc. 30:1822-1830.

2. Thind, I. S., and W. H. Price. 1969. The effect of cyclophosphamide treatment on experimental arbovirus infection. Am. J. Epidemiol. 90: 62-68.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6416	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	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
78 04 21	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NUMBERS	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	009
b. SECONDARY				
c. //////////	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code) (U) Determinants for virulence and attenuation of arbo- and arenavirus vaccine candidates

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	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	220.0
FISCAL YEAR CURRENT 79	0.5	119.5

19. RESPONSIBLE DOD ORGANIZATION
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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) Virulence; (U) Encephalitis, equine (VEE, WEE, EEE); (U) Lassa fever virus; (U) Chikungunya

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

23 (U) Compare pathogenesis for rodents and primates of virulent and attenuated strains of arbo- and arenaviruses by identifying target tissues destroyed and evaluating protective responses induced by vaccine strains. Biochemical and physical properties of strains are correlated with biological parameters determining virulence. Studies lead to improved vaccines for the protection of U. S. military forces.

24 (U) Direct and indirect effects of virus replication are assessed by a variety of techniques. Humoral and cellular immune responses to infection are measured. Key virus-cell interactions are studied in explant culture. Structural difference among virus strains are correlated with specific biologic functions.

25 (U) 77 10 - 78 09 - Hydroxylapatite column chromatography techniques were employed to classify VEE, EEE, and WEE virus strains based on virion surface charges. Utility of the technique for separating minority subpopulations of virus from apparently homogeneous virus stocks was exploited to isolate a virulent virus from stocks of attenuated VEE vaccine, TC-83, and attenuated viruses potentially useful as vaccines from virulent VEE, WEE, EEE and Chikungunya stocks. Development of animal models for lethal arenavirus disease has been initiated to test efficacy of candidate vaccines, passively administered antibody, and antiviral drugs. Preliminary studies using Lassa fever virus focused on optimizing assay systems for virus and antibody. Pathogenesis studies in primates and guinea pigs suggest that both animals are suitable models for human Lassa fever virus disease.

Publications: Anal. Biochem. 82:423-434, 1977; Am. J. Epidemiol. 106:408-417, 1977; Circ. Res. 43:398-405, 1978; J. Med. Virol. 2:109-116, 1978.

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 009: Determinants for Virulence and Attenuation of Arbo- and Arenavirus Vaccine Candidates

Background:

The virulence of a virus is a phenotypic characteristic determined in part by the biochemical and physical properties of the virus itself, and in part by the host responses, both beneficial and deleterious, to the infection. Our published studies using alphaviruses as models suggest that virion surface charge is critical in determining the interaction of alphaviruses with cells of the reticuloendothelial system (RES), which degrade ingested virions (1). Using techniques which separate virions on the basis of surface charge (2), virus populations can be separated into component populations of virulent and attenuated particles, which interact differently with the RES (3). Studies conducted during the past year extended these concepts to different alpha viruses (Chikungunya, CHIK) and to new virus groups, including the arenaviruses and especially Lassa fever (LAS) virus. The development of animal models for lethal arenavirus disease should further facilitate the efficacy testing of antiviral drugs and candidate arenavirus vaccines.

Progress:

Hydroxylapatite chromatography of alphavirus populations. The details of separating virulent and avirulent populations of alphaviruses by hydroxylapatite chromatography were published this year (2, 4) or are in manuscript form, and only the general conclusions are summarized here. Hydroxylapatite chromatography separates proteins and whole virus particles on the basis of differences in surface charge. Viruses were eluted from columns with characteristic profiles whose shape is determined by the dimensions of a phosphate of pH gradient applied to the column. Using conditions determined empirically to be optimal for the separations desired (2), 43 VEE-strains were classified into groups similar to those based on more cumbersome serologic techniques (4). A similar classification scheme was developed for EEE strains (manuscript in preparation). Column chromatography was also shown to be a sensitive indicator of heterogeneity within virus populations, and detected shifts in the composition of virus populations with passage (2). Thus the technique was used to detect subpopulations of virulent VEE virus suspected to be present in stocks of attenuated VEE virus vaccine, and to characterize viruses from those personnel who became sick following inoculation of this vaccine (manuscript in preparation). Attenuated virions were obtained from virulent stocks of WEE and EEE viruses, suggesting that the technique would be useful for obtaining candidate vaccine strains of alphaviruses. Therefore recent experiments have focused on differentiating virulent from attenuated CHIK virus. Once the conditions for maximizing the

separations of CHIK viruses have been identified, work will be initiated to derive an attenuated CHIK virus directly from a virulent virus population using the column technique.

Virulence of CHIK virus strains in primates. The efficacy testing of a candidate vaccine for CHIK virus will require development of a susceptible animal model for challenge. Toward this end, 3 strains were inoculated SC into groups of rhesus and squirrel monkeys. Viremias were determined and temperatures, hematocrits, and blood leukocyte levels were monitored daily, but did not fluctuate significantly. Two strains, BAH-306 and 15561, passage 1, produced moderate viremias in both rhesus and squirrel monkeys. Viremias peaked on day 2, and diminished to undetectable levels by days 4-5 (Table I). In contrast, CHIK strain 15561, passage 10, failed to produce

TABLE I. VIREMIAS AND ANTIBODY RESPONSES IN MONKEYS INOCULATED WITH CHIK VIRUS STRAINS

CHIK STRAIN DOSE (Log_{10} PFU)	MONKEY SPECIES	LOG_{10} PFU/ml PLASMA					RIA TITER day 36 (1:)
		1	2	3	4	5	
BAH-306 (4.5)	Rhesus	3.8	5.3	3.9	< 0.7	< 0.7	2560
		3.5	5.1	3.9	< 0.7	< 0.7	2560
		2.2	4.5	3.8	< 0.7	< 0.7	in progress
		2.9	5.1	4.2	< 0.7	< 0.7	in progress
	Squirrel	3.1	3.9	1.4	< 0.7	< 0.7	7230
		3.8	4.2	1.9	< 0.7	< 0.7	996
		2.5	2.8	2.6	1.4	< 0.7	in progress
		2.3	2.6	0.7	< 0.7	< 0.7	in progress
15561, p1 (5.4)	Rhesus	2.9	4.0	2.9	< 0.7	< 0.7	in progress
		2.0	4.2	3.7	< 0.7	< 0.7	in progress
	Squirrel	2.3	2.2	1.4	< 0.7	< 0.7	in progress
		3.0	2.5	1.4	< 0.7	< 0.7	in progress
15561, p 10 (preinactivation E-20 vaccine) (4.8)	Rhesus	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	10384
		< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	< 80
	Squirrel	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	< 80
		< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	< 80

viremias, although in one rhesus monkey, inoculation of this virus induced a high level antibody response. This virus suspension was the actual preformalin-inactivation material used to prepare the CHIK (E-20) vaccine. The observation that this virus might be attenuated suggests that it might be a

useful starting material for a live attenuated CHIK vaccine. Other markers of attenuateion, including plaque size, temperature sensitivity and elution profiles using hydroxylapatite chromatography columns, are being investigated to differentiate this and other candidate CHIK vaccine viruses from the virulent parent strains.

Development of models for lethal arenavirus disease in rodents. Pichinde virus (PIC), strain 4763, was adapted to kill inbred, strain 13 guinea pigs by sequential spleen-to-spleen passages. Within 4 passages, this virus was uniformly lethal for adult, strain 13 guinea pigs inoculated SC (Table II). This

TABLE II. RELATIONSHIP BETWEEN PASSAGE LEVEL OF PIC VIRUS AND LETHALITY FOR ADULT, STRAIN 13 GUINEA PIGS

SPLEEN PASSAGE	NO. DEAD/INOCULATION	MEAN DAY OF DEATH
0	1/20	15
1	3/10	17
2	6/10	15
3	8/10	16
4	10/10	15
8	10/10	14

virus was passed 4 additional times and a large quantity of passage 8 virus was prepared. Passage 8 virus (called adapted PIC) was totally benign for rhesus monkeys, but uniformly lethal for guinea pigs. Adapted PIC produced a relatively high viremia compared with the parent virus, in guinea pigs (Table III). This higher viremia reflected enhanced replication of the adapted virus in target tissues such as spleen, liver, bone marrow, pancreas, and adrenals (Table III). Adapted PIC replicated efficiently in these tissues while the parent did not. Adapted PIC apparently did not replicate in the brain.

Lesions associated with adapted PIC infection were found most commonly in the liver, adrenal cortex and spleen. The earliest lesions were found in the liver, beginning day 6. By day 9, liver sections obtained from all infected guinea pigs contained mulitple areas of necrosis throughout the parenchyma. These consisted of dilated sinusoids containing nuclear debris. Liver cords contained individual hepatocytes in various stages of degeneration; in advanced lesions, the hepatic cords were disrupted. A severely necrotic liver was a consistent finding in moribund guinea pigs. In addition, diffuse fatty change was usually observed late in infection.

In spleen, lesions were apparent beginning on day 9. PTAH staining revealed accumulations of fibrillar material within the sinusoids of the red pulp, and within perifollicular areas. By day 12, the fibrin deposition was accompanied by necrosis and eventually complete destruction of the red pulp architecture.

In adrenals, the lesions were limited to the cortex. Focal areas of necrosis consisted of dilated sinusoids containing nuclear debris, and a few granulocytes. This lesion was consistently found in moribund guinea pigs. In contrast,

TABLE III. COMPARISON OF ADAPTED VS PARENT PIC VIRUS REPLICATION IN TISSUES OF OUTBRED GUINEA PIGS (n = 3)

TISSUE	STRAIN	LOG_{10} PFU/ml or gm ± SE, BY DAY POSTINOCULATION				
		3	6	9	12	15
Plasma	Adapted	1.6 ± 0.15	2.1 ± 0.20	4.4 ± 0.13	5.4 ± 0.28	5.0 ± 0.23
	Parent	< 0.7 ± 0	1.0 ± 0.40	< 0.7 ± 0	< 0.7 ± 0	< 0.7 ± 0
Spleen	Adapted	6.0 ± 0.25	6.1 ± 0.24	6.6 ± 0.8	7.8 ± 0.26	7.5 ± 0.10
	Parent	3.7 ± 0.20	5.3 ± 0.14	4.4 ± 0.5	< 0.7 ± 0	< 0.7 ± 0
Liver	Adapted	5.4 ± 0.25	5.0 ± 0.15	5.8 ± 0.05	5.8 ± 0.05	6.4 ± 0.27
	Parent	2.8 ± 0.10	4.4 ± 0.65	4.0 ± 0.05	< 0.7 ± 0	< 0.7 ± 0
Thymus	Adapted	6.5 ± 0.15	5.9 ± 0.11	5.9 ± 0.6	6.6 ± 0.15	6.7 ± 0.25
	Parent	< 0.7 ± 0	3.0 ± 0.24	3.5 ± 1.05	< 0.7 ± 0	< 0.7 ± 0
Adrenal	Adapted	5.2 ± 0.55	4.7 ± 0.80	6.1 ± 0.17	7.5 ± 0.17	7.5 ± 0.09
	Parent	3.4 ± 0.10	1.9 ± 1.35	2.2 ± 0.16	< 0.7 ± 0	< 0.7 ± 0
Pancreas	Adapted	5.8 ± 0.35	5.5 ± 0.25	5.9 ± 0.03	6.7 ± 0.16	6.8 ± 0.23
	Parent	4.2 ± 0.20	5.2 ± 0.30	3.6 ± 0.55	< 0.7 ± 0	< 0.7 ± 0
Bone marrow	Adapted	3.7 ± 0.20	5.0 ± 0.57	6.9 ± 0.13	6.3 ± 0.18	6.4 ± 0.52
	Parent	< 0.7 ± 0	2.0 ± 1.45	1.9 ± 0.70	< 0.7 ± 0	< 0.7 ± 0

essentially no histologic changes were seen in tissues of guinea pigs infected with parent PIC. Using direct immunofluorescence, the distribution of antigens in tissues correlated closely with the recovery of infectious virus.

By FA in liver, individual hepatocytes and elongated cells, probably Kupffer cells, contained high concentrations of PIC antigen beginning day 6. By days 9-12, these areas had enlarged; clusters of cells contained antigen, suggesting a cell-to-cell spread of virus within liver. In spleen, virus antigen was clearly excluded from the white pulp, but was concentrated particularly in large cells (macrophages?) surrounding the white pulp, and was also distributed in individual cells throughout the red pulp. In adrenals, high concentrations of viral antigen were found early in individual cells within the cortex. Gradually these areas enlarged to include > 20 contiguous cells containing antigen.

Protective efficacy of ribavirin for lethal PIC infections. Ribavirin, administered daily in 2 doses of 5 mg/kg to guinea pigs, significantly reduced viremia. More detailed studies were conducted using inbred strain MHA hamsters. Ribavirin, administered daily for 14 days beginning day 0, afforded complete protection to MHA hamsters (Table IV). When ribavirin was delayed

TABLE IV. EFFECT OF RIBAVIRIN ON SURVIVAL OF HMA HAMSTERS INOCULATED WITH PIC VIRUS

DAYS AFTER INFECTION	% SURVIVAL		
	Virus only (n = 27)	Ribavirin[a] days 0-14 (n = 20)	Ribavirin days 4-14 (n = 10)
7	100	100	100
8	95	100	100
9	60	100	100
10	54	100	100
11	38	100	90
12	34	100	60
13	25	100	50
14	20	100	50
15	11	100	50
21	11	100	50

[a] Ribavirin inoculated SC twice daily, 5 mg/kg.

for 4 days, only 50% of treated hamsters survived. However, this still represents a significant degree of protection. Replication of virus in target tissues and viremia curves for treated and untreated groups of hamsters reflected the mortality data. No viremia was detected in hamsters receiving ribavirin beginning day 0 (Table V). Virus titers recovered from spleen and liver were markedly suppressed. In hamsters receiving drug beginning day 4, viremia and

TABLE V. COMPARISON OF PIC VIRUS CONCENTRATIONS IN TISSUES OF RIBAVIRIN-TREATED AND UNTREATED MHA HAMSTERS (n = 3)

TISSUE	TREATMENT (Days)	LOG_{10} PFU/ml or gm ± SE, BY DAY POSTINOCULATION					
		3	6	9	12	15	18
Plasma	None	3.3 ± 0.86	7.5 ± 0.10	6.8 ± 1.40	620 ± 0.15	5.3 ± 0.30	died
	0-14	< 0.7 ± 0	< 0.7 ± 0	< 0.7 ± 0	< 0.7 ± 0	< 0.7 ± 0	< 0.7 ± 0
	4-14	NT[a]	5.2 ± 0.18	3.8 ± 1.76	4.9 ± 0.43	3.9 ± 0.40	< 0.7 ± 0
Liver	None	5.5 ± 0.98	8.7 ± 0.03	5.7 ± 0.41	6.1 ± 0.32	5.0 ± 0.49	died
	0-14	3.4 ± 0.86	2.0 ± 1.03	3.1 ± 1.13	< 0.7 ± 0	< 0.7 ± 0	< 0.7 ± 0
	4-14	NT	7.3 ± 0.03	5.2 ± 0	5.8 ± 0.20	4.1 ± 0.76	< 0.7 ± 0
Spleen	None	6.0 ± 1.0	8.7 ± 0.20	6.8 ± 0.40	7.1 ± 0.14	6.4 ± 0.17	died
	0-14	5.2 ± 0.15	.6 ± 0.26	2.9 ± 1.93	3.1 ± 1.10	< 0.7 ± 0	< 0.7 ± 0
	4-14	NT	6.7 ± 0.20	5.6 ± 1.12	5.8 ± 0.15	5.1 ± 0.33	3.4 ± 0.03

[a] Not tested.

tissue levels were only moderately suppressed. Detection of virus antigen by FA paralleled infectious virus titrations. Development of histologic lesions in tissues of untreated hamsters were similar to those described above for PIC in guinea pigs. In ribavirin-treated hamsters, no lesions developed in tissues from the day-0-14 group, and only minimal lesions in the day-4-14 group.

Protective efficacy of ribavirin for other arenaviruses. The protection of hamsters and guinea pigs against lethal PIC virus infection by ribavirin suggested that this drug might be useful for treating other arenavirus infections. A preliminary screening procedure was established to measure the sensitivity to ribavirin in vitro. Ribavirin, in concentrations of $\geq$ 10 μg, completely inhibited plaque formation in BS-C-1 cells by PIC, Junin (JUN) and Lassa (LAS) viruses. When tested in Vero cells, higher concentrations of ribavirin were required (Table VI).

TABLE VI. EFFECT OF RIBAVIRIN ON PLAQUE FORMATION OF ARENAVIRUSES

VIRUS	RIBAVIRON (μg/ml)	LOG_{10} PFU/0.2 ml	
		BS-C-1	Vero
PIC	50	< 0.7	< 0.7
	10	< 0.7	5.1
	1	5.1	5.3
	0	5.2	5.4
JUN, #XJ Clone 3	50	< 0.7	< 0.7
	10	< 0.7	4.8
	1	5.3	5.8
	0	5.3	5.8
LAS	50	< 0.7	< 0.7
	10	< 0.7	4.5
	1	4.1	4.4
	0	4.0	4.4

Detection and assay of virus. Three strains were titrated by counting PFU on various cell lines (Vero, BS-C-1, MRC-5, FRhL-103) maintained in a 5% CO_2, humidified atmosphere under Eagle's basal medium with Earle's salts, containing HEPES buffer (8 gm/L), sodium bicarbonate (2.2 gm/L), and 0.5% agarose, pH 7.1. The highest titers were obtained using Vero cells. To compare the sensitivities of PFU assay vs. direct immunofluorescence (DFA), the LAS strains were assayed on Vero cells using both methods (Table VII); endpoints were reached in the same dilution by both methods for each virus tested. Direct FA staining of infected cell monolayers is thus no more sensitive than plaque enumeration under the conditions routinely employed. For detection of virus in clinical specimens, both methods would be equally sensitive. However, preliminary data suggest that DFA of spot preparations made from trypsinized, infected cell monolayers may be

TABLE VII. COMPARATIVE SENSITIVITIES OF DFA AND PFU ASSAYS FOR DETECTING LAS VIRUS IN VERO CELLS[a]

STRAIN	LOG_{10} DILUTION	DFA	AVERAGE NO. PLAQUES
Sierra Leone placenta	-4	+	56
	-5	±	5
	-6	0	0
	-7	0	0
Pinneo	-4	+	TNTC
	-5	+	21
	-6	+	3
	-7	0	0
Josiah	-4	+	TNTC
	-5	+	26
	-6	+	3
	-7	0	0

[a] Virus dilutions incubated with cell monolayers for 4 days. For DFA, cells were fixed with 70% acetone, and read directly using epiluminescence; + = $\geq$ 20% cells fluorescing; ± = a few cells fluorescing; 0 = no cells fluorescing. For PFU, cells were stained with neutral red and plaques counted after 4-6 hr of incubation.

slightly more sensitive than plaque enumeration. Titration of LAS virus strains by intracerebral inoculation of suckling hamsters yielded variable death patterns, which often did not correlate with the dilution. Suckling hamster titrations are therefore not useful for routine assay of LAS.

Sensitivity of LAS virus to environmental factors. To determine the stability under various conditions, LAS (strain Sierra Leone placenta) was diluted into medium containing 2% fetal calf serum, and subjected to sequential cycles of freezing/thawing or to various temperatures (Table VIII). Samples were titrated immediately after thawing or after the stated incubation period. Four cycles of freezing/thawing did not affect the virus titer. Likewise, incubation for 2 hr at 0, 25, and 37 C did not reduce infectivity, but incubation at 60 C for 15 min reduced infectivity to undetectable levels. In addition, exposure of this dilution of LAS to ultraviolet light (8 mW/cm^2) completely inactivated the virus within 15 sec. Dilution of the Josiah strain into the recommended working strengths of various common disinfectants, followed by incubation for 1 or 5 min resulted in complete inactivation (Table IX). When the disinfectants were diluted to 1/5 normal working strength, they still completely inactivated the virus, except for Novalsan-S, and formalin. Dilution of virus into tap water did not result in inactivation.

TABLE VIII. STABILITY OF LAS FEVER VIRUS TO ENVIRONMENTAL FACTORS

INCUBATION CONDITIONS		LOG_{10} PFU/0.2 ml BY TIME OF EXPOSURE			
		0	15 sec	15 min	120 min
Wet ice		5.2		5.3	5.2
25 C		5.2		5.1	5.2
37 C		5.2		5.1	4.7
60 C		5.2		< 0.7	< 0.7
UV light (8 mW/cm^2)		5.2	< 0.7	< 0.7	
Freeze/thaw	0 cycle	4.9			
	1 cycle	5.1			
	4 cycles	5.0			

Development of a model for studying the pathogenesis and treatment of lethal LAS fever virus infections in primates. The lethalities of 3 LAS strains, originally obtained from Dr. Karl Johnson, Center for Disease Control, were determined using rhesus monkeys (Table X). All virus seeds were passaged one additional time in Vero cell cultures, and adjusted to 6.0-6.2 log_{10} Vero PFU/0.5 ml and inoculated SC. The Josiah strain was the most virulent, killing 4 of 6 control rhesus monkeys. Monkeys previously exposed to JUN and/or Machupo (MAC) viruses were not significantly protected from LAS challenge, since 4 of 7 such monkeys died. None of 4 monkeys treated with ribavirin died. (Ribavirin studied were conducted in collaboration with investigators working under Work Unit A841 00 026.) Mean viremia levels between control and ribavirin groups, and between control and JUN/MAC-immune groups, were not significantly different on any day. However, when mean viremia levels were compared for lethally infected vs. nonlethally infected monkeys (pooled without reference to drug treatment group or prior immune status), lethally infected monkeys exhibited viremias that were significantly ($P < 0.05$) higher beginning on days 9 and 10 and persisting until death (mean day 12.5) as shown in Table XI.

To determine which organs were involved in the replication of LAS, tissues freshly obtained from rhesus monkeys immediately after death were homogenized, clarified by centrifugation (10,000 g, 30 min) and titrated for PFU on Vero cell monolayers (Table XII). Virus concentrations in all visceral tissues tested were clearly higher than in the contained blood, implying significant virus replication in all these tissues. Virus concentrations in nervous tissue were lower than in visceral tissues; virus retrieved from the cerebrum may represent virus in the contained blood, but virus in the brain stem and spinal cord probably represent replicated virus. All tissues were processed for routine histologic and immunofluorescence staining, but have not been examined to date.

TABLE IX. INFECTIVITY OF LAS VIRUS FOLLOWING INCUBATION IN COMMON DISINFECTANTS DILUTED IN TAP WATER

DISINFECTANT	RECOMMENDED WORKING STRENGTH (%)	INCUBATION AT 25 C (minutes)	NO. PLAQUES BY DILUTIONS[a]			
			-2	-3	-4	-5
Lysol	5	1	0	0		
		5	0	0		
Staphene	2	1	0	0		
		5	0	0		
Germ Warfare	2	1	0	0		
		5	0	0		
Novalsan-S	1	1	TNTC	TNTC	217	25
		5	TNTC	TNTC	86	9
Formalin	10	1		TNTC	192	23
		5		9	2	
Roccal	1	1	0	0		
		5	0	0		
Chloramine T	1	1	0	0		
		5	0	0		
Water	100	1			TNTC	39
		5			TNTC	71
Control diluent		1			TNTC	64
		5			TNTC	48

[a] Disinfectant was used at 1/5 working strength. At full working strength, no plaques were detected in the -3 dilutions of virus/disinfectant mixtures; -2 dilutions were not tested because the disinfectants alone were toxic for Vero cells at that concentration.

TABLE X. LETHALITY OF LAS VIRUS STRAINS[a] AND VIREMIA PRODUCED IN RHESUS MONKEYS TREATED WITH RIBAVIRIN[b]

DAYS	MEAN VIREMIA, LOG_{10} PFU/ml SERUM ± SE[a]					
	Sierra Leone placenta		Pinneo Control[c]	Josiah		
	Control	Ribavirin		Control	Ribavirin	JUN/MAC Immune[d]
3	< 0.7 ± 0	< 0.7 ± 0	< 0.7	1.2 ± 0.22	1.6 ± 0.41	1.0 ± 0.39
5	1.4 ± 0.49	< 0.7 ± 0	< 0.7	2.9 ± 0.20	3.3 ± 0.10	2.6 ± 0.24
7	3.2 ± 0.16	< 0.7 ± 0	1.8	3.8 ± 0.19	3.4 ± 0.15	3.4 ± 0.23
9-10	3.8 ± 0.30	1.4 ± 0.46	1.5	4.3 ± 0.43	3.2 ± 0.29	4.5 ± 0.46
11-13	3.8 ± 0.73	1.6 ± 0.60	1.4	4.7 ± 0.81	4.0 ± 0.80	4.8 ± 0.64
14-15	3.1 ± 0.12	1.9 ± 0.45	1.4	2.4 ± 0.35	2.2 ± 0.57	3.9 ± 0.55
16-17	3.0 ± 0.17	0.9 ± 0.37	2.0	2.6 ± 0.35	1.6 ± 0.51	2.3 ± 1.34
19-20	2.2 ± 0.17	< 0.7 ± 0	1.6	2.2 ± 0.75	2.0 ± 0.44	1.4 ± 0
23	< 0.7	< 0.7	< 0.7			
Dead/Total	1/4	0/4	0/2	4/6	0/4	4/7
Day of death	12			10,11,12,12		12,12,14,17

[a] LAS virus seeds received from CDC were passaged one additional time in Vero cell culture and adjusted to 6.0-6.2 log_{10} PFU/0.5 ml, then inoculated SC.

[b] Ribavirin treatment was initiated with a loading dose of 50 mg/kg on day 5 postinfection, followed by 3 doses daily of 10 mg/kg, SC.

[c] No SE is given since n = 2.

[d] Monkeys were immune to JUN and/or MAC viruses having been exposed to them 1-6 mon previously, and having demonstrable antibody immediately prior to LAS challenge.

TABLE XI. COMPARISON OF VIREMIAS PRODUCED BY LAS VIRUS (STRAIN JOSIAH) IN LETHALLY VS. NONLETHALLY INFECTED RHESUS MONKEYS[a]

DAYS	MEAN LOG_{10} PFU/ml SERUM ± SE	
	Lethally infected (n = 8)	Nonlethally infected (n = 8)
3	1.1 ± 0.17	1.2 ± 0.17
5	3.0 ± 0.12	2.8 ± 0.23
7	4.2 ± 0.12	3.3 ± 0.06
9-10	5.2 ± 0.13[b]	3.2 ± 0.17
11-13	6.0 ± 0.32[b]	3.1 ± 0.38
14-15	5.2 ± 0.25[b]	2.5 ± 0.28
17	5.4	1.7 ± 0.32
19	died	1.5 ± 0.28

[a] Based on pooled viremias from lethally infected monkeys in control and JUN/MAC groups in Table X, and nonlethally infected monkeys from all 3 groups.

[b] $P < 0.05$.

TABLE XII. CONCENTRATIONS OF INFECTIOUS LAS VIRUS IN TISSUES OF RHESUS MONKEYS DYING AFTER VIRUS INOCULATION (n = 8)[a]

TISSUE	LOG_{10} PFU/ml or gm
Serum[b]	5.6 ± 0.36
Outer cerebrum	5.3 ± 0.30
Brain stem	6.0 ± 0.36
Spinal cord	6.6 ± 0.32
Axillary lymph node	6.6 ± 0.27
Spleen	6.8 ± 0.29
Liver	7.6 ± 0.49
Pancreas	7.1 ± 0.29
Lung	7.3 ± 0.30
Kidney	6.6 ± 0.22
Adrenal	7.6 ± 0.38
Pleural	7.3 ± 0.43

[a] Tissues obtained from the 8 lethally infected monkeys whose mean viremias are shown in Table XI.

[b] Obtained at time of death. Mean day of death = 12.5.

The handling of significant numbers of rhesus monkeys, especially under the total containment conditions required for LAS research, presents serious logistical problems, which would be reduced if a small rodent model host were available. Guinea pigs and inbred strain MHA hamsters were tested for susceptibility to Josiah strain. No MHA hamsters died, nor did any adult guinea pigs (weighing $\geq$ 500 gm) following SC inoculation of 5.7 $\log_{10}$ PFU. However, 3 of 5 young guinea pigs (260-300 gm) died. Virus, isolated from spleens of dead guinea pigs, will serve as starting material for producing a guinea pig-adapted strain, by serial passage (spleen-to-spleen) in guinea pigs (analogously to the adaptation of PIC guinea pigs).

Measurement of antibody to LAS virus. Plasma, obtained from a convalescent patient following LAS infection, was titrated for antibody in a conventional serum dilution, PRN test using Vero cells (Table XIII) No plaque

TABLE XIII. PROTEIN A-MEDIATED PRN OF LAS VIRUS BY IMMUNE SERUM

ANTISERUM DILUTION (1:)	% OF CONTROL PLAQUES	
	No protein A	With protein A
10	86	58
20		16
40	81	17
80		29
160	95	49
320		83
640	86	84
1280		95
Control	100	92

reduction was observed in any dilution of plasma tested. However, when a protein a-bearing Staphylococcus reagent was added to precipitate infection virus/antibody complexes, significant plaque reduction (i.e., > 80%) was observed in the 1:20, 1:40 and 1:80 dilutions. Using indirect fluorescent antibody (IFA) technique, this plasma had a titer of 1:64 against LAS. Whether antibody, detectable only by IFA or protein A precipitation techniques but not by conventional PRN test, has any protective efficacy, remains to be determined.

Presentations:

1. Jahrling, P. B. Characterization of virulent Venezuelan encephalitis viruses isolated from throat wash fluids obtained from TC-83 vaccine reactors. Presented, American Soc. Trop. Med. Hyg. Meeting, 8-11 Nov 77, Denver, CO.

2. Jahrling, P. B. Pathogenesis and treatment of lethal arenavirus diseases in rodents and primates. Presented, International Congress for Virology, The Hague, The Netherlands, 3 Aug 1978.

Publications:

1. Marker, S. C., P. Melby, and P. B. Jahrling. 1977. Resolving power in a practical application of preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis: preparation of eastern encephalitis virus proteins. Anal. Biochem. 82:423-434.

2. Jahrling, P. B., and G. A. Eddy. 1977. Comparisons among members of the Venezuelan encephalitis virus complex using hydroxylapatite column chromatography. Am. J. Epidemiol. 106:408-417.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV 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
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10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
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b. CONTRIBUTING				
c. //////////	STOG 78-7.2.1, 3, 6			

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(U) Cellular responses in lymphatic tissues following immunization

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19. RESPONSIBLE DOD ORGANIZATION

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

23 (U) 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) 77 10 - 78 09 - Studies with the cytoskeletal probes cytochalasin-A and colchicine have defined the role of microfilaments and microtubules in regulating lymphocyte homing and recirculation which will be useful in future studies of antigen-specific homing. Preliminary studies of lymphocyte chemotaxis under agarose suggest that lymphocytes are attracted to mediators which do not attract acute inflammatory cells and vice versa, while monocytes and immunoblasts are attracted to all of these mediators. HEV endothelial cells actively remove protein-receptor complexes from the surfaces of recirculating lymphocytes without damaging the cells. This process may expose receptors and render the cell more responsive to subsequent antigenic stimulation in vivo. The local and regional response to complete Freund's adjuvant (CFA) was studied and compared to that induced by muramyl dipeptide (MDP) injection. While CFA potentiates both cellular (CMI) and humoral immunity, MDP appears to favor CMI. In addition, MDP appears to be suppressive to the primary immune response while potentiating anamnestic responses to VEE C-84 antigens.

Publications: Fed. Proc. 37:591(2x), 1978.
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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 010: Cellular Responses in Lymphatic Tissue Following Immunization

Background:

Induction of immunity depends upon a complex series of cellular interactions including: antigen processing and presentation by phagocytic cells bearing "self" antigens; T and B cell cooperation in the presence of these macrophages; proliferation, differentiation and maturation of immunocompetent cells to form effecter cells; and feedback regulation by the products of helper and suppressor cells augmented by local release of lymphokines, monokine and other cell products. It is believed that all of these immune functions take place within the microenvironments of lymphatic tissues. Since lymph nodes are antigen traps through which a constant traffic of immunocompetent cells occurs, in vitro manipulation of one or more of these cooperating cell systems may provide information which can be used in the development of better immunological adjuvants.

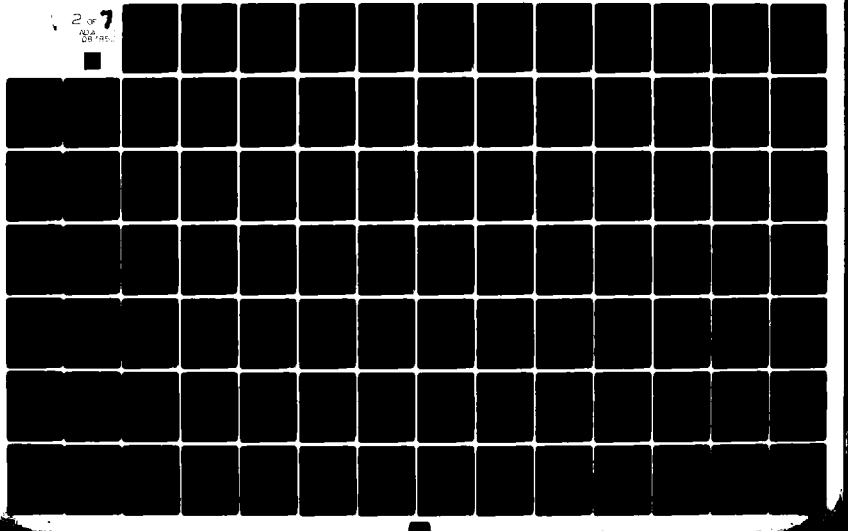
Progress:

Lymphocyte migration. Studies of basic mechanisms of lymphocyte homing and emigration into lymphatic tissues have reached a summation point with regard to cytoskeletal control of cell surface receptors. Recirculating lymphocytes emigrate from the blood into lymph nodes by selectively crossing high endothelial venules (HEV). This process may depend upon the ability of lymphocytes to attach to endothelium via receptors and on the capacity of these cells to actively propel themselves across venule walls. The role of cytoskeletal structures in membrane recognition and cellular locomotion was investigated using cytochalasin A (cyto A) and colchicine treated thoracic duct lymphocytes. Cyto A was used because it causes prolonged effects on microfilaments without altering hexose or nucleoside membrane transport. The effects of colchicine on microtubule polymerization are well known. Random movement of lymphocytes was assessed by measuring the numbers of cells at centrifugal distances from the well edge after the lymphocytes had migrated beneath agarose. After 18 hr incubation, migrating lymphocytes produced a distance-distribution which ranged from sessile to highly mobile cells. Pretreatment with cyto A for 30 min produced a dose-dependent reduction in locomotion. Doses above 4 ug/ml markedly reduced migration without affecting viability. Suspensions of these cells were viewed by scanning electron microscopy (SEM). Untreated lymphocytes had numerous short surface microvilli. Cyto A treatment reduced the number, yielding cells with smooth pitted surfaces interspersed with zeiotic blebs. Colchicine- and lumicolchicine-treated lymphocytes had few surface changes except for slight elongation of microvilli in the former. Transmission electron microscopy (TEM) of cyto A-treated cells which were fixed at physiological temperature, revealed irregular clumps of aggregated 5-nm microfilaments near margins of vesicles

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and at bases of zeiotic blebs; occasionally aggregates were found trapped between vesicle membranes. These changes may represent release of microfilaments from connections with membrane proteins, and accumulation of aggregates at residual anchoring sites. Effects of colchicine on lymphocyte microtubules were monitored by TEM of the pericentriolar regions of cells fixed at 37°. Untreated lymphocytes had approximately 12 segments of 25-nm microtubules within 1 μ of the centrioles. Increasing doses of colchicine from 10^{-8} to 10^{-2} M caused progressive reduction in the number of visible microtubules in treated lymphocytes.

TABLE I. IN VITRO EFFECTS OF CYTO A ON LYMPHOCYTES

PHASE MICROSCOPY, 37°C				SEM AND TEM CHANGES		
Dose μg/ml	% viable	% motile	% with active blebbing	% loss of microvilli	Aggregated microfilaments	% cells with blebs
0	98.6	15.6	0	0	0	0
0.2	95.8	6.1	8.9	17.1	±	8.3
2.0	96.4	3.7	11.4	51.7	+	33.3
4.0	96.7	0.3	12.9	77.3	++	65.0
6.0	97.2	0	21.7	80.5	++++	61.2
8.0	94.8	0	35.7	89.7	++++	65.1

TABLE II. IN VITRO EFFECTS OF COLCHICINE ON LYMPHOCYTES

PHASE MICROSCOPY, 37°C				SEM AND TEM CHANGES		
Dose M	% viable	% motile	% blebbing	% loss of microvilli	Microvilli length (nm)	Microtubule no.[a]
0	98.0	15.6	0	0	500	12.0
10^{-10}	94.4	16.3	0	0	548	11.0
10^{-8}	95.3	14.9	0	0	560	9.9
10^{-6}	98.2	15.7	0	0	645	6.7
10^{-4}	94.6	14.7	0	0	814	1.3
10^{-3}	78.8	0.3	8	5	ND[b]	0.2
10^{-2}	64.3	0	21	10	ND	0.5
Lumicolchicine						
10^{-4}	96.8	15.9	0	0	599	11.9

[a]Within 1 μm of centriole.
[b]Not done.

Lymphocyte traffic was studied in vivo by whole tissue uptake and autoradiographic techniques following IV infusion of [^{3}H] uridine-labeled lymphocytes. In the tissue uptake studies there was early accumulation of label in lung, liver and spleen after infusion which correlated with blood flow to these organs. Traffic of untreated lymphocytes was indicated by persistent label in the spleen which tapered off in time. There was progressive selective accumulation of these cells in lymph nodes which reached peak levels in 18 hr. In contrast, lymphocytes treated with cyto A, did not show appreciable accumulation in lymph nodes and splenic uptake was markedly depressed after 4 hr. Absence of alterations in the distribution of label to the liver attested to the viability of cyto A-treated lymphocytes, since most heat-killed cells were sequestered in liver sinusoids after infusion. Lymph node uptake of colchicine-treated lymphocytes was moderately reduced until 8 hr after infusion. In reciprocal experiments, where rats were treated by IP injection of colchicine, normal lymphocytes failed to home to spleen and lymph nodes.

Quantitative autoradiography of lymph nodes following infusion of untreated lymphocytes showed rapid clearance of labeled cells from the lumens of vessels, passage into the walls of HEV and accumulation in the cortical parenchyma. Labeled cyto A treated lymphocytes accumulated on luminal surfaces of HEV, but did not enter node parenchyma by 4 hr postinfusion.

Colchicine-treated cells did not adhere to or migrate across HEV into lymph nodes; blood samples indicated that most of these cells were circulating in the peripheral blood.

TABLE III. INTRANODAL DISTRIBUTION OF LABELED LYMPHOCYTES AFTER IV INFUSION (EFFECTS OF CYTO A AND COLCHICINE)

Time (min)	MEAN NO. LABELED LYMPHOCYTES/10 HPF								
	HEV lumen			HEV wall			Node cortex		
	TDL	Cyto A	Col.	TDL	Cyto A	Col.	TDL	Cyto A	Col.
3	10.1	27.6	1.8	15.8	7.6	0.3	1.0	0.5	0
30	1.8	27.1	3.6	27.8	36.1	4.1	28.8	19.3	1.4
60	0.4	18.3	4.2	12.1	21.5	6.4	150.8	15.6	11.7
240	2.2	14.5	0.8	12.0	19.1	0.9	302.8	57.8	10.0

SEM of lymph node vessels (which had been flushed by perfusion prior to fixation in order to remove nonadherent cells) was used to measure the adhesive properties of lymphocyte-homing receptors. Untreated lymphocytes adhered at a ratio of about 2 lymphocytes/10 endothelial cells in these preparations. The adherent cell index was increased to 5 in lymph nodes

from animals receiving transfusions of cyto A treated cells prior to flushing. Some of these adherent lymphocytes resembled cyto A-treated cells shown earlier in in vitro studies. This observation confirmed the capacity of some cyto A treated cells to recognize and adhere to HEV surfaces. In contrast, no lymphocytes were found adhering to HEV surfaces in perfused lymph nodes from rats which were given IP colchicine.

TABLE IV. DETERMINATION OF ADHERENT LUMINAL LYMPHOCYTES

PERFUSION CONDITIONS	ACI[a] ± SE
Normal rat nodes (no cells added)	
Flushed with dextran/saline (DS)	0.235 ± 0.03
Normal rat nodes (+ transfused TDL)	
Flushed with DS 30 min after cyto A-TDL, IV	0.535 ± 0.09
Flushed with DS 30 min after Col-TDL, IV	0.150 ± 0.01
Nodes from rats given 1 µg/gm Col., IP (no cells added) Flushed with DS	0 ± 0

[a]Adherent cell index (ACI) = $\frac{\text{No. adherent lymphocytes}}{\text{No. endothelial cells}}$

In EM studies of normal nodes, lymphocytes were found which appeared to be in the process of migrating when they were fixed at 37°. A circumferential indentation and bilateral bundles of thickened microfilaments were seen which suggested the contraction ring proposed by Lewis in 1931 (1).

While normal migrating lymphocytes had symmetrical bundles of microfilaments at contraction rings, cyto A-treated cells had eccentric distributions of microfilaments. In addition, clustered filaments were found at membrane sites where these lymphocytes made contact with the endothelium.

Interendothelial lymphocytes contain microtubules within their cytoplasm, beneath the microfilament mat, and adjacent to the perinuclear cystern. Parallel arrays of microtubules were also seen beneath the membrane of adjacent endothelial cells.

The HEV endothelium from colchicinized rat lymph nodes lacked its normal complement of microtubules and fasces of 10-nm filaments were found randomly coiling through the cytoplasm as early as 30 min after IP injection.

These studies have attempted to define the role of microfilaments and microtubules in lymphocyte recirculation. Cyto A clearly blocks lymphocyte locomotion in vitro and in vivo without completely preventing lymphocyte homing to endothelial surfaces, while colchicine treatment of the lymphocytes or the microenvironment prevents recognition and attachment of these cells to endothelium at doses which did not reduce motility.

When taken in context of recent models of transmembrane control of cell surface receptors, these findings suggest that lymphocyte homing receptors are somehow linked through the membrane to actinomyosin microfilaments. The distribution or stabilization of these recognition receptors in strategic locations on the cell surface may be controlled through direct or indirect connections with microtubules. Stabilization of receptors on HEV endothelium by microtubules or via connections with 10-nm filaments may provide the surface against which the force of lymphocyte receptor-redistribution may be translated into forward movement.

The ability to control the motile apparatus of the lymphocyte without affecting the ability to recognize and adhere to sites of emigration will become a useful tool in future studies of antigen-specific homing. Stabilization of cells attached by their homing receptor sites will allow us to make quantitative assessments of the numbers of "specific" cells called to a region of the lymph node by antigen or other cell products.

Lymphocyte chemotaxis. Previous studies of lymph node microvasculature suggested that the structure of the HEV and physiological alterations of the lymph node microenvironment might establish a chemotactic gradient for lymphocytes (2, 3). During this past year, we have been examining the feasibility of testing this hypothesis in vitro using a recently developed chemotaxis system (4). In this system random migration (RM) was quantitated by measuring centripital migration perpendicular to the median axis of the chemotactic gradient and chemotactic migration (CM) was measured in line with the gradient. (CM-RM) = chemotactic differential (CD) in microns. (CM/RM) = chemotactic index (CI); CI must be $\geq$ 2 for chemotaxis to be proven. Data summarized in Tables V-VII reflect our progress so far.

TABLE V. DIFFERENTIAL CHEMOTAXIS BY MATURE SMALL LYMPHOCYTES

AGENT	CD (μm)	CI
Endotoxin activated serum	620.2	4.35
Activated C_{3a} + C_{3b}	780.8	5.60
Activated C_{5a} + C_{5b}	4.0	1.02
Low MW leukocyte extracts	680.1	5.00
N-Formyl-Met, Leu, Phe	2.8	1.01
N-Formyl-Met Met, Met	3.0	1.10
Activated T cell supernatant	71.3	1.35
Activated B cell supernatant	-16.0	0.91

The data (Table V) suggest that mature small lymphocytes of the type found in thoracic duct lymph (TDL) are selectively attracted by stimuli associated with chronic inflammation or immunization but are not attracted toward factors associated with acute inflammation. This is consistent with previous observations of cellular traffic across HEV which is restricted to lymphoid cells and excludes PMN except when the lymphatic tissue is acutely inflamed (3).

TABLE VI. CHEMOTACTIC RESPONSES OF NORMAL AND STIMULATED LYMPHOCYTES TOWARD ENDOTOXIN-ACTIVATED RAT SERUM

TYPE OF CELLS	CD (μm)	CI
Normal TDL, 90% T, 10% B	132	4.3
T-Cells		
Concanavalin A-stimulated TDL		
24 hr	16	1.2
48 hr	1410	6.9
B-Cells		
LPS-stimulated TDL		
24 hr	3	1.0
48 hr	21	1.4

These data (Table VI) reveal the remarkable mobility of the lectin-stimulated T cell which is attracted toward a gradient of mixed inflammatory mediators produced by endotoxin activated serum. During activation with concanavalin A these cells go through a refractory stage which may correlate with microtubule polymerization and membrane receptor stabilization since colchicine treatment permits these cells to migrate normally. Normal and activated B cells are by nature phlegmatic cells and do not migrate great distances even when stimulated.

We next investigated whether normal or stimulated lymphocytes would be attracted to factors released by other lymphocytes. These data are shown in Table VII.

TABLE VII. CHEMOTAXIS TOWARD FACTORS SECRETED BY OTHER LYMPHOCYTES

MIGRATING CELL TYPE	SUPERNATANT: LPS-stimulated[a] spleen cells CD (μm)	LPS-stimulated[a] spleen cells CI	Con A-stimulated TDL CD	Con A-stimulated TDL CI
B-blasts	30	1.1	-48	0.8
T-blasts	960	3.8	10	1.0
TDL	14	1.4	18	1.2

[a]Lipopolysaccharide

In these experiments, T-blasts appear to be attracted to factors released by LPS-stimulated B cells while they do not respond to factors released by Con-A-stimulated T cells. Normal TDL are weakly attracted to factors released by both T and B cell blasts.

In summary, the chemotaxis studies are adding to our understanding of the role of diffusable factors in regulating traffic of lymphocytes into lymph nodes. Some of these studies point to mechanisms by which antigenic stimulation may alter the composition of lymphoid cells in a lymph node by causing stimulated cells to release factors capable of selecting specific classes of lymphocytes.

Lymphocyte scrubbing. During EM studies of lymphocyte recirculation, unusual structural relationships between lymphocytes and endothelial cells were occasionally seen. The most obvious of these included phagocytosis and degradation of anti-lymphocyte globulin-treated lymphocytes. Occasionally fragments of migrating lymphocytes were pinched off by the adjacent endothelial cells, while sparing the lymphocyte. On other occasions active pinocytosis and membrane ruffling was associated with endothelial membranes which were in contact with anti-immunoglobulin-treated lymphocytes. Rarely, smooth membrane arrays of tubules were seen emptying into the space

between endothelial cell and lymphocyte,

These peculiar relationships between endothelial cells and lymphocytes, whose surfaces had been altered, suggested that the endothelium of HEV may remove something from or "scrub" the surfaces of migrating lymphocytes. To test this hypothesis, we treated TDL with anti-immunoglobulin FAB_2 fragments to which horseradish peroxidase (HRP) had been conjugated and injected these cells IV into groups of Lewis rats. At varying time intervals postinfusion lymph nodes were excised, fixed, and incubated 3,3'-diaminobenzidine tetrahydrochloride solution to develop the HRP reaction product. These tissues were embedded in Epon and examined by EM. The HRP reaction product was occasionally seen distributed as patches along the surfaces of adherent lymphocytes within the lumens of HEV. Occasionally capping and endocytosis of the HRP conjugate were seen in lymphocytes which had commenced migrating at the moment of fixation. Closer examination of the space between migrating lymphocytes and HEV endothelial cells revealed active pinocytosis involving the endothelial cell membrane. The HRP reaction product was clearly being removed from the lymphocyte surface through the action of the endothelial cell membrane. It is not known whether secretion of an enzyme by the endothelial cells may have preceded pinocytosis or if the endothelial cells were merely mechanically stripping the conjugate from the lymphocyte surface. This concept of lymphocyte scrubbing is attractive and has been suggested by other investigators who used it to explain some of the discrepancies associated with clonal selection, immune deviation and failure to respond to chronic viral infection. This is the first EM demonstration that this phenomenon may actually take place at the HEV.

In addition to peroxidase-conjugated anti-immunoglobulin, ferritin-Con-A conjugates were scrubbed from the surface of migrating lymphocytes. Lymphocytes with Con-A-ferritin patches may still be seen in the lumens of HEV up to 2 hr suggesting impeded emigration until the Con-A is removed.

Future studies of lymphocyte scrubbing will deal with removal of anti-idiotypic antibodies, anti-IgM and anti-IgD. These studies will establish the immunological importance of scrubbing, since these antibodies profoundly influence in vivo immune reactivity without injuring the coated cells: this effect is gradually lost as would be expected if the complexes were removed or degraded.

Adjuvant studies. Complete Freund's adjuvant (CFA) is still the most effective immunopotentiating agent, despite many years of research into possible replacements. It has the remarkable ability of potentiating both delayed hypersensitivity (DTH) and humoral antibody production, which is a feat that most synthetic adjuvants lack. It is not clear, however, whether this ability is beneficial. This is underscored by the fact that CFA has been associated with induction of arthritis, thyroiditis, orchitis and encephalitis of allergic etiology in experimental animals. Since CFA is capable of inducing immunity to a nonreplicating viral antigen which competes for potency and longevity with immunity induced by

the same virus in a live-attenuated form, CFA should be positive standard against which candidate adjuvants should be measured. When this is done, even the putative minimal molecular congener of CFA pales by comparison. The following recent studies have attempted to establish a baseline effect of CFA on the immune architecture of the injection site and the regional lymph node with emphasis on the cell populations involved and on the dynamic flux of cells through these tissues. Simultaneously, the ability of CFA to potentiate an immune response to C-84 inactivated VEE vaccine was studied in normal and thymectomized rats. Thymectomy of mature rats is thought to remove the source of immature immunoregulator T cells while leaving the animal with a normal complement of mature recirculating T cells.

The effects of SC injection of CFA on the local site and the regional lymph node were studied sequentially in 80 female Lewis rats. These tissues were studied using histologic, morphometric, microvascular perfusion and radiokinetic techniques. The initial local response to the deposit of CFA on day 0-1 was acute inflammation and hyperemia of existing vessels. PMN and fibrin exudate surrounded the lipid droplets. Between days 2 and 4 the PMN in and around the oil droplets began to degenerate and release their granules. On day 2 the surrounding vessels became tortuous. Multibranched, "cork screw"-like vessels penetrated the oil droplets on day 3. New vessel growth peaked in the injection site on day 7 as lymphocytes, monocytes and foam cells began to appear in the oil droplet wall. The PMN reaction was then finished. On day 14, lymphoid cell clusters and macrophages predominated; rare epitheleoid cells were present in the interstitium. New vessel growth was nearing completion as specialized tortuous vessels forming hairpin loops began to appear. Multiple small tuberculoid granulomas were first seen in the interstitium outside of oil droplets by day 21. Foam cells and lymphoid cell clusters were also seen; more specialized "hairpin" vessels were present. By 28 days new vessel growth had ceased although the "hairpin" vessels remained at sites of granulomas. Dense connective tissue appeared to wall off the oil droplets while the granulomas remained prominent in the interstitium. The oil droplets were completely walled off by 56 days. Large aggregates of lymphoid cells formed nodules in the interstitium near persisting granulomas and foam cells. Lymphocytes were seen crossing venous sinuses lined by flat endothelium in both the histological and the perfusion preparations.

These local changes were impressive and the fact that vascular proliferation at the local site corresponded with degeneration and lysis of PMN exudates suggested that factors in the PMN granules might influence vascular proliferation. In addition these PMN may have ingested mycobacteria and subjected them to lysozyme degradation to produce, endogenously, one or more muramyl dipeptide analogs. Although systemic adjuvant-like effects were already taking place, tuberculoid granulomas did not appear at the local site for at least 21 days. This suggests that granuloma formation may be the result of adjuvant effects rather than the cause. The effects of CFA on the regional lymph nodes from this study were quantitated and are presented in Table VIII.

TABLE VIII. EFFECTS OF CFA ON THE REGIONAL LYMPH NODE.

DAYS AFTER SC INJECTION	Node weight (mg)	MASS (mg) T Cortical	MASS (mg) B Follicle	LMI[a]	Traffic[b] index	HEV Total length (μm)
0	18	9.0	0.39	0.92	0.94	613
1	38	21.2	0.30	1.43	1.86	618
3	41	26.2	1.19	1.72	3.03	1199
7	53	37.0	1.22	1.35	2.24	1269
14	47	30.5	1.60	1.48	2.87	1251
28	38	22.8	2.17	1.50	1.03	3145
56	34	19.0	1.70	1.30	1.17	1740

[a] LMI = $\frac{\text{No. migrating lymphocytes}}{\text{No. endothelial cells}}$

[b] Traffic index = $\frac{\text{CPM regional lymph node}}{\text{CPM contralateral lymph node}}$

It is clear from these studies that the principal effects of CFA on the regional node lay in prolonged enlargement of the T cell cortex. These changes appear to correlate with both persistently increased lymphocyte traffic and activation of cortical T cells. In previous studies of antigen stimulation of regional lymph nodes using skin grafts or particulate microbial antigens, enlargment of the T-reactive cortex only persisted until the day 14 and subsequently gave way to germinal center expansion and plasma cell differentiation. CFA also induced marked proliferation of HEV which provided expanded endothelial surface for lymphocyte emigration. Since vascular proliferation in the regional nodes paralleled angiogenesis at the CFA injection site, it is attractive to speculate that leukocyte products and/or lymphokines generated locally may initiate and augment HEV proliferation in the regional lymph node.

When CFA was injected SC in combination with 0.3 ml of C-84 vaccine in normal and thymectomized rats marked potentiation and prolongation of peak titer was produced by CFA in thymectomized rats (Table IX).

TABLE IX. EFFECT OF THYMECTOMY AND CFA ON C-84 VEE TITERS

DAYS	C-84 Alone	+ Thymectomy	+ CFA	+ CFA + Thymectomy
0				
10	80 ± 1.2	320 ± 1.0	453 ± 3.0	1520 ± 1.2
18	106 ± 1.0	1470 ± 1.0	3378 ± 1.0	13195 ± 1.0
26	557 ± 2.1	1463 ± 1.7	6629 ± 1.2	15229 ± 1.1
40	403 ± 1.0	422 ± 3.1	5090 ± 1.3	8830 ± 1.1
53	640 ± 1.1	840 ± 6.0	3599 ± 1.5	7687 ± 1.1
82	320 ± 1.5	277 ± 4.1	3578 ± 1.4	5072 ± 1.2
160	64 ± 2.2	53 ± 6.1	538 ± 1.4	970 ± 3.2

Thymectomy produced at least one or two tube dilutions more titer in CFA-C-84 vaccinated rats. Thymectomy produced significant titer increases up to 26 days in control rats vaccinated only with C-84 vaccine; however, these titers returned to normal levels for the duration of the experiment. It may be possible that adult thymectomy, which selectively removed the source of short-lived T cells but not long-lived recirculating cells, will be a useful tool in identifying the population of T cells which are responsible for limiting the magnitude of an immune response.

Collaborative studies with LTC Ascher have shown that muramyl dipeptide (MDP) augmented delayed cutaneous hypersensitivity (DCH) to viral and bacterial skin test antigens in a manner similar to CFA when MDP was administered SC to guinea pigs as a water-in-oil emulsion. MDP did not augment DCH when given in media. Although a systematic morphological study was not performed, MDP in oil appeared to produce a fibroblastic and granulomatous inflammatory infiltrate in foot pads which had been inoculated 14-21 days before.

We achieved a parodoxical result when trying to assess the effect of varying the dose of MDP in media or in a water-in-oil suspension on the production of antibody. The results tabulated in Table X show a reciprocal relationship of MDP dose to level of antibody on day 14. These data suggest that there is a dose-related suppression of antibody production following primary immunization with C-84 antigen in combination with MDP. This somewhat disturbing observation is not without precedent.

TABLE X. EFFECT OF MURAMYL DIPEPTIDE DOSE ON THE PRN_{80} TITER OF VEE (C-84) VACCINE AT 14 DAYS

MDP (µg)	GEOMETRIC MEAN TITER + SD	
	In Oil	In Media
0	-	638 ± 1.2
0.2	1431 ± 1.5	640 ± 1.1
2.0	279 ± 3.2	452 ± 1.3
20.0	10	71 ± 1.8
100.0	ND	17 ± 1.3
500.0	ND	9 ± 1.2
0 (CFA)	1270 ± 1.8	-

None of the published studies claiming adjuvanticity of MDP shows potentiation of antibody levels following primary immunization. Significant elevations of antibody were only seen after secondary boost with antigen. In their zeal to show the adjuvanticity of MDP those authors neglected to observe an important difference between the effects of CFA and MDP, its putative minimal molecular congener. Primary immunization with CFA + antigen elevated both the primary and secondary immune responses, while MDP only augmented secondary immune responses. This effect could be produced if MDP produced a change in the differentiation of stimulated lymphocytes. It is customary to think of antigen-induced lymphocyte proliferation as productive of a large number of plasma cell precursors and T helper cells with relatively few B and T memory cells. It is possible that MDP causes memory cell production at the expense of plasma cell differentiation. This hypothesis could explain both the DCH observations shown by LTC Ascher in guinea pigs and the necessity for secondary boost for antibody augmentation.

When the studies of adjuvant effects of doses of MDP in media or oil on antibody titers to C-84 VEE vaccine were continued into secondary vaccination stages, none of the primary responses equaled the levels obtained by inclusion of the vaccine in Freund's complete adjuvant. In the secondary responses the results formed 2 groups, (2) equal or better than the CFA group and (b) equal to or less than the C-84 control group. The high secondary titers were produced by 2.0 µg MDP/saline + C-84, 0.2 µg MDP/oil + C-84, CFA and C-84, and 20.0 µg MDP/saline. Control secondary titers were produced by C-84 alone, 2.0 µg MDP/oil + C-84, and 0.2 µg MDP/saline + C-84. The heterogeneous data suggest that MDP has an inconsistent dose-effect on immunity and requires further study with respect to mechanisms before its utility as a human adjuvant can be predicted (Table XI).

TABLE XI. EFFECT OF MDP IN SALINE OR IN OIL ON IMMUNITY OF VEE C-84 VIRUS

	GEOMETRIC MEAN TITER OF C-84 + MDP VACCINE + SALINE (OIL)				
DAY	C-84 ALONE	0.2 µg MDP	2.0 µg MDP	20.0 µg MDP	C-84 + CFA
Primary:					
7	113	40 (105)	95 (22)	31 (<10)	31
14	97	253 (557)	452 (63)	63 (<10)	2100
31	861	1015 (1463)	905 (100)	806 (<10)	5034
42	430	1269 (1280)	1280 (63)	403 (<10)	3422
56	640	1015 (1463)	1280 (63)	640 (<10)	3778
86	430	403 (1273)	1522 (50)	320 (<10)	2085
Secondary: (Day 86)					
7	2549	2539 (5095)	10000 (4031)	8000 (15)	6370
14	3026	2015 (6660)	8458 (2539)	6349 (14)	8000
28	2544	1600 (8746)	10000 (4031)	8000 (20)	6349
42	2152	1007 (8746)	10000 (2015)	5039 (14)	8000
56	1280	640 (5059)	5079 (1600)	1612 (10)	2560
86	905	806 (3598)	5079 (1600)	1600 (7)	3200

Presentations:

1. Anderson, A. O. Cells and tissues of the immune system, Lecture 8 of Principles of Immunology. Presented, The Johns Hopkins University School of Medicine, Baltimore, MD, 2 Dec 77.

2. Anderson, A. O. T and B cells in lymphatic tissues. Presented, Hood College Masters Program Lecture in Pathogenesis, Frederick, MD, 30 Jan 78.

3. Anderson, A. O. Lymphocytes, recirculation, lymph node structure, T and B cells, and cell cooperation. Presented, The Johns Hopkins Immunology Council Survey Course on Pathogenesis of Allergic and Infectious Diseases, Lecture 10, The Johns Hopkins University School of Medicine, Baltimore, MD, 24 Feb 78.

4. Anderson, A. O. Immunosurveillance of tumor immunity. Presented, Hood College Masters Course on Pathogenesis, 15 Mar 78.

5. Anderson, A. O., N. D. Anderson, R. D. Hoffman, and J. D. White. Cytoskeletal control of lymphocyte recirculation. Presented, American Association of Pathologists, Atlantic City, NJ, 9-14 Apr 78 (Fed. Proc. 37:591, 1978).

6. Anderson, A. O. Lymphocyte homing and recirculation. Presented, Washington Pathology Society, Bethesda, MD, 23 May 78.

7. Anderson, A. O. Control of lymphocyte recirculation. Presented, Department of Bacteriology and Immunology, University of Glasgow School of Medicine, Glasgow, Scotland, 7 June 78.

8. Anderson, A. O., N. D. Anderson, and J. D. White. Basic mechanisms of lymphocyte recirculation. Presented, VI International Conference on Lymphatic Tissues and Germinal Centers in Immune Reactions, Damp/Kiel, Germany, 11-16 June 78 (Z. Immun. Forsch. 154:298, 1978).

9. Anderson, A. O. Importance of transmembrane cytoskeletal control of cell surface receptors in lymphocyte homing and emigration. Presented, Gordon Research Conference on Immunochemistry and Immunobiology, Plymouth, NH, 4 Aug 78.

Publications:

1. Anderson, N. D., A. O. Anderson, R. D. Hoffman and J. D. White. 1978. Cytoskeletal control of lymphocyte recirculation. II. Studies with colchicine. Fed. Proc. 37:591.

2. Anderson, A. O., N. D. Anderson, and J. D. White. 1979. Basic mechanisms of lymphocyte recirculation. Advan. Exp. Biol. Med. In press.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 04 21	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
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FISCAL YEAR CURRENT 79	1.0	123.9

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NAME:

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POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Arboviruses; (U) Vaccine development; (U) Dengue virus

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual 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) 77 10 - 78 09 - Studies with selected clones of dengue (DEN-1) continued. Data obtained with both parent viruses and selected clones indicate that with DEN-1 the mouse virulence "marker" will be of no value in selecting avirulent subpopulations for possible human vaccine use, since neither parents nor clones kill suckling mice with regularity via the IC route. Much emphasis has been placed on the development of a highly sensitive assay procedure for detection of viremia in rhesus monkeys, since a decreased ability to elicit viremia in monkeys is the one "marker" of presumed avirulence that has yet to be disproved. The parameters defined thus far include: (a) sampling sera daily for 14 days postinoculation; (b) direct plaquing on LLC-MK-2 cells using both undiluted serum and 1:2 or 1:5 dilution of serum to bypass serum inhibitors, and (c) concurrently inoculating cells (LLC-MK-2 or SW-13) under liquid medium to allow amplification of low level virus in these serum specimens. Initial studies incorporating all of these parameters are underway in 14 rhesus monkeys.

Publication: In: Ebola Virus Hemorrhagic Fever, pp. 237-242, Elsevier/North-Holland Biomedical Press, 1978.

*Available to contractors upon originator's approval

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 011: Development of Arbovirus Vaccines for Disease of 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).

Our DEN-1 program is at an earlier stage of development than are the others. Thus far, we have adapted 3 human DEN-1 isolates to acceptable cells (DBS-103), performed plaque-to-plaque passages to obtain pure subpopulations and carried out some temperature sensitivity and mouse and monkey virulence studies. Much emphasis was placed this year on increasing the sensitivity of the monkey viremia assay system, due to the singular importance of this marker in documenting decreased virulence.

Progress:

As reported last year (1) 3 DEN-1 strains, isolated from human serum specimens and adapted to grow to ~ 10^6 PFU/ml in DBS-103 cells, were subjected to plaque-to-plaque passage in these cells to select small plaque subpopulations (or variants). More than 30 such subpopulations were isolated from the DEN-1, #1, #2 and #3 "parents" (7th passage material in DBS-103 cultures). During the year baseline data were accumulated on the 3 "parent" strains and selected plaque variants, with emphasis on the PL-2 variant from the DEN-1 #1 "parent." The PL-2 variant is a consistently small (1-2 mm) plaque former which had shown the greatest promise on preliminary evaluation (1).

Temperature sensitivity (ts) studies. Two types of ts studies are employed to determine "markers" of presumed, altered virulence. In the first type, direct plaquing at temperatures of 35 to 40°C in selected cultures, e.g., BS-C-1 or LLC-MK_2, is employed. In this test one is simply comparing the ability of "parent viruses and selected clones to form plaques at given temperatures. In theory, attenuated clones should be restricted at higher temperatures in comparison with the "parent." In the second ts test, growth curves are conducted in selected, fluid-overlaid cell cultures, e.g., BS-C-1, LLC-MK_2, DBS-103, IMR-90 and WI-38, at the temperatures noted above. Again, it is anticipated that the less virulent clones or variants will be restricted in terms of peak virus release as temperatures are increased vis-a-vis "parent" virus which should exhibit higher permissive temperatures. It should be noted, however, that neither ts test is an absolute "marker" of altered

in vivo virulence, although experience with DEN-2 and DEN-3 strains indicated that either test may be used to screen selected clones for further study (2; P. K. Russell, WRAIR, personal communication).

The PL-2 vaccine candidate strain has been assayed for ts by the direct plaquing methods. As shown in Table I all 3 "parent" viruses were plaqued at 35°C in new LLC-MK_2 cells obtained from WRAIR. (These new cells vary significantly from the MK_2 cells that previously were used at USAMRIID, but are now available for general use.) In addition DEN-1 #1 "parent" and PL-2 were tested both at 35 and 39.3°C. Both exhibited about 1 $\log_{10}$ drop in titer when assayed at 39.3°C. Clearly, PL-2 which is a plaque-purified, small plaque variant, is not ts in terms of this test as compared with parent virus.

TABLE I. PLAQUE FORMATION BY PARENT DEN-1 VIRUS AND THE SMALL PLAQUE, PL-2 STRAIN, IN LLC-MK_2 CELLS AT 35 AND 39.3°C

VIRUS	PFU/ml	
	35°C	39.3°C
DEN-1 #1 "parent"	1.9×10^6	8.7×10^4
DEN-1 #2	1.2×10^7	N.T.[a]
DEN-1 #3	4.8×10^6	N.T.
PL-2	6.6×10^6	2.9×10^5

[a] N.T. = not tested.

Due to mechanical failure of one of our water bath's temperature controller/circulator, data are still being accumulated from growth curve-type ts evaluations of DEN-1 #1 parent vs. PL-2 in a variety of cell cultures at 35 and 39°C. However, in evaluating several types of cell cultures for their applicability, certain information has been accumulated. Table II summarizes typical qualitative results.

TABLE II. EVALUATION OF CELL CULTURES FOR USE IN GROWTH CURVE STUDIES OF PARENT DEN-1 VIRUS AND THE SMALL PLAQUE, PL-2 STRAIN AT 35 and 39°C

CELL TYPE	TEMP °C	CPE BY DAY POSTINOCULATION[a] (Parent/PL-2) 3	4	5	6	7
DBS-103	35	N.R.[b]	1+/±	2+/1+	3+/2+	3+/3+
	39	N.R.	1+/-	1+/ -	1+/ -	1+/ -
LLC-MK-2	35	-/-	±/±	1+/1+	1+/1+	1+/1+
	39	-/-	±/±	±/ ±	1+/1+	1+/1+
WI-38	35	-/-	N.R.	3+/2+	4+/4+	4+/4+
	39	±/-	N.R.	2+/1+	3+/1+	3+/1+
BS-C-1	35	1+/2+	1+/2+	1+/1+[c]	-	-
	39	1+/1+	-/ -[c]	-/ -[c]	-	-

[a] A given cell type was inoculated with the same virus input and then randomly placed at either 35 or 39°; inputs ranged from 10^5-10^6 PFU.

[b] N.R. = not recorded.

[c] By days 4 to 5 postinoculation control cultures of BS-C-1 have shown 2+ to 4+ CPE, thus negating results.

In all cases CPE readings of control cultures have been subtracted from those for infected cultures. As a result of these evaluations the following generalization can be made: (a) BS-C-1 cultures are unsuitable for these studies since 3+ to 4+ CPE occurs in controls by day 4 or 5, particularly at 39 or 40°C, (b) WI-38 and DBS-103 controls hold up better than other cultures at higher temperatures and also exhibit differences in degree of CPE between parent and PL-2 at the higher temperatures; and (c) LLC-MK_2 cultures fall between the other 2 groups of cells in terms of heat lability. That is, 1+ or occasionally 2+ CPE occurs at higher temperatures, but no differentiation can be made between parent and PL-2 in terms of CPE produced. Further studies of this ts "marker" system will be made using both WI-38, a human cell strain and LLC-MK_2 cells. Fortunately, a broad data base is available for DEN-2 and DEN-3 using this last cell system, which should prove useful for comparative purposes.

Suckling mouse (SM) virulence marker. In an earlier report we noted that in one test a clear difference was seen between PL-2 and DEN-1 #1 parent virus in terms of titer in SM inoculated intracerebrally (IC) (1). These tests have been repeated using a variety of mouse sources, including the WRAIR mouse colony, upon which all of their DEN-2 and 3 data are based. In no case could we confirm our earlier data. In fact, upon switching to WRAIR mice exclusively it became quite apparent that with DEN-1 parents and clones, one can expect

only scattered deaths that are not dose-related. This held true even for the new seed viruses prepared in primary green monkey kidney (PGMK). Thus the mouse virulence marker cannot be used for the isolates with which we are working. It should be noted that this marker is not crucial to DEN vaccine programs. For example, the other programs such as that of DEN-3 at WRAIR has the same problem while the University of Hawaii DEN-4 program suffers from the reverse problem, viz. both parent and vaccine candidates kill SM via the IC route with no appreciable differences being documented between the "parent" and "avirulent clones."

Preparation of new "parent" virus seeds in PGMK culture. During the course of this year's studies we became concerned that our original DBS-103-grown DEN-1 #1, #2 and #3 "parent" viruses may have been attenuated simply due to the 7 serial passages made in these cells from the original human serum specimens. We therefore carried out 3 serial passages in PGMK cell cultures to produce new "parent" viruses which would hopefully retain their presumed ability to induce viremia in monkeys. These new "parent" viruses were intended simply to be used as controls in monkey viremia, mouse virulence and ts studies. Shown in Table III are the results of plaque assays on the first passage of the 3 isolates.

TABLE III. REISOLATION OF 3 DEN-1 VIRUSES FROM HUMAN SERUM SPECIMENS IN PGMK CELL CULTURE

ORIGINAL SERUM NO.	DAY POST INOCULATION	PFU/ml (SIZE) BS-C-1	PFU/ml (SIZE) SW-13
DEN-1 #1	7	6.0×10^3 (0.5 mm)	
	10	6.5×10^5 (0.5 mm)	
	14	1.8×10^6 (0.5 mm)	1.8×10^6 (0.5 mm)
DEN-1 #2	7	3.6×10^2 (0.5 mm)	
	10	1.4×10^4 (0.5 mm)	
	14	1.2×10^6 (0.5-6.7 mm)	7.5×10^5 (0.5 mm)
DEN-1 #3	7	0	
	10	5.8×10^3 (0.5 mm)	
	14	6.8×10^4 (0.5 mm)	1.9×10^4 (0-5 mm)
DEN-1 #3 P 7, 103	-	2.3×10^6 (0.5-4.0 mm)	4.9×10^6 (0.5-5.0 mm)

As indicated, high titers were achieved with DEN-1 #1 and #2 by day 14 postinoculation; DEN-1 #3 reached nearly 5 $\log_{10}$ PFU/ml. In the subsequent 2 passages in PGMK culture preliminary titrations showed that titers were similar with the exception of a slight increase to >5 $\log_{10}$ PFU/ml for #3 isolate. Of some interest here is the fact that all 3

PGMK-grown isolates exhibited very small plaques (0.5 mm) both on BS-C-1 and SW-13 cultures. The DEN-1 #3 p-7, DBS-103 control is indicative of the plaque sizes and range we have always seen with the DBS-103-grown "parents," the preponderance being in the 1-2 mm range. Recently, similar observations have been made with the new Caribbean isolates of DEN-1 (Dr. Brandt, WRAIR, personal communication). As indicated above these newly isolated strains did not exhibit any more virulence for SM via the IC route than did the same isolates when grown out on DBS-103 cultures.

Rhesus monkey viremia (marker) studies. Due to a lack of sufficient biological containment holding space for monkeys (until just recently) only 2 monkey studies were taken to completion, with 2 others in progress now that containment restrictions have been modified. All studies are being conducted in cooperation with MAJ Stephen, AA Division.

One of the generally accepted "markers" of decreased virulence for attenuated DEN virus strains is a depression of viremia in rhesus monkeys as compared to parent viruses from which the strains were derived. To test this in vivo "marker" for one small plaque variant (PL-2), 2 monkeys each were inoculated SC with approximately 10^5 PFU of either parent virus (DEN-1 #1, 7th DBS-103 passage) or the PL-2 variant. Serum specimens were obtained on days 0 and 1-10 postinoculation for viremia determinations and on days 0, 14, 30 and 60 for 80% plaque reduction serum neutralization PRN_{80} tests. Clinical observations were made daily during the course of this first study.

By day 30 postinoculation all 4 animals had converted serologically showing PRN_{80} of 1:40 and > 1:160 for those given parent virus and 1:10 and 1:40 for those given PL-2. As tested by direct plaquing on LLC-MK_2 cells no virus was detected in any specimens taken on days 1-10 postinoculation; a repeat test confirmed this. Since WRAIR at the time had experienced similar difficulties in direct plaquing from human specimens (Dr. Brandt, personal communication) it was agreed that all future viremia determinations would be conducted not only by direct plaquing, but also by inoculation of appropriate liquid-overlaid cell cultures with subsequent plaque assay of culture fluids (allowing 7-10 days for outgrowth). With regard to clinical evaluation, as expected the monkeys presented no signs of dengue infection.

After extensive discussion of these earlier results with WRAIR personnel, a consensus was reached that the "parent" virus, which was obtained by direct passage of DEN-1 #1 from human serum into DBS-103 cells (a total of 7 passages were made), may have been attenuated for monkeys to a large degree simply through environmental pressures peculiar to this virus/cell combination. If this were indeed the case, it would have explained the absence of viremia in monkeys given the "parent" virus. Thus, it was possible that we had a candidate vaccine strain but could not prove it at least in terms of decreased monkey virulence. As a result we carried out the serial passage from the original human serum specimens in PGMK cell cultures as described above. At the time, this decision was based on the fact that WRAIR's DEN-2 "parent" strain produced viremia in rhesus monkeys even after 6 serial passages in PGMK culture

Thus, a second study was initiated in which 3 monkeys each were inoculated SC with the new PGMK-grown parents. On days 1-10 serum was obtained to determine viremia by 2 methods: (a) the direct plaque technique on LLC-MK_2 and/or BS-C-1 cultures, and (b) a qualitative method in which monkey serum specimens are inoculated onto SW-13 cultures (highly susceptible cells) under liquid medium with subsequent plaque assay of the culture fluids after 8-15 days outgrowth. Additional serum specimens were obtained on days 0, 14, 30 and 60 postinoculation for PRN_{80} tests. Monkeys were observed daily for signs of illness.

As shown in Table IV, all monkeys were infected as indicated by their day 30, PRN_{80} titers; these tests are being repeated in new, low-passage BS-C-1 cultures to get absolute titers. Viremia results were more encouraging than any we have previously observed. Although occasional spurious plaques were obtained in the BS-C-1 systems, the LLC-MK_2 system clearly detected virus, most often on days 3-7. Only one monkey, T-302, was negative for virus on all 10 days when tested in this manner. Significantly, all direct plaquing with undiluted serum yielded negative results, whereas dilution of serum 1:2 prior to testing yielded the positive results indicated. Further the indirect outgrowth methods in SW-13 cultures not only confirmed the viremias detected by the direct plaquing technique but also detected virus that was not demonstrable by the former technique. It should be noted that with the direct plaquing method in no case were 2 to 3 $\log_{10}$ PFU/ml obtained as was the case with DEN-2. In this second study most titers were between 1.0-1.8 $\log_{10}$ PFU/ml. Confirmatory titrations are being made on low passage BS-C-1 and LLC-MK_2 cultures by the direct plaque technique. Upon completion reliable quantitative data will be reported in detail.

TABLE IV. RESPONSES OF RHESUS MONKEYS TO SC INOCULATION OF PGMK-GROWN DEN-1 VIRUSES

MONKEY NO.	INOCULUM Identification	INOCULUM Titer (PFU)	VIREMIA (Days 1-10)	RECIP. PRN_{80} TITER (Day 30)
T-307	DEN-1 #1 p1 PGMK	$10^{5.8}$	+	$\geq$ 20
T-303			+	$\geq$ 20
B-7050			+	$\geq$ 20
T-309	DEN-1 #2 p1 PGMK	$10^{5.7}$	+	$\geq$ 20
T-302			-	$\geq$ 20
4837			+	$\geq$ 20
T-308	DEN-1 #3 p1 PGMK	$10^{4.2}$	+	$\geq$ 20
T-277			+	$\geq$ 20
B-6973			+	$\geq$ 20

It should be emphasized that the acquisition of a monkey "marker" is of great importance to the DEN-1 program. At the recent Dengue Vaccine Task Force Meeting (WRAIR, June 1978) the concensus was that the ultimate test of avirulence for man is the inability of vaccine candidate strains to elicit viremia in rhesus monkeys. Mouse virulence, plaque size and ts markers were considered excellent for use as "screening" aids, but could not be accepted as absolute markers of avirulence in the absence of the monkey "marker." Extensive discussions at this meeting also brought to light problems encountered by others in detecting both viremia in monkeys and man. Unfortunately no data base or DEN-1 virus specimens with known monkey virulence were available from any of the acknowledged dengue experts in attendance. It was once again emphasized, however, that both direct plaquing and outgrowth (or enrichment) procedures would be routinely employed; also, sampling for viremia as late as day 14 was also mentioned as a possible improvement on the heretofore standard 10-day viremia sampling period. In an effort to aid us in evaluating and refining our assay systems, WRAIR recently provided us with two DEN-1 strains, which may cause viremia in monkeys (#13802 and Corec A). Recently these have been inoculated into 2 rhesus monkeys each. Serum samples taken on days 1-14 will be tested as described above for virus content; routine serology will also be performed. To reiterate, it is of utmost importance that: (a) "parent" viruses be capable of producing detectable viremias in monkeys, so that we can demonstrate the lack of this characteristic in vaccine candidates and (b) the most sensitive virus assay systems available be developed or modified to this end. Much effort will be spent on this portion of the DEN-1 program for the next few months.

Additional "marker" and other studies. In an attempt to define additional "markers" for the small plaque variant (PL-2) candidate vaccine strain, growth curves at 35°C were obtained for the PL-2 strain in the following cell cultures: DBS-103, WI-38, and IMR-90. Parent viruses (DEN-1, #1, #2 and #3) were used as controls in this study. It was hoped that some differences would be seen between the parents and the small plaque variant either in terms of degree of CPE produced during a 7-day incubation period, or in terms of virus yields. All cultures received approximately 10^5 PFU of the respective viruses. Samples for subsequent titration in BS-C-1 cultures were taken on days 3-7, at which times CPE was recorded (Table V). Considerably less CPE was elicited by the PL-2 strain in both WI-38 and IMR-90 cell cultures through day 6, although clearly visible differences could be seen by day 3. In a repeat study using only IMR-90 cultures it was again possible to clearly distinguish the degree of CPE produced by the PL-2 strain and the parent virus, DEN-1, #1. Interestingly, no consistent marked differences were noted in the virus yields of the 4 DEN-1 strains in the 3 culture types tested. Although further confirmation must be obtained in a "double-blind" study it would appear that a useful *in vitro* marker has been defined.

TABLE V. CPE PRODUCED IN CELL CULTURES BY 4 DEN-1 STRAINS

DAY POST-INOCULATION	VIRUS STRAIN	CPE[a] BY CELL TYPE		
		DBS-103	WI-38	IMR-90
3	DEN-1 #1	±	1+ to 2+	1+ to 2+
	" #2	±	1+ to 2+	1+ to 2+
	" #3	±	1+ to 2+	1+ to 2+
	PL-2	±	±	±
4	DEN-1 #1	1+	2+ to 3+	2+ to 3+
	" #2	1+	3+ to 4+	3+ to 4+
	" #3	1+	3+ to 4+	3+ to 4+
	PL-2	1+	± to 1+	±
5	DEN-1 #1	2+	3+ to 4+	3+ to 4+
	" #2	2+	3+ to 4+	3+ to 4+
	" #3	2+	3+ to 4+	3+ to 4+
	PL-2	2+	1+	1+
6	DEN-1 #1	3+	4+	4+
	" #2	3+	4+	4+
	" #3	3+	4+	4+
	PL-2	2+	2+	2+
7	DEN-1 #1	4+	4+	4+
	" #2	4+	4+	4+
	" #3	4+	4+	4+
	PL-2	3+	3+	3+

[a]Control cultures appeared normal throughout the incubation period.

As reported previously (1) the DEN-1 #3 "parent" has not plaqued consistently in DBS-103 cultures. However, in an effort to obtain more clones to screen as vaccine candidates, we are again attempting to perform plaque-to-plaque purification of this isolate using the 7th DBS-103 passage ("parent") as starting material. On the first plaquing attempt 19 plaques were detected by microscopic examination, and placed in suspension with 50% FCS and HBSS for subsequent sizing and titration in BS-C-1 and LLC-MK_2 cells and to use for the 1st plaque-to-plaque passage. Unlike plaque suspensions obtained from DEN-1 #1 and #2, these suspensions exhibited extremely low titers, 1-3 PFU/ml to a maximum of approximately 10^3 PFU/ml in only 2/19 plaque pick suspensions. In the BS-C-1 plaque system virtually all the plaques observed are 2 mm in diameter, with very few at the 3-mm level. The first plaque-to-plaque passage attempt yielded no plaques; however, due to the low titers of the suspensions and the low

plaquing efficiency of the virus in these cells, limited success was expected. Repeated plaquing from these suspensions proved fruitless.

It should be noted that additional cloning is being attempted concurrently from the other 2 DEN-1 "parents" as well as from plaque suspensions of both "parents" at various stages of plaque purification. All clones thus obtained will be screened for the direct plaquing ts "marker." Those possessing the ts marker will be further evaluated.

Work with the hydroxylapatite column chromatographic technique described last year (1) was discontinued early in this reporting period. The main reasons were: (a) a wide range of pH and phosphate buffer molarities failed to produce pure large or small plaque separation, although extremely shallow gradients were admittedly not evaluated; and (b) in view of the many other markers employed in all the DEN vaccine programs, the need for and significance of this marker were subject to reappraisal. No further collaborative effort with Dr. Jahrling in this area is anticipated.

In-progress and future studies under this work unit, in addition to those already mentioned will include the following: (a) isolation and characterization of additional clones from large volume Caribbean serum specimens to be supplied by COL Russell; (b) reevaluation of the DBS-103 pass-7 "parents" and selected clones in rhesus monkeys using the improved viremia assay system described herein; (c) establishment of additional markers of avirulence such as sensitivity to polyanions such as heparin or dextran sulfate and (d) use of alternate passage of selected parent viruses in CEC and DBS-103 culture in an effort to attenuate the viruses by adapting to cells from an unnatural host (i.e., chickens). Such an approach has shown promise in the Machupo vaccine program (3).

Presentations:

1. Cole, F. E., Jr. Progress with Dengue-1 vaccine. Presented at the Dengue Virus Vaccine Workshop, WRAIR, Washington, DC, 6 Jun 78.

2. Cole, F. E., Jr. Problems in viral vaccine development. Presented at Program Status Review, USAMRDC, Fort Detrick, MD, 9 Aug 78.

Publication:

Eddy, G. A. and F. E. Cole, Jr. 1978. The development of a vaccine against African hemorrhagic fever, pp 237-242. In Ebola virus hemorrhagic fever (S. R. Pattyn, ed.). Elsevier/North-Holland Biomed Press, New York.

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1. U.S. Army Medical Research Institute of Infectious Diseases, 1 Oct 1977. Annual Progress Report, FY 1977, pp. 107-116. Fort Detrick, MD.

2. Eckels, K. H., W. E. Brandt, V. R. Harrison, J. M. McCown, and P. K. Russell, 1976. Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. Infect. Immun. 14:1221-1227.

3. U.S. Army Medical Research Institute of Infectious Diseases, 1 Oct 1977. Annual Progress Report, FY 1977, pp. 157-164, Fort Detrick, MD.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OD6415	78 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
77 10 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	012
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(U) Studies in immunization of the respiratory tract

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
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17. CONTRACT GRANT

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18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.0	155.0
	CURRENT 79	1.0	152.7

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21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede 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 (Furnish individual 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 operation, 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) 77 10 - 78 09 - Respiratory inoculation established chronic Pseudomonas pseudomallei infections in mice which were limited primarily to the lungs and persisted at least 80 days without overt signs of illness. Twenty intracerebral followed by 5 IP passages enhanced the low virulence for mice to less than 20 organisms/median lethal dose. Hamsters vaccinated with either formalin-killed virulent P. pseudomallei or a live avirulent strain developed significant levels of humoral antibody; 38% of those vaccinated with the inactivated preparation survived respiratory challenge which killed all live-cell vaccinated hamsters. Four replicate vaccinations with killed cells increased the survival rate to 70%. No protection followed repeated vaccination with live, avirulent strain.

Publication: Antimicrob. Agents Chemother. 13:284-288, 1978.
Lab. Anim. Sci. 28: in press, 1978.

*Available to contractors upon originator's approval

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 012: Studies in Immunization of the Respiratory Tract

Background:

Pseudomonas pseudomallei, causative organism of melioidosis, has been considered a potential threat agent requiring medical defense considerations. It also produces natural infections of significant military importance. The organism is easily produced at high concentrations in large volumes, and its production requires no sophisticated facilities. It is infective by the aerosol route as well as through skin abrasions, wounds or ingestion. The disease is endemic in tropical and subtropical areas of SE Asia where the organism exists as a free-living saprophyte; 30% or more of the adult populations of these areas have had subclinical exposure to melioidosis (1).

By contrast, the disease has been seen in the U.S. primarily in people who have returned from countries where it is known to exist. It seems reasonable to predict high casualty rates in such an unprotected population as a consequence of exposure to aerosols of this agent. There are at present no immunoprophylactic procedures recommended for the disease.

Its management depends almost entirely on antibiotic therapy. However, severely ill patients require large antibiotic doses which must be maintained until after pulmonary lesions are resolved (2), and even then, bacteriological relapse may occur.

Studies were initiated to increase fundamental knowledge on the mechanisms of pathogenesis and immunogenesis of respiratory melioidosis that would be useful in developing means for its management.

Progress:

Development of an infection model in mice and hamsters. Since our studies are concerned primarily with respiratory melioidosis, we sought a suitable small animal susceptible to infection by the respiratory route. Pathogenesis of 2 strains of P. pseudomallei obtained from ATCC, 23343 and 11668, was studied.

Strain 11668 proved essentially avirulent for both mice and hamsters. Both species survived large doses administered by either the IN or IP route without overt signs of illness. Strain 23343 was extremely virulent for hamsters, but not mice; 50% of the hamsters given 20 CFU by either the IP, IN, or aerosol route died within 2-3 days.

Bacteriological assays of tissue from hamsters exposed to aerosols of 23343 demonstrated that the bacteria multiplied rapidly in the lungs (Table I).

TABLE I. REPLICATION OF P. PSEUDOMALLEI IN HAMSTERS FOLLOWING INHALATION OF 300 CFU (n = 2)

HOURS AFTER EXPOSURE	LOG_{10}/ORGAN			
	Lung	Blood (1 ml)	Liver	Spleen
8	1.1	0	0	0
24	5.7	0.7	2.9	0
48	8.4	6.0	6.9	6.8
72	7.0	5.7	8.0	7.5

There was a progressive pulmonary spread of the infection with concentrations reaching 10^8 organisms/lung at 2 days. Blood stream invasion was apparent by 24 hr. Replication in liver and spleen, although lagging slightly behind that in the lung, reached similarly high levels within 3 days. None of the hamsters survived beyond 3 days.

During this rapidly fatal infection, macroscopic lesions often were not apparent in organs other than the lung. However, in a few of the hamsters given high doses, in addition to almost complete hemorrhagic pulmonary consolidation, spleen and liver had many small discrete lesions: occasionally a purulent dissolution of the eyes occurred.

Because of the rapid course of the disease in hamsters, they are of only limited usefulness in studies of chronic melioidosis, immunizing procedures or evaluation of therapeutic agents. Although mice are resistant to this organism, Nigg et al. demonstrated that its virulence for mice could be enhanced by serial intracerebral (IC) passage (3). Based on these procedures, a series of IC passages of 23343 was initiated in mice. Data in Table II indicated that 20 IC passages of infected brain tissue reduced the LD_{50} from >10,000 to <20 organisms when administered by the IC route, but no change in virulence was apparent when the same material was administered IP. However, 5 subsequent passages of

infected spleen tissues by the IP route resulted in a dramatic increase in virulence when administered by the IP, IN or aerosol route: 50% of the mice died within 5-7 days after receiving a dose of <20 CFU.

TABLE II. SERIAL PASSAGE OF P. PSEUDOMALLEI (ATCC 23343) IN MICE

ROUTE AND PASSAGE NO.			LOG_{10} ORGANISMS/LD_{50} IN MICE			
IC	IP	INOCULUM	IC	IP	IN	AEROSOL
0	0	Culture	>4.7	>4.7	>4.7	
2	0	Brain	4.0			
11	0	Brain	2.5			
20	0	Brain	<1.3	>4.7		
20	1	Spleen		4.0		
20	5	Spleen		<1.3	<1.3	2.4

Data in Table III indicate that the rapid multiplication of the adapted strain in mice and the gross pathology observed were similar to what was seen in hamsters.

TABLE III. REPLICATION OF PARENT AND MOUSE-ADAPTED P. PSEUDOMALLEI IN MICE AFTER IN INOCULATION OF 200 CFU (n = 2)

DAYS AFTER INOCULATION	LOG_{10} CFU/ORGAN					
	MOUSE-ADAPTED			PARENT		
	Lung	Liver	Spleen	Lung	Liver	Spleen
1	4.9	0	0	3.0	1.6	1.7
2	7.8	6.9	0	4.7	4.1	0
3	8.3	6.6	6.7	4.0	0	0
7	Died day ≤4			2.9	0	0
23				2.9	1.0	0
55				2.6	0	0
76				3.2	1.0	0

The parent strain replicated; significant numbers persisted in the lungs of mice, but peak concentrations were 3-4 logs lower than those seen with the adapted strain and apparently did not reach critical levels. Viable organisms were detected only sporadically in other organs; mice exhibited no ill effects of the infection. None of the mice died by 76 days after exposure; preliminary histopathological examinations have not revealed any significant pathological lesions. In anticipation of its usefulness as a chronic disease model, these experiments will be expanded to characterize pathologic changes induced by this apparently benign infection.

Immune response of hamsters to vaccination. While the infection model work was progressing, experiments were initiated to obtain baseline data on immune response patterns in hamsters vaccinated with either formalin-killed virulent organisms (strain 23343) or the live, but avirulent, strain 11668. Groups of Golden Syrian hamsters were inoculated with either 10^9 killed cells or 10^6 live, avirulent cells either singly or as 4 replicates given at weekly intervals. In each case, 1/2 the dose was given IN and 1/2, IP. At selected intervals after inoculation, samples of whole blood were assayed for lymphocyte transformation based on the incorporation of tritiated thymidine; sera and bronchoalveolar lavage fluids were assayed for antibody by IFA procedures; spleen lymphocytes were collected from each hamster for measurement of migration inhibition factor (MIF) activity.

As shown by data in Table IV, a single dose of either antigen stimulated development of significant levels of humoral antibody. Titers in hamsters vaccinated with the killed preparation peaked at 3 weeks, then started to diminish.

TABLE IV. IFA ANTIBODY TITERS OF HAMSTERS FOLLOWING PRIMARY INOCULATION WITH KILLED LIVE P. PSEUDOMALLEI

VACCINE	GEOMETRIC MEAN IFA TITER BY DAYS					
	3	7	14	21	28	35
Formalin-killed, 23343	0	156	195	319	135	94
Live, avirulent 11668	0	177	135	102	102	ND

Antibody titers in live-cell vaccinated animals peaked even earlier, but by 4 weeks were only slightly lower than in hamsters vaccinated with the killed preparation. We were unable to detect

significant levels of antibody in concentrated bronchoalveolar lavage fluids from any vaccinated hamsters.

The role of lymphokines in host defense against P. pseudomallei is largely unknown. However, these infections are known to provoke a state of delayed hypersensitivity in some cases. Thus, it seemed important to assay for increased MIF or blastogenic factors in vaccinated animals. Unfortunately, efforts to assay these factors have not been successful. Despite manipulations of the assay procedures, including varying cell concentrations and testing the effects of various concentrations of homologous antigens, as well as the T-lymphocyte mitogen, PHA, no increased blastogenesis was detected; results of MIF assays have been equivocal. Guinea pigs will be employed instead of hamsters in subsequent efforts to examine the role of lymphokines in host defense against P. pseudomallei.

Four weeks after vaccination, groups of hamsters given each type of vaccine as a single dose or as 4 replicate doses were challenged with 50 LD_{50} of virulent strain 23343 presented as an aerosol. A group of hamsters given a placebo consisting of heart infusion broth as a dual IN and IP inoculation was challenged in a similar way. Table V shows the geometric mean antibody titer of each group at the time of challenge, an indication of gross lung pathology observed in hamsters that became moribund after challenge, and the estimated survival rates.

TABLE V. EFFECT OF VACCINATION ON RESISTANCE OF HAMSTERS TO SPA CHALLENGE WITH 50 RESPIRATORY LD_{50} OF P. PSEUDOMALLEI

REGIMEN	VACCINE	N	IFA TITER[a] AT CHALLENGE	MEAN LUNG LESION SCORE	% SURVIVAL
Single	Saline, control	40	0	3+	0
	Live, 11668	20	102	3+	0
	Killed, 23343	40	135	0	38
Replicate (4)	Live, 11668	10	135	3+	0
	Killed, 23343	10	216	0	70

[a]Geometric mean of reciprocal

All of the sham-vaccinated hamsters and those that received the live avirulent vaccine were dead within 4 days postchallenge. Abscesses of varying size, some surrounded by areas of hemorrhagic consolidation, were distributed over approximately 75% of the lungs.

Thirty-eight percent of those that received a single dose of the killed preparation survived even though their antibody levels were only slightly higher than those of live-cell vaccinated hamsters. Repeated vaccination with both preparations stimulated a small increase in antibody titer obtained, and significantly increased protection in those given killed vaccine; however, the live-cell vaccinated hamsters were not protected. Apparently, antibodies stimulated by the attenutated strain are not directed against virulence factors of the virulent strain. IFA procedures may not be sufficiently discriminating to detect these differences.

Publications:

Scott, G. H., E. L. Stephen, and R. F. Berendt. 1978. Activity of amantadine, rimantadine, and ribavirin against swine influenza in mice and squirrel monkeys. Antimicrob. Agents Chemother. 13:284-288.

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1. South East Asia Treaty Organization. 1970. Melioidosis. In SEATO Medical Research Laboratory Annual Progress Report. Bangkok, Thailand.

2. Eickhoff, T. C., J. V. Bennett, P. S. Hayes, and J. Feeley. 1970. Pseudomonas pseudomallei:susceptibility to chemotherapeutic agents. J. Infect. Dis. 121:95-102.

3. Nigg, C., J. Ruch, E. Scott, and K. Woble. 1956. Enhancement of virulence of Malliomyces pseudomallei. J. Bacteriol. 71:530-541.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6416	78 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
78 04 21	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	62776A	3M162776A841	00	013
b. CONTRIBUTING				
c. ~~XXXXXX~~ STOG 78-7.2.1, 3, 6				

11. TITLE (Precede with Security Classification Code)

(U) Enhancement of the 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	CONT	DA	C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.0	260.0
	CURRENT 79	1.0	118.9

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence considered

POC:DA

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 (Furnish 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) 77 10 - 78 09 - Several potential immunologic adjuvants have been evaluated with formalin-inactivated Venezuelan equine encephalomyelitis (VEE) in laboratory rodents and monkeys. The interferon inducers, lysine-stabilized poly(I)-poly(C) [poly(ICLC)] and tilorone HCl (analog 11,567) are highly effective adjuvants when given in combination with inactivated VEE vaccine to laboratory rodents and rhesus monkeys. Adjuvant doses of 11,567 as low as 15 mg/kg given with VEE vaccine significantly enhanced resistance in mice against homologous virus challenge compared to vaccine controls. A metabolized lipid emulsion (LE) has been prepared for potential use with inactivated aqueous virus vaccines. The LE, when given with VEE vaccine was an effective adjuvant for conferring mice significant enhanced resistance to virus challenge. Monkeys given 2 doses of the emulsified water-in-oil VEE vaccine developed 3- to 7-fold higher VEE virus serum neutralizing antibody titers than those given vaccine alone.

Publications: Am. J. Trop. Med. Hyg. 26:1191-1198, 1977; Abstr. Annual Mtg., Am. Soc. Microbiol., p. 47, 1978; Lab. Anim. Sci. 28(3):339-342, 1978.

*Available to contractors upon originator's approval

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 013: Enhancement of Inactivated Viral Vaccines of Military Importance

Background:

Prophylactic immunization using vaccines is generally considered one of the most effective methods for control of infectious diseases. However, when inactivated vaccines are used, adjuvants, which act nonspecifically to enhance immune responsiveness to a specific antigen, are frequently needed to achieve long-lasting effective immunity. Certain substances which induce interferon appear to be promising antiviral agents as well as effective modulators of host immunity. Previous reports (1) have described the successful use of the interferon inducers, lysine-stabilized poly(I)·poly(C) [poly(ICLC)] and poly(A)·poly(U) [poly(AULC)] as adjuvants with several marginally antigenic virus vaccines. Emphasis is presently directed towards completing poly(ICLC) adjuvant studies and identifying other compounds with adjuvant activity for use with inactivated vaccines.

Progress:

Adjuvant studies with poly(ICLC) in hamsters given inactivated alphavirus vaccines. Last year, we reported that poly(ICLC) combined with either inactivated EEE or WEE virus vaccines was an effective adjuvant for conferring in hamsters significant dose-related temporal protection against homologous virus challenge (1). To extend these observations, the last of a 3-part study was completed to assess poly(ICLC) as an adjuvant for enhancing the immunogenicity of 3 inactivated alphavirus vaccines, WEE, EEE and VEE in hamsters. Hamsters were considered the animal model of choice for conducting protection studies, since all 3 viruses cause lethal infections following IP virus challenge. Groups of hamsters were inoculaed with VEE virus vaccine and graded doses (25-200 µg/kg body weight) of poly(ICLC) in accordance with previously described methods (1). Adjuvant doses of poly(ICLC) given in conjunction with VEE vaccine were shown to be highly effective in conferring hamsters significant ($P < 0.05$) enhanced resistance against VEE virus challenge when compared to vaccinated controls (Table I).

Poly(ICLC) is known to be a potent interferon inducer in several species of laboratory animals (1). Accordingly, we investigated the poly-(ICLC)-induced interferon responses in hamsters given adjuvant plus the 3 inactivated alphavirus vaccines. Groups of 12 hamsters each were inoculated SC with either EEE, WEE, or VEE virus vaccine alone, or combined with 200 µg/kg of poly(ICLC). Controls were given poly(ICLC) only. Serum interferon (IF) titers in hamsters were measured at 8, 24, 48, and 72 hr postinoculation (Table II). Ten of 48 hamsters given either EEE, VEE or WEE vaccine plus poly(ICLC), or poly(ICLC) alone, had detectable levels of IF 8 hr postinoculation, while 45 of these animals had titers ranging

TABLE I. EFFECT OF POLY(ICLC) ON SURVIVAL OF SC VACCINATED HAMSTERS (9/POINT) CHALLENGED TEMPORALLY WITH VEE VIRUS

VEE VACCINE[a]	POLY(ICLC) (μg/kg)	% SURVIVORS[b] BY CHALLENGE DAY							
		1	3	5	7	10	14	17	21
0.3 ml	200	56*	67*	100***	100***	89**	44	56	89
0.3 ml	100	22	67**	100***	67**	89**	44	56	56
0.3 ml	25	22	38[c]	78**	67**	33	44	22	33
0.3 ml	0	0	0	0	0	0	11	22	33
0	200	0	0	11	0	0	0	11	11
0 (saline controls)	0	0	11	0	0	0	11	0	0

[a] 1:4 dilution

[b] Hamsters challenged IP with ~ 125 hamster $IPLD_{50}$/0.3 ml of VEE(Trinidad) virus and observed for 21 days

[c] n = 8

*P < 0.05; **P < 0.01; ***P < 0.001 vs. vaccine controls.

from 12-69 IF units by 24 hr postinoculation. It appears that EEE vaccine alone may induce low levels of IF in hamsters, since 3 of 12 animals had detectable IF titers postinoculation.

TABLE II. SERUM IF TITERS IN HAMSTERS GIVEN VEE, EEE, OR WEE VIRUS VACCINE ALONE OR COMBINED WITH 200 μg/kg POLY(ICLC) (N=12)

VACCINE[a] (0.3 ml)	POLY(ICLC) (200 μg/kg)	NO. RESPONDERS/GROUP (GM UNITS IF)[b] BY HR 8	24	48	72
EEE	-	0 (<20)	3 (12)	0 (<20)	0 (<20)
	+	3 (12)	12 (69)	0 (<20)	0 (<20)
VEE	-	0 (<20)	0 (<20)	0 (<20)	0 (<20)
	+	3 (12)	12 (45)	0 (<20)	0 (<20)
WEE	-	0 (<20)	0 (<20)	0 (<20)	0 (<20)
	+	2 (11)	10 (36)	0 (<20)	0 (<20)
None	+	2 (12)	11 (34)	0 (<20)	0 (<20)

[a] 0.3 ml undiluted

[b] Reciprocal serum IF titers < 20 were assigned a value of 10 for calculations.

These data suggest that poly(ICLC)-induced endogenous IF may likely account for the early enhanced nonspecific resistance seen in hamsters challenged on day 1 and 3 following inoculation with vaccine plus adjuvant.

Adjuvant studies with tilorone analog 11,567, Bru-Pel, and a lipid emulsion in mice and monkeys. Tilorone hydrochloride, a low molecular weight orally and parenterally active interferon inducer in rodents, has been reported to have antiviral, antitumor, and adjuvant properties (2). Tilorone analog (11,567) (supplied by Merrell-National Laboratories) was chosen for investigation for adjuvant activity with inactivated viral vaccines, since it has been reported to have biological activity similar to tilorone, but with diminished toxicity. Bru-Pel (3), a nonviable aqueous ether-extracted preparation of Brucella abortus (kindly supplied by Dr. J. S. Youngner, School of Medicine, University of Pittsburgh) was also screened for adjuvant activity. Previous reports have described its ability to induce interferon in mice and enhance nonspecific resistance to viral infections (3).

A series of experiments were performed to assess the adjuvant effects of 11,567 and Bru-Pel in mice given inactivated VEE virus vaccine. Other adjuvants including poly(ICLC), poly(AULC), Freund's complete (FCA) and Freund's incomplete (FIA) were included for comparison.

Results are shown in Table III. Clearly, groups of mice given 11,567 and poly(ICLC) as adjuvants with a marginally antigenic dose of vaccine had significantly ($P < 0.05$) higher survival rates compared to the Freund's adjuvant groups and vaccine controls. Detectable levels of serum neutralizing (SN) antibody were present only in the 11,567, poly(ICLC) and poly(AULC) adjuvant-vaccine groups. Antibody responses and % survivors in groups of mice given Bru-Pel plus vaccine were not different from vaccine controls.

TABLE III. EFFECT OF 11,567 AND OTHER ADJUVANTS ON ANTIBODY RESPONSE AND SURVIVAL OF VEE VACCINATED MICE CHALLENGED ON DAY 14 WITH 2,400 MIPLD$_{50}$ VEE VIRUS

TREATMENT			GM SN Ab TITER	% SURVIVORS
Adjuvant	Vaccine[a]	DOSE/kg	Day 14 (n=5)	Day 35 (n=20)
11,567	Vaccine	62 mg	5	70**
		250 mg	6	63** (n=19)
	Saline	250 mg	<4	0
Poly(ICLC)	Vaccine	62 µg	3	80**
		250 µg	8	75**
	Saline	250 µg	<4	0
Poly(AULC)	Vaccine	62 µg	5	25
		250 µg	ND[b]	ND
	Saline	250 µg	4	0
Bru-Pel	Vaccine	62 mg	<4	20
		250 mg	<4	5
	Saline	250 mg	<4	0
FCA (1:1)	Vaccine		<4	15
	Saline		<4	0
FIA (1:1)	Vaccine		<4	5
	Saline		<4	5
None	Vaccine controls		<4	0
	Saline controls		<4	0

[a] VEE vaccine (0.25 ml, 1:4 dilution)
[b] ND = not done
$P < 0.001$ vs. vaccine controls

Table IV shows the results of an adjuvant-dose response study with 11,567 in VEE vaccinated mice. Detectable levels of antibody were present on day 14 postvaccination in all groups given 11,567 plus vaccine. Percentage survival was significantly enhanced in 6 of 7 groups given 11,567 plus vaccine compared to vaccine controls. The minimum adjuvant

dose of 11,567 required for significant potentiation of VEE SN antibody responses in mice, was 125 mg/kg. However, adjuvant doses of the compound as low as 15 mg/kg were shown to enhance survival significantly in challenged mice compared to vaccine controls. Survival rates among mice given either 250 or 500 mg/kg of 11,567 alone without vaccine, were not different from saline controls. The only signs of toxicity seen in any of the treatment groups were localized skin reactions at the inoculation sites in mice given $\geq$ 250 mg/kg body weight. Studies were also conducted to determine the effects of adjuvant dose and route of 11,567 administration on temporal antibody responses and resistance of vaccinated mice to homologous virus challenge (data not shown). Mice given either 10 or 100 mg/kg of 11,567 SC plus VEE vaccine, were afforded significantly ($P < 0.05$) greater resistance to VEE virus challenge on days 3, 7, and 21 postvaccination than vaccinated controls. Antibody responses and percent survivors were highest in mice given 100 mg/kg of 11,567 plus vaccine. While the combined SC administration of 11,567 with vaccine was shown to significantly enhance resistance in mice to VEE virus challenge, no adjuvant effects were observed when 11,567 was given by the IP route, separate from vaccine.

TABLE IV. EFFECT OF DOSE OF 11,567 ON ANTIBODY RESPONSE AND SURVIVAL OF VEE VACCINATED MICE CHALLENGED ON DAY 14 WITH 1,000 $MIPLD_{50}$ VEE VIRUS

TREATMENT (SC)	DOSE OF 11,567 (mg/kg)	GM SN Ab TITER DAY 14	% SURVIVORS DAY 35
Vaccine[a]	7	6	56
+	15	6	69*
11,567	31	4	94**
	62	7	94**
	125	27**	100**
	250	13*	100**
	500	3	88**
Vaccine Controls	0	<4	19
11,567 Controls	250	<4	0
	500	<4	0
Saline Controls	0	<4	0

[a]VEE vaccine (0.25 ml: 1:4 dilution)
* $P < 0.05$; **$P < 0.001$ vs. vaccine controls.

Di Luzio and Riggi (4) have described the development of a lipid emulsion for the measurement of reticuloendothelial function. In collaboration with CPT Jack A. Reynolds (Work Unit BS03 00 025) a lipid emulsion (LE) was prepared containing highly refined peanut oil, glycerol

and lecithin, for testing as a potential adjuvant for use with inactivated aqueous virus vaccines. We believe this formulation meets most established criteria for water-in-oil adjuvants.

Because of the earlier reported success in the use of 11,567 as an adjuvant with VEE vaccine, additional investigations were undertaken to further assess the adjuvanticity of 11,567 and LE with VEE vaccine in mice and monkeys. The results of these studies are shown in Tables V and VI. Mice given a single SC dose of LE adjuvant vaccine were afforded 88% protection against VEE virus challenge compared to 38% in vaccine controls. Survival rates for mice given both 11,567 and LE with vaccine were not different from those in mice given either 11,567 or LE alone with vaccine. The combined SC administration of either 11,567 or LE with vaccine, effectively enhanced resistance in mice to virus challenge. No additive adjuvant effect, as evidenced by increased survival rates, was seen when the 2 substances were given concurrently with vaccine.

TABLE V. EFFECTS OF 11,567 LIPID EMULSION (LE) AND FREUND'S COMPLETE ADJUVANT (FCA) ON SURVIVAL OF VEE VACCINATED MICE CHALLENGED ON DAY 21 WITH 425 $MIPLD_{50}$ VEE VIRUS

TREATMENT (SC)		% SURVIVORS
VACCINE[a]	ADJUVANT[b]	DAY 42 (n=16)
VEE	LE	88*
	11,567	75
	11,567 + LE	81*
Saline	11,567 + LE	6
VEE	FCA (1:1)	6
VEE	None	38
None	None	6

[a] VEE vaccine (0.25 ml; 1:4 dilution).
[b] LE (0.1 ml; 1:2 dilution); analog 11,567 (100 mg/kg).
* $P < 0.05$ vs. vaccine controls.

VEE virus SN antibody titers in rhesus monkeys vaccinated on day 0 and boosted on day 28 with 0.5 ml of VEE vaccine alone, or combined with either 10 or 100 mg/kg of 11,567, or LE are shown in Table VI. Primary antibody responses in the group of monkeys given the 10 mg/kg dose of 11,567 were 4- to 5-fold higher than titers in vaccine controls. Furthermore, following boosting on day 28, an 8- to 16-fold increase in SN antibody titers was seen between the low-dose 11,567 adjuvant-vaccine group and vaccine controls. In contrast, primary antibody responses in monkeys given LE plus vaccine were not different from vaccine controls.

TABLE VI. ADJUVANT EFFECTS OF TILORONE HCl (ANALOG 11,567) AND LIPID EMULSION (LE) IN RHESUS MONKEYS GIVEN INACTIVATED VEE VIRUS VACCINE[a]

GROUP	PREPARATION, DAYS 0, 28		GEOMETRIC MEAN PRN_{80} ANTIBODY TITERS (N=3) BY DAYS								
	VEE Vaccine	Adjuvant	7	14	21	28	32	35	42	56	70
1	+	11,567 (100 mg/kg)	40	40	20	16	20	203	256	323	128
2	+	11,567 (10 mg/kg)	102	203	64	51	51	2580	4096	1625	645
3	+	LE (0.5 gm)	32	64	32	25	51	645	2048	1024	362
4	+	None	32	51	13	13	20	256	323	161	81

[a]VEE vaccine (0.5 ml, undiluted) inoculated SC alone or combined with 11,567 and LE on day 0 and 28.

However, secondary antibody responses in monkeys given 2 doses of vaccine combined with LE were 3- to 7-fold higher than titers in controls. Since antibody titers in monkeys given 10 mg/kg of 11,567 were much higher than titers in the group given the higher dose, we believe lower adjuvant doses of the compound may also be effective. Additional studies in monkeys are warranted to determine the lowest possible adjuvant dose of 11,567 and LE to minimize potential adverse reactions and to determine the least amount of antigen compatable with efficient immunization.

Presentations:

Harrington, D. G., D. E. Hilmas, C. L. Crabbs, and T. A. Casciola. Adjuvant effects of tilorone hydrochloride (analog 11,567) in mice given inactivated Venezuelan equine encephalomyelitis virus vaccine. Presented, 18th Annual Meeting, Am. Soc. Microbiol., Las Vegas, NE, 14-19 May 1978 (Abstracts, p. 47, 1978).

Publications:

1. Harrington, D. G., D. E. Hilmas, M. R. Elwell, R. E. Whitmire, and E. L. Stephen. 1977. Intranasal infection of monkeys with Japanese encephalitis virus: clinical response and treatment with a nuclease-resistant derivative of poly(I).poly(C). Am. J. Trop. Med. Hyg. 26: 1191-1198.

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2. Munson, A. E., J. A. Munson, W. Regelson, and G. L. Wampler. 1972. Effect of Tilorone hydrochloride and congeners on reticulo-endothelial system, tumors, and the immune response. Cancer Res. 32: 1397-1403.

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4. Di Luzio, N. R., and S. J. Riggi. 1964. The development of a lipid emulsion for the measurement of reticuloendothelial function. J. Reticuloendothel. Soc. 1:136-149.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6415	78 10 01	DD-R&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
78 04 21	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	62776A	3M162776A841	00	016
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 78-7.2.1, 3, 6			

11. TITLE (Procede with Security Classification Code) (U) Interaction of viruses with peripheral host leukocytes as an index of immunity against infections of BW 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 08	CONT	DA	C. In-house

17. CONTRACT GRANT
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e. KIND OF AWARD — f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 78	1.0	155.0
FISCAL YEAR CURRENT 79	1.0	33.9

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21. GENERAL USE
Foreign intelligence considered

POC: DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Arboviruses; (U) Leukocytes; (U) Viral diseases; (U) Human status; (U) Phagocytosis

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

23 (U) Examine the interaction of human peripheral leukocytes with viruses of military importance with regard to the growth of the virus and its effect on the host cells. Determine the role of the immune state induced by military vaccines on virus-leukocyte interaction.

24 (U) Examine in detail the virus adsorption and growth characteristics of each type of peripheral blood leukocytes from both vaccinated and nonvaccinated individuals. Measure the effect of these cells on the infectivity of these viruses.

25 (U) 77 10 - 78 09 - Studies conducted by this laboratory to investigate the interaction of VEE virus (strain TC-83) with human peripheral blood leukocytes (PBL) have revealed that the PBL are indeed capable of supporting TC-83 virus growth with the macrophage being the primary replicative cell for virus growth in vitro. Mononuclear and macrophage cells from immune donors differ in their reactivity to this virus. Exposure to TC-83 antigen, even formalin-killed vaccine, apparently conveys to the vaccinees' macrophages a greater affinity for TC-83 virus growth in vitro. A study employing human vaccinees revealed that nonimmune donor macrophages convert to an immune donor macrophage state about 28 days postvaccination. The differences between immune and nonimmune macrophages may reflect a difference in cell surfaces. Supportive evidence for such surface differences was revealed in preliminary adsorption studies employing radioatively labeled virus.

Publication: Teratology 16:285-295, 1977.

*Available to contractors upon originator's approval

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 016: Interaction of Viruses with Peripheral Host Leukocytes as an Index of Immunity Against Infections of BW Importance

Background:

Selected viruses differ in their ability to replicate in diverse types of human leukocytes. Virus replication within cultured human monocytes has been demonstrated for vesicular stomatitis (1), 17D yellow fever (YF) (2) and measles (3) viruses. Several viruses have been shown to propagate in human peripheral blood lymphocytes.

With the exception of dengue (DEN) viruses and YF vaccine virus, little information is available on the interaction of arborviruses and human peripheral leukocytes. Studies with nearly homogeneous cultures of each leukocyte type revealed that 17D virus replicated to high titer and induced interferon in human monocytes. A small amount of virus replication took place in unstimulated lymphocytes while phytohemagglutinin (PHA)-stimulated lymphocytes were capable of supporting virus replication. No virus growth was observed in polymorphonuclear (PMN) cultures.

DEN viruses have been shown to replicate in both monkey and human peripheral leukocyte cultures. In a recent study (4), it was demonstrated that DEN-2 virus replicates in B-type cultured human lymphoblastoid cell lines (HCL), B lymphocytes, and in macrophages, but not in T type HCL, T lymphocytes and PMN.

Since VEE virus shows a predilection for the reticuloendothelial system during human infection (5), a study of this virus interaction with the peripheral blood leukocytes (PBL) was undertaken.

Progress:

Virus-leukocyte interaction. In previous annual reports, we described the in vitro interaction of VEE virus vaccine strain TC-83 with human peripheral blood leukocytes (PBL). PBL are capable of supporting TC-83 virus growth with the macrophage being the primary replicative cell for virus growth. Evidence was presented which showed differences in the in vitro interaction of TC-83 virus with PBL from immune and nonimmune individuals.

Our laboratory has been involved in the evaluation of the inactivated VEE vaccine (C-84-1, made from TC-83 strain) in regard to its effect on human PBL. Immunization of humans with C-84-1 induces in PBL an enhanced avidity for this virus that appears approximately 1 month later. Results of this ongoing study are presented in Table I. Cells from nonimmune donors did not support growth of the virus when tested prior to receiving

TABLE I. VIRUS GROWTH IN PBL FROM INDIVIDUALS IMMUNIZED WITH KILLED VEE VIRUS VACCINE (C-84-1)

VOLUNTEER NO.	VIRUS TITER[a] (Log_{10}PFU/ml)			
	DO	D_{28}	D_{180}	D_{270}
1	0	6.0[a]	5.5	4.0
2	0	6.0	6.0	ND
3	0	7.0	ND[b]	ND
7	0	6.0	ND	5.5
9	0	6.0	4.5	ND
10	0	6.0	4.5	ND

[a] 72-hr virus growth.
[b] ND = not done.

the vaccine. When cells from these same donors were tested with a similar virus dose 28 days after vaccination, virus growth was observed in all cases. It can also be seen that this conversion of the PBL has continued through the 270-day testing period. It appears that the PBL are altered sometime after exposure to TC-83 virus antigen. This enhanced virus-cell interaction, we theorize, may play a role in the removal of infectious virus from the circulation and subsequent protection of the host from infection.

VEE virus does not infect macrophages from nonimmune individuals *in vitro* when serum is absent from the growth medium. Presently, we are attempting to determine what factor or factors in serum are responsible for this enhancement of virus susceptibility, and at what stage of infection, i.e., adsorption, eclipse, etc., it is required.

Experiments were performed to determine if serum *per se* or serum from a particular species (human) was required to observe this enhanced cell susceptibility. It can be seen (Table II) that sera from all 5 animal species enabled virus to propagate in these nonimmune cells. In contrast, the cells inoculated and incubated in medium with human serum albumin were refractory to virus infection. Results of an experiment titrating the concentration of human serum required for enhanced growth are found in Table III. The enhanced growth effect was not observed when medium contained < 1% human serum. In a separate experiment, sera shown in Table II heated at 56°C for 1 hr, demonstrated no significant decrease in enhancement activity. Apparently, the factor or factors in serum which aid growth of TC-83 virus in macrophages is heat-stable and not species-specific.

TABLE II. EFFECT OF VARIOUS SERA ON GROWTH OF TC-83 VIRUS IN NONIMMUNE MACROPHAGES

MEDIUM SUPPLEMENT	VIRUS GROWTH (PFU/ml) x 10^3	
	Day 0	Day 3
Human serum albumin	0.3	0.46
Fetal calf serum	0.06	2000
Human serum	4.5	10,000
Guinea pig serum	0.5	10
Rabbit serum	0.45	6000
Mouse serum	0.50	1000

TABLE III. TITRATION OF SERUM REQUIRED FOR GROWTH OF TC-83 VIRUS IN NONIMMUNE MACROPHAGES

% HUMAN SERUM IN MEDIUM	VIRUS GROWTH (PFU/ml) x 10^3	
	Day 0	Day 3
0 (HSA)	0.1	0
0.01	0.03	0.065
0.05	0.025	0.02
0.10	0.03	0.02
1.0	0	400
5.0	0.05	1000
10.0	0.10	300
20.0	0.02	10,000

We are presently investigating complement, more specifically C3, as a possible factor in this enhancement phenomenon. To better understand the role of complement, experiments utilizing purified C3 fraction, radioactive virus, complement-depleted serum and trypsin-treated macrophages are currently in progress.

Efforts were directed toward demonstrating the possible existence of cytophilic, VEE virus-specific antibody on the surface of macrophages from VEE-immune donors. To accomplish this, we attempted to extract immunoglobulin from the cell surface. Several procedures were tried, (1) 37°C extraction for 1 hr in HBSS, (2) 56°C extraction, and (3) low pH. On occasion only the 37°C extraction appeared to yield sufficient quantities of immunoglobulin detectable in the highly sensitive protein A precipitation test. Wash material taken prior to the extraction showed no protein A

activity, thus eliminating humoral antibody as a source of contaminating activity. When extracted material was incubated with labeled VEE virus, 90% of the counts were precipitated in the protein A test. This indicated the presence of specific VEE immunoglobulin in the extracted cell material which may provide the enhanced virus avidity observed in the PBL cultures from immune donors. Presently, we are endeavoring to (a) improve the extraction procedure, (b) identify the class of cytophilic antibody involved, and (c) determine the role of cytophilic antibody in immunity to, and pathogenesis of, VEE virus infection.

Collaborative Study. Our laboratory has been involved in a collaborative study with National Institutes of Health (NIH) for the past several years for the purpose of examining the teratogenicity of selected arboviruses. Virus isolations and serology were performed by our laboratory on specimens sent from NIH. Preliminary results indicate that VEE, DEN-2, WEE and YF vaccine viruses are teratogenic for rhesus monkeys. The first of a series of papers was published in 1977.

Publication:

London, W. T., N. H. Levitt, S. G. Kent, V. G. Wong, and J. L. Sever. 1977. Congenital cerebral and ocular malformations induced in rhesus monkeys in Venezuelan equine encephalitis virus. Teratology 16:285-296.

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5. Ehrenkranz, N. J., and A. K. Ventura. 1974. Venezuelan equine encephalitis virus infection in man. Ann. Rev. Med. 25:9-14.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6420	78 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
77 10 01	D. CHANGE	U	U	NA	NL	X YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	017
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede 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	CONT	DA	C. In-house

17. CONTRACT GRANT
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e. KIND OF AWARD — f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING	78	0.8	190.0
FISCAL YEAR — CURRENT	79	1.0	104.8

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

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22. KEYWORDS (Precede EACH 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 (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Continue attempts to develop vaccines against Bolivian hemorrhagic fever (BHF) and Argentine hemorrhagic fever (AHF). Investigate cross-protection between the 2 diseases. Determine if a vaccine against one will protect against both. Both viruses produce illnesses of high morbidity and mortality. Both are related to highly fatal Lassa fever of Africa. Vaccines and methods of treatment are essential for these highly militarily important diseases.

24 (U) Obtain or develop attenuated strains of Machupo (MAC) and/or Junin (JUN) viruses and determine ability of immunization with one to protect against virulent challenge of the other in monkeys and guinea pigs.

25 (U) 77 10 - 78 09 - Experiments were carried out in support of other work units to study the effect of the drug ribavirin on experimental models for BHF and Lassa fever. Preliminary evidence was obtained that the virus of AHF, JUN virus, protected monkeys against experimental lethal challenge with the virus of BHF (MAC). Similar data were developed in a lethal guinea pig model of BHF. The JUN virus used was clone 3 strain, an attenuated virus which has been used experimentally in 600 volunteers in Argentina, as a possible AHF vaccine.

Publications: Ebola Virus Hemorrhagic Fever, pp. 237-243, 1978.
J. Gen. Virol. 41:183-188, 1978.
Am. J. Trop. Med. Hyg. 27: in press, 1978.

* Available to contractors upon information's approval

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 017: South American Hemorrhagic Fevers: Pathogenesis and Vaccine Development

Background:

Earlier research on Bolivian hemorrhagic fever (BHF) reported under this work unit has described the development of a primate model; the demonstration of passive prophylaxis by serum antibody; the demonstration of a therapeutic effect of passive serum antibody; the partial attenuation of the etiologic virus (Machupo) of BHF by chick embryo cell culture passage; development of an experimental inactivated vaccine; and the role of cross-protection against experimental BHF in monkeys by other members of the Tacaribe virus complex. We have defined both a primate model in the form of a rhesus monkey and other monkey models and a predictable hemorrhagic guinea pig model for BHF.

A major effort of our research during this past year has been concentrated on 2 specific aspects; one, the use of the drug ribavirin in the therapy of the disease (see Work Unit A841 00 026 of MAJ Stephen) will not be reported here in detail; and two, exploration of the relative ability of Junin virus, particularly the attenuated clone 3 variant to protect against BHF.

Progress:

The support provided to MAJ Stephen under Work Unit A841 00 026 may be summarized briefly. The lethal rhesus monkey model which profices a predictable lethal model of BHF was used to assess the efficacy of ribavirin given either at the time of infection with Machupo (MAC) virus or at the time of onset of clinical signs. In general, the results of those studies were very favorable and strongly supported the hypothesis that ribavirin is effective against BHF.

Cross-protection against BHF by Junin virus. During the period from 1969 to 1971 a group headed by Parodi in Argentina used attenuated variant of Junin (JUN) virus, clone 3, to immunize approximately 600 persons against Argentine hemorrhagic fever (AHF). The results of that study were reasonably well documented and showed that the virus was relatively safe and apparently effacious. The vaccine recipients developed antibody and although there was some mild illness there were no severe reactions to immunization with the clone 3 virus. However, because of its obscure passage history and because the vaccine itself was an attenuated agent grown in mouse brain there was considerable sentiment in Argentina that further use of this product should be discontinued. Since 1971 there have been no immunizations with clone 3 virus. Recently, however, the interest in this

attenuated virus has surfaced once again because of the obvious need for a vaccine against AHF. Because of this interest, we undertook certain studies with clone 3 virus to determine its efficacy against BHF.

Two studies are reported here; one was carried out in guinea pigs and the second, in rhesus monkeys. In both instances the animals were immunized with the clone 3 variant of JUN virus. The results in Table I show the anomalous results for guinea pigs immunized with either attenuated MAC or attenuated JUN, clone 3. Guinea pigs were challenged with either low-passage MAC virus (Parent) or a guinea pig variant substrain (M-SP5) of that same virus which had been adapted to guinea pig spleen through 5 successive spleen-to-spleen passages. The results show that both the attenuated MAC and JUN viruses protected relatively well against the low-passage MAC. Curiously, however, it appears that the clone 3 of JUN protected better against the highly guinea pig lethal, spleen-passaged variant of MAC virus than did the attenuated MAC virus itself. We are at a loss to explain these results but they will be repeated in future experiments and reported in subsequent annual reports.

TABLE I. IMMUNIZATION OF GUINEA PIGS AGAINST MACHUPO VIRUS BY ATTENUATED MACHUPO OR ATTENUATED JUNIN VIRUS

IMMUNIZING VIRUS	CHALLENGE[c] VIRUS AT 42 DAYS	TOTAL	ILL	DEAD
None	Parent	5	4	3
	M-Sp5	6	6	6
MAC, C-35[a]	Parent	5	1	0
	M-Sp5	6	4	4
JUN, Clone 3[b]	Parent	6	0	0
	M-Sp5	6	4	0

[a] Attenuated by 35 chick embryo cell culture passages of Parent.

[b] Attenuated by mouse brain passage of the XJ strain: $\sim 2.5 \log_{10}$ of inoculum used to immunize.

[c] Parent = 1st cell culture passage of Malale strain MAC virus.

M-Sp5 = 5th guinea pig spleen passage of Parent.

In addition to the studies in guinea pigs we have also assessed the ability of clone 3 to protect rhesus monkeys (n = 8) against lethal MAC challenge. Following inoculation of 5-6 logs of the virus, 6 of 8 monkeys developed fever of one day duration; all developed transitory leukopenia; and there were no serious effects or reactions. There was one death, but it was due to causes unrelated to the inoculation of the JUN. The 7 surviving monkeys all developed specific neutralizing antibody at titers >1:256 against the immunizing virus. These same monkeys also developed antibody titers against other strains of JUN.

Three of the monkeys from that group of 8 were challenged with MAC ∿3 mon after immunization with JUN. The antibody titers to JUN virus were ∿1:1000 at the time of challenge and changed very little during the subsequent 2 mon. The monkeys were also tested for the presence of specific neutralizing antibody against MAC virus at the time of challenge. One of the monkeys showed a titer of 1:8 and the other 2 monkeys were negative for MAC virus. Following challenge none of the monkeys became ill nor showed significant clinical signs of illness. There was a brief period during which one of the monkeys showed a possibly diminished appepite but otherwise there were no signs of disease typical of those seen in unprotected monkeys challenged with MAC. It is probably quite significant that all of the monkeys had specific neutralizing antibody against MAC by day 7, 2 of them developed antibody by day 4 and the antibody titers in all the monkeys rose to high levels by day 21. Our previous studies have shown that normal control rhesus monkeys do not develop specific neutralizing antibody against MAC until day 28, if they live that long. The results of the study clearly show the monkeys were strongly immune to MAC virus 3 mon after JUN immunization. Subsequent groups of similarly immunized monkeys will be reported upon in succeeding annual reports. The possibility that both BHF and AHF can be immunized against with a single attenuated vaccine is highly encouraging. It is our intent to pursue this possibility further and to assist Argentine investigators in the development of AHF vaccine.

Presentations:

1. Eddy, G. A. Alterations in Machupo virus resulting from selective passages. Presented, Annual Meeting Am. Soc. Trop. Med. Hyg., Denver, CO, 8-11 Nov 77.

2. Eddy, G. A. and F. E. Cole, Jr. The development of a vaccine against African hemorrhagic fever. Presented, Proceedings of an International Colloquium on Ebola Virus Infection and Other Haemorrhagic Fevers, Antwerp, Belgium, 6-8 Dec 77.

Publications:

1. Gangemi, J. D., R. R. Rosato, E. V. Connell, E. M. Johnson, and G. A. Eddy. 1978. Structural polypeptides of Machupo virus. J. Gen. Virol. 41:183-188.

2. Eddy, G. A., and F. E. Cole, Jr. 1978. The development of a vaccine against African hemorrhagic fever, pp. 237-242. In Ebola Virus Haemorrhagic Fever (S. R. Pattyn, ed.). Elsevier/North Holland Biomedical Press, New York.

3. Scott, S. K., R. L. Hickman, C. M. Lang, G. A. Eddy, D. E. Hilmas, and R. O. Spertzel. 1978. Studies on the coagulation system and blood pressure during experimental Bolivian hemorrhagic fever in rhesus monkeys. Am. J. Trop. Med. Hyg. 27: in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6428	78 10 01	

3 DATE PREV SUMMARY	4 KIND OF SUMMARY	5 SUMMARY SCTY	6 RDTE SECURITY	7 REGRADING	8A DISSEM INSTR	8B SPECIAL DATA CONTRACTOR RESS	8C TECH EVAL
78 04 21	D. CHANGE	U	U	NA	NL	X YES NO	A. WORK UNIT

10 NAT'L INTEREST	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	020
b. CONTRIBUTING				
c. 777777	STOG 78-7.2.1, 3, 6			

11 TITLE (Precede 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	CONT	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. FUNDING (Thousands)
FISCAL YEAR PRECEDING 78	1.0	120.0
FISCAL YEAR CURRENT 79	3.0	241.1

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ASSOCIATE INVESTIGATORS

NAME Johnson, A. D.

NAME Lewis, Jr., G. E. POC:DA

21 GENERAL USE

Foreign intelligence considered

22 KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Botulism, (U) Toxoid; (U) Clostridium botulinum

23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish 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-G. Use purified toxoids to develop a polyvalent toxoid. Prepare immune globulin (human) for treatment of botulism.

25 (U) 77 10 - 78 09 - Milligram amounts of C. botulinum neurotoxin type A have been produced and toxoided. When adsorbed to aluminum hydroxide, a satisfactory immunogen for development of antibodies was produced. Fifty liters of Botulism Immune Plasma (Human) were collected and an AIDRB submission was prepared. Fermentor kinetics of the release of type A toxin have been investigated.

Publication: Lancet 2:634-635, 1978.

*Available to contractors upon originator's approval

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 020: Microbial Toxins and Their Role in the Pathogenesis 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 completely prevents 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 5 antigens to types A-E neurotoxins. At the time of its preparation full knowledge of the neurotoxin was not available; the preparation contains < 10% of the desired immunogen. Mild side reactions including tenderness, redness, heat, and swelling at the site of the injection are common. The basic course to produce satisfactory antibody levels 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 animals.

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 widespread human sensitivity to horse proteins. (The only U.S. commercial source of equine antiserum notified Center for Disease Control (CDC) in July 1978 that they would no longer provide this product.) Although a human antiserum for the treatment of botulism has been proposed for many years, no real effort has been expended to develop such a product.

Progress:

C. botulinum strains producing types A-G were obtained from CDC and ATCC. These strains have been assayed in static culture and are producing lower amounts of neurotoxin than originally described.

Small lots (10-20 mg) of C. botulinum type A neurotoxin have been prepared by column chromatography. The neurotoxin is completely nicked and displays 2 subunits on SDS gels with reduction. The MW of the subunits are ~ 100,000 and 50,000. The purified neurotoxin was treated for 35 days with 0.6% formaldehyde to achieve toxoiding. The toxoid was adsorbed to Al $(OH)_3$ gel and was antigenic in rabbits. On Ouchterlony gel diffusion, the toxoid produced 3 lines to crude culture supernatants while the purified neurotoxin produced only a single line indicating a high degree of purity of the neurotoxin.

The national supply of botulism antitoxin is finite, of questionable quality and efficacy, was produced in horses, and will need to be replenished in the near future. A safe, human-derived botulism antitoxin is highly desirable for potential use in individuals exposed to one or more of the botulinal neurotoxins. The mouse serum neutralization test, used for determination of botulinal neurotoxin titers in human sera, was established at USAMRIID in March of this year. Potential donors of botulism immune plasma were recruited and screened. Those selected are being plasmapheresed at Walter Reed Army Medical Center. As of 30 July 1978, 10 civilian donors have contributed a combined total of 54 L (174 units, average 305 ml/unit) of Botulism Immune Plasma (Human). The current goal is 60 L, 50 of which will be converted into a Botulism Immune Globulin (Human), pentavalent product. Ten liters of hyperimmune serum will be maintained at USAMRIID for immediate emergency utilization, if needed. Each plasma unit collected has been tested for RPR card test reactivity and hepatitis B surface antigen reactivity by R. I. A. An application outlining the collection, testing, certification, and proposed use of Botulism Immune Plasma (Human) and requesting the issuance of an investigational new drug member has been submitted through U.S. Army Medical Research and Development Command to the Food and Drug Administration Bureau of Biologics IND #1332 was assigned to this product. In July, 1978, a plasmapheresis unit was established at USAMRIID.

Three lots of Botulinum Toxoid Absorbed Pentavalent (A,B,C,D,E) were recently packaged for USAMRIID by the Michigan Department of Public Health. This toxoid was prepared in 1971 according to the original 1958 protocol. We have requested the CDC, Atlanta, GA to submit data on the 3 lots of toxoid to the Bureau of Biologics as an addendum to BB-IND 161, which is held by CDC.

The production of toxin by C. botulinum type A grown in a 50-L fermenter under various conditions was examined (Table I). Maximum levels of toxins were produced within 30 hr, in all cases. In contrast, incubation times of 4-5 days are employed for toxin production by static cultures of this organism.

Under fermentation conditions 1 and 2 (Table I), the maximum concentration of toxin was reached only after cell lysis had occurred. However, using condition 4, the maximum toxin level was reached before the culture had lysed significantly. The level of toxin did not increase with cell lysis, and remained constant during 126 hr of incubation (condition 4).

When the concentration of glucose in the medium was increased to 1.0% (condition 3), toxin concentration reached its maximum in 18 hr. The culture did not lyse significantly under these conditions. In contrast to previously published reports, these data (conditions 3 and 4) indicate that lysis of C. botulinum is not essential for the release of toxin.

TABLE I. TOXIN PRODUCTION BY CLOSTRIDIUM BOTULINUM TYPE A[a] AS MEASURED IP IN MICE

HOURS	LD_{50}/ml (x 10^3) AT 4 LEVELS OF GLUCOSE			
	0.5%[b]	0.5%[c]	1.0%[c]	0.5%[d]
0	4.2	0.63	6.3	6.3
6	6.3	6.30	42.0	63.0
12	63.0	420.00	200.0	200.0
18	120.0	420.0	630.0	-
24	200.0	420.0	630.0	630.0
30	420.0	630.0	630.0	630.0

[a]Medium: 2% peptone, 1% yeast extract, 0.1% thioglycolate, glucose as indicated.
[b]100 rpm agitation, 10 L N_2/min sparge.
[c]50 rpm agitation, 5 L N_2/min sparge.
[d]50 rpm agitation, 5 L N_2/min overlay, 0.5% glucose.

The efficacy of homologous and heterologous antitoxin in the prevention of type A botulism was evaluated in guinea pigs. Antitoxins were administered IM at various time intervals before or after challenge with type A neurotoxin. Groups of 4-6 guinea pigs were passively immunized with 1 of 4 sequential log dilutions of either homologous (guinea pig) or heterologous (equine) type A antitoxin. Both antitoxins were adjusted to contain 1.6 IU of activity in 0.5 ml of their lowest dilution. Each animal was challenged SC with 4.5 guinea pig LD_{50} of type A neurotoxin. Immunization with 1.6 U of homologous antitoxin at 2 hr, or at 24 hr before or at 2 hr after challenge assured 100% survival. The majority (5 of 8) of guinea pigs administered 1.6 U of either antitoxin at 18 hr after challenge survived.

Those guinea pigs previously immunized at -24 hr were again challenged, 18 days after the initial challenge. Half of the animals

received an identical (4.5 LD_{50}) second challenge of neurotoxin. The remaining half were challenged with a 4-fold greater dose (18 LD_{50}) of neurotoxin. Guinea pigs previously immunized with 1.6 U of homologous antitoxin survived both levels of challenge. All animals previously administered 1.6 U of heterologous antitoxin died of acute botulinal intoxication within 4 days after this 2nd challenge.

Likewise, guinea pigs immunized with 1.6 U of homologous antitoxin and not previously challenged, were immune to challenge 14 days after immunization. However, those immunized with 1.6 U of heterologous antitoxin, and similarly challenged did not survive the 4th day.

Immunization with only 0.16 U of either antitoxin at 24 hr before or 18 hr after challenge did not provide adequate protection. All +18hr recipients died within 5 days of challenge. However, a majority of those immunized at -24 hr, survived through day 8. Two homologous and 1 heterologous recipient survived the 10th day.

Thus the following conclusions concerning the efficacy of homologous and heterologous (equine) antitoxin in the prevention of type A botulism in guinea pigs may be made: (a) homologous and heterologous antitoxin are similarly efficacious when administered 2 or 24 hr before, or 2 hr after, exposure to lethal neurotoxin; (b) the administration of either antitoxin 24 hr before challenge is efficaciously superior to similar doses of antitoxin administered 18 hr after challenge; (c) 14 days after passive immunization, homologous antitoxin is protective and immunologically active at a concentration that is at least 10-fold greater than that of heterologous antitoxin. Thus the effective half-life of homologous antitoxin is much longer than that of heterologous antitoxin; and (d) passive immunization with less than optimal protective amounts of antitoxin can prolong survival time by a factor of at least 2. This additional time would allow for the implementation of intensive supportive care to human patients.

Publications:

Lewis, Jr., G. E., and J. F. Metzger. 1978. Botulism Immune Plasma (Human). Lancet 2:634-635.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OG6425	78 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
78 04 21	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	62776A	3M162776A841	00	025
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code) (U) Effect of protease inhibitors upon kinin system activation during Salmonella typhimurium infection in monkeys

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	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		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.0	85.0
	CURRENT 79	1.0	118.9

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21. GENERAL USE
Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME:
NAME:

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Kallikrein; (U) Kinins; (U) Bacterial sepsis; (U) Protease inhibitor; (U) Aprotinin; (U) Monkeys

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

23 (U) Investigate the effects of a protease inhibitor (aprotinin) upon activation of the kinin system during Salmonella typhimurium infection in monkeys. It is hoped that this new approach to therapy will prove useful in a wide variety of infections with hemorrhagic and/or hypotensive complications which are potential causes of military casualties from either natural or BW disease.

24 (U) Determine pharmacology and appropriate routes of administration of aprotinin to monkeys. Use aprotinin in monkeys infected with S. typhimurium as prophylaxis against kinin activation and disseminated intravascular coagulation.

25 (U) 77 10 - 78 09 - Aprotinin was labeled with 125-I and injected into 8 healthy rhesus monkeys to study plasma turnover. Disappearance of the injected label from plasma was fit to a triple exponential equation. The 3 half-lives were 4.20, 41.50 min and 57.09 hrs. Thirteen monkeys injected with 40,000 KIU/kg of aprotinin showed an average increase of 47.3% in the activated partial thromboplastin time (APTT). This effect was short-lived and by 1 hr the APTT had returned to normal.

These data indicate that aprotinin has a very short half-life in the plasma of rhesus monkeys and has to be administered by continuous IV infusion. When circulating in high levels, aprotinin can inhibit the initial phase of intravascular coagulation.

*Available to contractors upon originator's approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, NOV 68 AND 1498-1, MAR 69 (FOR ARMY USE) ARE OBSOLETE. * U.S. GPO 1974-540-833 8631

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 025: Effect of Protease Inhibitors Upon Kinin System Activation During Salmonella typhimurium Infection in Monkeys

Background:

The infection of rhesus monkeys (Macaca mulatta) with Salmonella typhimurium results in the clinical syndrome of disseminated intravascular coagulation (DIC). DIC is characterized by intravascular activation of Factor XII and consequent activation of the pathways for coagulation, fibrinolysis and kinin generation (1).

Theoretically, the use of protease inhibitors would block the sequential activation of the proteolytic enzymes in these pathways. If this effect is proved to be clinically significant, these agents will be a novel approach in the therapy of infectious diseases (2, 3). Protease inhibitors would be adjuvant therapy in the severe bacterial, rickettsial and viral infections associated with DIC (3).

Aprotinin (Trasylol^{R}) is a protease inhibitor obtained from bovine lung. Among others it is a kallikrein, trypsin and plasmin inhibitor. In vitro, it has been shown to inhibit early reactions of blood coagulation and to prolong clotting time. In view of these properties, aprotinin has been proposed as therapy for DIC. However, in vivo studies were contradictory as to the effect of aprotinin in human coagulation and in clinical trials the effect of aprotinin was found to be beneficial in uncontrolled studies (4, 5).

Progress:

Initial efforts were directed toward the establishment of laboratory procedures. To study the potential of aprotinin as therapy in DIC we started by determining the plasma turnover of it in rhesus monkeys.

Pharmacology of aprotinin. Samples (1 ml) were iodinated by microdiffusion. The labeled product maintained the inhibitory properties of stock material as demonstrated by successful inhibition of the esterase activity of kallikrein as measured by the benzoyl arginine ethyl ester and tosyl-L-argine methyl ester assays.

Eight monkeys were injected (via saphenous vein) with 1 ml of [^{125}I]aprotinin (10,000 KIU/ml) and 40,000 KIU/kg of "cold" aprotinin. Two min after the end of the injection, blood was sampled from the contralateral femoral vein· this sample was considered the 0 time sample. Subsequent samples were obtained at ∿3, 6, 9, 15, 20, 60, 120, 180 and 270 min and 24, 48 and 72 hr.

The radioactive content of these plasma samples was measured in a gamma counter and plotted on a logarithmic scale against time. In each case, the disappearance curve of the injected label was fit to a triple exponential equation:

$$y = \sigma_1 e^{-\lambda_1 t} + \sigma_2 e^{-\lambda_2 t} + \sigma_3 e^{-\lambda_3 t}$$

Where y represents disintegrations per minute, t represents time, $\lambda_{1,2,3}$ are the time constants and $\sigma_{1,2,3}$ the constants of the exponentials. The equation that best fits the data for each test was calculated by linear regression analysis.

The calculated half-lifes are represented in Table I. In view of such short half-lifes it was decided that the only effective route of administration would be continuous intravenous infusion.

TABLE I. TURNOVERS OF [^{125}I]APROTININ IN PLASMA OF RHESUS MONKEYS

MONKEY	HALF-LIFE ($T\text{-}1/2 = \frac{1n2}{\lambda}$)		
	$\lambda 1$ (min)	$\lambda 2$ (min)	$\lambda 3$ (hrs)
1	6.5	60	41
2	6.0	67	68
3	3.2	34	33
4	3.8	36	102
5	5.0	53	68
6	3.2	25	48
7	3.5	34	44
8	2.4	23	52
Mean $\pm$ SD	4.2 $\pm$ 1.4	42 $\pm$ 16	57 $\pm$ 22

Aprotinin effect in coagulation. Thirteen monkeys were injected with 40,000 KIU of aprotinin; blood samples were obtained at $\sim$2, 5, 10, 15, 20, 30, 45, 60, and 120 min after injection. The activated partial thromboplastin time (APTT) peaked at 4.61 $\pm$ 1.05 min (SE) after the injection with an increase of 47.30 $\pm$ 4.95% without any significant change in the prothrombin time. This effect was short-lived and by 1 hr APTT values had returned to normal (Table II).

TABLE II. EFFECT OF APROTININ IN APTT AFTER IV INJECTION OF 40,000 KIU OF APROTININ

MEAN ± SD	
Min after end of injection	% Increase in APTT
2.3 ± 0.6	36.5 ± 15.0
6.4 ± 1.0	38.9 ± 19.6
12.3 ± 0.6	31.2 ± 18.2
15.8 ± 4.0	26.9 ± 18.2
26.3 ± 1.4	20.3 ± 16.3
33.1 ± 2.4	15.9 ± 16.5
46.6 ± 2.2	17.8 ± 16.6
61.2 ± 4.1	11.0 ± 14.1
125.3 ± 6.1	9.3 ± 23.6

In summary, the data obtained indicate that aprotinin has a very short half-life in the plasma of rhesus monkeys and when circulating in high levels it can inhibit the initial phase of DIC.

Effect of aprotinin in DIC. A protocol for the study of aprotinin in DIC has been established; 6 groups of 4 monkeys each will be studied (Table III). The following parameters will be determined: (a) general conditions, i.e., temperature, rash, etc.; (b) bacteremia; (c) plasma prekallikrein, fibrinogen, fibrin degradation products, plasminogen, APTT and prothrombin time and (d) hematocrit and WBC and platelet counts. We have studied 15 monkeys so far, but results are being analyzed.

Comparative study of plasminogen activation. The plasminogen activator used in routine assays of clinical samples is streptokinase. However, streptokinase is not an activator of rhesus monkey plasminogen. In our assays we have used urokinase for that function.

TABLE III. EXPERIMENT DESIGN - EFFECT OF APROTININ IN DIC INDUCED IN MONKEYS (4/GROUP) BY S. TYPHIMURIUM INFECTION.

GROUP	INFECTION QUANTITY	APROTININ[a]
Saline	0	-
		+
S. typhimurium (heat-killed)	10^9/ml	-
		+
S. typhimurium (live)	10^9/ml	-
		+

[a] Initial dose: 4 ml/kg (40,000 KIU) and maintenance dose: 15 ml/kg/24 hr (150,000 KIU).

We compared streptokinase and urokinase activation in different primate species. In human samples 776 U of urokinase or the standard 2083 U of streptokinase hydrolyze similar amounts of the substrate Na-CBZ-6-lysine-p-nitrophenyl ester (CLN). Using these amounts of activators, we tested plasma from humans and the following subhuman primates: rhesus (M. mulatta), cynomolgus (M. fascicularis), bonnet (M. radiata) and African green (Cercopithecus aethiops) monkeys. Two different substrates were studied: CLN and H-D-Val-Leu-Lys-pNa.2 HCl (Ortho Chromogenic Substrate S-2251). The results are shown in Table IV.

Kininogen metabolism. (Collaboration with Drs J. J. Pisano and J. V. Pierce, National Institutes of Health). The objective of these studies is to determine whether urinary kininogen has a plasma (glomerular filtration) or renal (tubular secretion) origin. We injected radiolabeled ^{125}I-labeled kininogen in monkeys and applied their plasma and urine samples to Sephadex columns.

The peaks of radioactivity in the urine in no experiment ever corresponded to the peak in the plasma sample attributable to the ^{125}I-labeled kininogen. If further studies corroborate these findings, the source of urinary kininogen must be renal secretion.

TABLE IV. PLASMINOGEN DETERMINATIONS IN PRIMATE SPECIES (ALL DETERMINATIONS RUN IN DUPLICATE)

SPECIES (n)	SUBSTRATE[a]	MEAN ± SE			
		Urokinase, 776 U	Streptokinase, 2083 U	None	UROKINASE/ STREPTOKINASE
Human (5)	CLN	1.22 ± 0.12	1.44 ± 0.18	0.30 ± 0.03	0.87 ± 0.06
	S-2251	0.16 ± 0.02	0.25 ± 0.04	0.02 ± 0.00	0.66 ± 0.09
Rhesus (5)	CLN	1.18 ± 0.16	0.38 ± 0.05	0.28 ± 0.03	3.15 ± 0.02
	S-2251	0.16 ± 0.02	0.08 ± 0.02	0.02 ± 0.00	2.78 ± 1.17
Cynomolgus (5)	CLN	1.17 ± 0.20	0.49 ± 0.11	0.34 ± 0.04	2.48 ± 0.13
	S-2251	0.14 ± 0.03	0.25 ± 0.04	0.01 ± 0.00	0.53 ± 0.09
Green (4)	CLN	1.03 ± 0.21	0.78 ± 0.17	0.26 ± 0.04	1.38 ± 0.09
	S-2251	0.17 ± 0.04	0.15 ± 0.02	0.02 ± 0.00	1.10 ± 0.20
Bonnet (5)	CLN	1.21 ± 0.20	0.52 ± 0.09	0.27 ± 0.03	2.40 ± 0.12
	S-2251	0.17 ± 0.03	0.13 ± 0.02	0.02 ± 0.01	1.33 ± 0.08

[a] CLN units, ml^{-1} (extinction coefficient at pH 5.5 = 5302 M^{-1} cm^{-1}).
S-2251, ΔOD = 405 nm.

Publications:

None

LITERATURE CITED

1. Wing, D. A., T. Yamada, H. B. Hawley, and G. W. Pettit. 1978. Model for disseminated intravascular coagulation: bacterial sepsis in rhesus monkeys. J. Lab. Clin. Med. 92:239-251.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6423	78 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
78 04 21	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	62776A	3M162776A841	00	026
b. CONTRIBUTING				
c. ~~XXXXXX~~/	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(U) Effectiveness of selected antiviral compounds against diseases of BW 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
70 12	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		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.0	230.0
	CURRENT 79	1.0	285.9

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

21. GENERAL USE

Foreign intelligence considered

ASSOCIATE INVESTIGATORS
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POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Chemotherapy (U) Togaviruses; (U) Arenaviruses; (U) Metabolism; (U) Bioavailability; (U) Monkeys

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

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) 77 10 - 78 09 - Lysine-stabilized poly(I)-poly(C) [poly(ICLC)] was effective in delaying the onset of viremia and time to death of Pichinde-infected guinea pigs; however, percent survival was not increased. Ribavirin significantly increased survival as well. Ribavirin was evaluated in vitro against Machupo virus and found to have significant antiviral activity. In addition, Machupo virus-infected rhesus monkeys were treated successfully with ribavirin, even when therapy was delayed until after the onset of clinical illness. Ribavirin was also shown to be effective in the treatment of Rift Valley infection in mice and hamsters.

Publications: Current Chemotherapy, Vol. 1, p. 317-319, 1978;
Fed. Proc. 37:379, 1978;
Abstr., Ann. Mtg. Am. Soc. Microbiol. - 1978, p. 228(2).
In Current Veterinary, Therapy VI, pp. 1345-1348, 1977.
J. Infect. Dis., in press, 1979
Anthropod-Borne Virus Information Exchange 34:179, 1978.
In Respiratory Disease Symposium, pp. 1700-182, 1978.

* 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 * U.S. GPO 1974-540-843/8691

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 026: Effectiveness of Selected Antiviral Compounds Against Diseases of BW Importance

Background:

Many potentially debilitating viral diseases exist in geographic locales of military significance for which acceptable vaccines are unavailable, either because the viruses are newly isolated and acceptable vaccines have thus far not been developed, as in the case of the hemorrhagic arenaviruses (1), or because of the existence of several variant species of one virus, as with dengue (DEN) or sandfly fever (SF) virus (2). Additionally, as many of these viral pathogens (e.g., SF) cause only transient illness in the local populace with insignificant mortality, there has been little concerted effort for development of vaccines for their control (2.3). Diseases of this nature, however, are of vital concern to the military, as the potential exists for morbidity rates of as high as 80% in susceptible troops, thus, neutralizing military effectiveness.

Antiviral chemotherapy is recognized as an acceptable tool for control of certain viral diseases which are as yet unmanageable through routine vaccine application (1). The development of animal models for the study of human diseases has resulted in many potentially useful tools for investigating the effectiveness of antiviral chemotherapeutic agents. Examples of representative virus infections include yellow fever (YF) in rhesus monkeys (flavivirus infection) (4), VEE in mice and monkeys (alphavirus infection) (5), Rift Valley fever (RVF) in mice and monkeys (Bunyamwera virus infection) (6), and Machupo (MAC) virus in guinea pigs and rhesus monkeys (arenavirus infection) (7).

In addition, practical evaluation of potential antiviral agents can be done in tissue culture using these same viruses. This approach can be used most effectively to evaluate analogs of a candidate antiviral compound for either increased efficacy or decreased toxicity.

Progress:

As a screening procedure, studies were conducted to evaluate the antiviral properties of several potential antiviral compounds. Butylated hydroxytoluene (BHT), an antioxidant used in food preservation, was evaluated against VEE virus infection in mice. BHT was given once daily, either per os or SC, in dosages ranging from 0-400 mg/kg. The virus ranged from 10-1000 PFU/mouse. Mice given oral (100, 200 or 400 mg/kg) BHT and no virus had 11, 22, and 56% mortality, respectively, whereas, mice given similar doses SC were not affected by drug toxicity. In infected mice there was no effect of drug treatment on time to death or survival regardless of dose or route of administration. Since higher doses of BHT are not possible because of toxicity, it appears that this compound will not be useful in the prevention or treatment of VEE virus infection.

The antiviral activity of 5 other compounds (actinomycin, rubiflavin, griseorhodin-A, bostrycoidin, daunorubicin) obtained from the Natural Substances Branch (National Cancer Institute) was evaluated in vitro against YF and VEE viruses. Drug concentrations ranging from 0.0008 to 10 μg/ml were used. No significant activity was noted by the plaque reduction assay technique with any of the drugs tested. In addition, rubiflavin, daunorubicin, and bostrycoidin were moderately to severely cytotoxic to Vero and LLC-MK_2 tissue culture cells.

Ribavirin, rimantadine, and poly(ICLC) were evaluated against a guinea pig-lethal variant of Pichinde (PIC) virus developed by Dr. Jahrling, Virology Division. Guinea pigs were infected with 10,000 PFU of PIC virus, one-half dose IP and one-half dose SC, and then treated by IM injection for 5 days beginning on day 0, using triacetyl ribavirin (20 mg/kg/dose b.i.d.), rimantadine hydrochloride (20 mg/kg/dose, b.i.d.), or poly(ICLC) (1.0 mg/kg/dose, b.i.d.). In rimantadine-treated guinea pigs there was no effect on the level of viremia or % survival. In poly(ICLC)-treated guinea pigs there was no change in viremia, and although mean time-to-death (MTD) was extended, there was no change in survival rate. Ribavirin triacetate, however, delayed onset of viremia, reduced peak viremia, and increased survival.

Based on the results of the PIC virus study, the antiviral effect of ribavirin was evaluated against MAC virus, the causative agent of Bolivian hemorrhagic fever and another arenavirus. Ribavirin at levels of 10, 25, and 60 μg/ml was evaluated in vitro in MAC virus-infected Vero cells. Ribavirin treatment reduced the yield of virus as much as 5 logs at the highest drug concentration.

Ribavirin and its triacetyl analog were then evaluated against MAC virus in vivo. Guinea pigs were infected with ~ 1000 PFU of MAC virus and then treated with ribavirin or triacetyl ribavirin, 25 mg/kg b.i.d., for 10 days. Percent survival was significantly increased ($P < 0.001$) in both the ribavirin (87.5% survival) and triacetyl ribavirin-treated groups (78.7% survival) when compared to the virus control group (0 survival).

Ribavirin was further evaluated in MAC virus-infected rhesus monkeys. Three groups of monkeys, 4/group, were infected SC with 32,000 PFU of MAC virus. Group I was a virus control group and received virus plus saline. Group II received virus plus ribavirin, 10 mg/kg, t.i.d. IM. Treatment began on day 0 and was repeated daily for a total of 17 days. Group III received virus plus ribavirin treatment, 20 mg/kg, t.i.d. on the same schedule of treatment as group II. Viremia titers were significantly reduced ($P < 0.001$) in both the 10 and 20 mg/kg-treated groups (Table I). Additionally, the viremia titer for the 20 mg/kg-treated group was significantly less ($P < 0.001$) than that of the 10 mg/kg-treated group, indicating that ribavirin produced a direct, dose-related effect. MTD and % survival were increased in the treated groups (Table I).

TABLE I. VIREMIA AND DAY OF DEATH OF RHESUS MONKEYS CHALLENGED WITH MACHUPO VIRUS AND TREATED WITH RIBAVIRIN.

GROUP DOSE (mg/kg)	MONKEY NO.	VIREMIA BY DAYS (Log_{10} PFU/ml) 5	7	9	12	14	16	19	21	28	DAY OF DEATH
	1	2.18	2.86	2.78	4.48	4.52	3.13	2.68	1.70	-	33
I	2	-[a]	2.70	3.31	5.66	5.75	5.46	4.26	3.67	3.29	29
(Saline)	3	2.97	5.16	4.11	4.58	5.83	5.27	DEAD			17
	4	2.60	4.06	3.39	4.50	5.79	5.64	4.33	2.78	DEAD	25
	1	3.13	3.63	3.01	2.00	1.88	-	-	-	-	
II	2	3.14	3.58	3.13	2.51	3.39	2.60	-	-	-	
(10)	3	-	1.88	3.08	2.68	3.24	3.17	2.24	-	-	43[b]
	4	1.88	3.61	3.06	3.19	3.43	2.98	1.88	-	-	64[b]
	1	1.40	2.10	3.15	2.00	-	-	-	1.40	-	
III	2	-	1.70	2.76	-	-	-	1.40	1.40	-	75[b]
(20)	3	1.40	2.30	1.70	-	-	-	1.40	1.70	-	38[b]
	4	1.88	2.83	3.37	-	1.70	1.40	-	-	-	41[b]

a < 2.5

b Killed when paralyzed.

The effect of ribavirin treatment begun after onset of clinical signs was evaluated. Baseline temperatures were taken for 8 rhesus monkeys and the results averaged for each monkey. They were inoculated with MAC virus; temperatures were taken daily thereafter. Treatment for each monkey was begun after its temperature exceeded its baseline mean daily value by more than 2 SD. Treatment was begun as early as 4 days postinfection in some animals, and as late as 7 days in others. All treated animals cleared their viremia by day 10, while controls had a steadily increasing viremia up to $10^{4.2}$ PFU/ml (Table II). Shortly after reaching peak viremia, all controls died, with a MTD of 22.7 days. The treated group had a MTD of 37.0 days, with a survival rate of 50% (2/4). Deaths in the treated monkeys were characterized by chronic, progressive loss of neuromuscular function. This can perhaps be explained by the fact that the virus strain used was previously passed 21 times in suckling hamster brain, and may have developed a high degree of neurotropism, which presents clinically only in those monkeys which have survived the initial acute hemorrhagic phase of the disease. The neurological syndrome has not been reported as a prominent feature in the naturally-acquired form of the disease in man.

To determine the effects of parenterally administered ribavirin on clinical pathological values, 8 rhesus monkeys were given ribavirin IM (20 mg/kg b.i.d.) for 17 days. The monkeys were bled twice weekly for 6 weeks. Blood samples were monitored for changes in hematocrit, WBC, platelet and RBC count and differential. Serum samples were evaluated for changes in globulin, albumin, SGOT, glucose, Ca, creatinine phosphokinase, cholesterol, total protein, creatine, BUN, SGPT, phosphate, LDH, Cl, K, total bilirubin, Na, and alkaline phosphatase. Ribavirin significantly decreased hematocrit and increased the platelet count ($P < .001$). These 2 parameters quickly returned to baseline values after ribavirin treatment was stopped. There was no significant change observed in any of the other hematological or serum chemistry determinations.

The antiviral activity of ribavirin was also evaluated against YF virus in rhesus monkeys. Previous *in vitro* work had shown good antiviral activity against YF virus grown on LLC-MK_2 cells with a plaque reduction of 50% at a concentration as low as 10 µg/ml. YF-infected monkeys were given a loading dose of ribavirin (50 mg/kg) either 16 or 72 hr after virus inoculation followed by 5 mg/kg t.i.d. for 10 days. Viremia titers, MTD, and % survival were similar in all groups regardless of treatment. Asibi strain YF virus concentration peaks in the liver and blood well before onset of clinical signs of illness; therefore, it is doubtful that this disease could ever be treated on a realistic basis in monkeys. Survival of 2 of 4 infected rhesus monkeys was observed when treatment was initiated 8 hr postinfection.

In vitro studies of ribavirin against RVF virus indicated marginal, if any, activity by the plaque reduction assay. *In vivo* ribavirin was evaluated against RVF, Zagazig 501, in golden Syrian hamsters and C57/B16 mice. Treatment was initiated the day of virus inoculation and

TABLE II. VIREMIA AND DAY OF DEATH OF RHESUS MONKEYS CHALLENGED WITH MACHUPO VIRUS AND TREATED WITH RIBAVIRIN

GROUP	MONKEY NO	VIREMIA BY DAYS (Log_{10} PFU/ml) 3	5	7	10	12	14	17	19	DAY OF DEATH
I (control)	1	-[a]	-	2.86	3.78	3.94	4.17	3.70	2.51	28
	2	-	-	2.40	4.18	6.30	DEAD			13
	3	-	2.48	4.17	2.74	3.97	4.38	4.70	3.13	23
	4	-	1.88	3.76	2.44	4.37	5.08	3.88	4.17	27
II (Treated)	1	1.38	2.38[c]	1.70	-	-	-	-	-	31[b]
	2	-[c]	-	3.01[c]	-	-	2.10	-	-	
	3	-	-	-	-	-	-	-	-	
	4	-	2.40[c]	2.86	-	-	-	-	-	43[b]

a < 2.5
b Killed when paralyzed.
c Initial day of treatment.

continued for 10 successive days. Hamsters received 20 mg/kg ribavirin b.i.d. and mice received 25 mg/kg t.i.d.. In the RVF-infected hamsters, ribavirin treatment delayed MTD from 2.9 to 14.5 days, and increased survival from 0 to 80%. All animals in the virus-control group became viremic with a mean peak viremia of 3.96 $\log_{10}$ PFU/ml. Only 1 of 5 in the treated group was viremic, with a titer of 3.70 $\log_{10}$ PFU/ml. Ribavirin treatment delayed MTD from 4.4 to 10.5 days, and increased survival from 4 to 92% in mice.

In order to develop a stock of SF virus for eventual testing of ribavirin, 2 stock pools (A-SF-WR-Sicilian: 1st FD SMO Pass., 10% HBSS-HE, 0.25% HSA, 16 May 1972 B-SF-BHK-21 S/1, 50% Stab. Med., 19 Mar 1972) of virus were inoculated in suckling hamster brains. Infected brains were harvested on days 10 and 11, centrifuged, stabilized in 50% fetal calf serum, and titered on SW-13 tissue culture cells. Virus titers of the pools ranged from 3.0 to 5.0 $\log_{10}$ PFU/ml. These pools will be used to evaluate the antiviral activity of ribavirin against SF virus both *in vitro* and *in vivo*.

Presentations:

1. Kuehne, R. W., W. L. Pannier, R. R. Rosato, and E. L. Stephen. Treatment of Tacaribe virus infection of mice using various antiviral compounds. Presented,Annual Meeting, Am. Soc. Microbiol., Las Vegas, NV, 14-19 May 1978 (Abstr. Annual Meeting - 1978, p. 228).

2. Jones, D. E., E. L. Stephen, and P. B. Jahrling. Evaluation of the antiviral activity of triacetyl ribavirin, rimantadine, and poly (ICLC) in a model arenavirus infection. Presented, Annual Meeting, Soc. Microbiol., Las Vegas, NV, 14-19 May 1978 (Abs. Annual Meeting - 1978, p. 228).

3. Stephen, E. L., G. H. Scott, and R. F. Berendt. Experimental infection of mice and squirrel monkeys using swine influenza virus: effect of amantadine, rimantadine, and ribavirin. Presented, Annual Meeting, FASEB, Atlantic City, NJ, 9-14 Apr 1978 (Fed. Proc. 37:379, 1978).

4. Stephen, E. L., and R. F. Berendt. Aerosol therapy for bacterial lung disease. Presented, Scientific Seminar, American College of Veterinary Internal Medicine, Dallas, TX, 16 Jul 1978 (Proceedings, Respiratory Disease Symposium, p. 170-182).

5. Stephen, E. L. Antiviral chemotherapy: rational approach for development of drugs for use in man. Presented, Lecture, University of Alabama Medical Center, Birmingham, AL, 17 Jul 1978.

Publications:

Hilmas, D. E., E. L. Stephen, R. O. Spertzel, H. B. Levy. 1978. Use of poly(ICLC) for the prophylaxis and treatment of Venezuelan equine encephalomyelitis virus infection in nonhuman primates, pp. 317-319. In Current Chemotherapy, Vol. 1 (W. Siegenthaler and R. Luthy, eds.). American Society for Microbiology, Washington, DC.

LITERATURE CITED

1. Pfau, C. J. 1975. Arenavirus chemotherapy - - retrospect and prospect. Bull. WHO 52:737-744.

2. Bartelloni, P. J., and R. B. Tesh. 1976. Clinical and serologic responses of volunteers infected with Phlebotomus fever virus (Sicilian type). Am. J. Trop. Med. and Hyg. 25:456-462.

3. Tesh, R. B., S. Saidi, E. Javadian and A. Nadim. 1977. Studies on the epidemiology of sandfly fever in Iran. I. Virus isolated obtained from Phlebotomus. Am. J. Trop. Med. Hyg. 26:282-287.

4. Hilmas, D. E., E. L. Stephen, P. C. Kosch, and R. O. Spertzel. 1976. Experimental yellow fever virus infection of rhesus monkeys. IRCS Med. Soc. Exp. Anim., etc. 4:487.

5. Kuehne, R. W., W. L. Pannier, and E. L. Stephen. 1977. Indirect mouse model for the evaluation with potential antiviral compounds: results with Venezuelan equine encephalomyelitis virus. Antimicrob. Agents Chemother. 11:683-687.

6. Easterday, B. C. 1965. Rift Valley fever. Adv. Vet. Sci. 10:65-127.

7. Eddy, G. A., S. K. Scott, F. S. Wagner, and O. M. Brand. 1975. Pathogenesis of Machupo virus infection in primates. Bull. WHO 52:517-521.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OG6410	78 06 27	DD-DR&E(AR)636

3. CAT. PREV. SUMRY	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
77 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	027
b. CONTRIBUTING				
c. 777777 STOG 78-7.2.1, 3, 6				

11. TITLE (Enter title with Security Classification Code) (U) Arenavirus-rodent models for use in cross-protection, antiviral chemotherapy and vaccine studies

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 01	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	0.8	94.9
	CURRENT 79	0	0

19. PERFORMING ORGANIZATION
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NAME: Cole, Jr., F. E. POC:DA
NAME:

GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Rodents; (U) Cross-protection; (U) Vaccines; (U) Antiviral chemotherapy; (U) Tacaribe complex

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

23 (U) Develop and define rodent models of lethal infection for use in arenavirus studies; investigate cross-protection conferred by Tacaribe (TCR) complex viruses against lethal challenge in the models; obtain basic data pertinent to cross-protection for vaccine development, employ lethal model as an indirect system for testing the efficacy of antiviral compounds against virus diseases of potential BW importance.
24 (U) Adult rodent models of lethal arenavirus infection are developed by sequential passage of infected brain material to obtain increased lethality. Time of onset, degree and duration of protection afforded by members of the TCR complex against challenge with lethal strains will be examined.
25 (U) 77 10 - 78 06 - Protection studies using formalin-inactivated TCR virus in a 2-dose schedule showed that the killed vaccine was effective (60% survival); addition of adjuvant increased effectiveness. Effectiveness will increase with increasing antigenic mass. Cross-protection studies were done with live viruses in which both the immunizing and challenge doses were given IC. Homologous TCR protection was complete. Pichinde virus afforded good protection with a single dose which decreased as the number of doses increased. The results with Tamiami virus were inconclusive. Studies in homozygous (nu/nu) and heterozygous (Nu/+) nude mice to determine the protective effect of passively administered TCR virus antibodies for Nu/+ mice and the possible potentiation of lethality or immunopathology in nu/nu mice were done. This work unit was officially terminated in May of 1978, when the principal investigator was assigned to Work Unit A841-00-063, "Rapid diagnosis of viral diseases of military importance."
Publication: Arch. Virol. 57: in press, 1978.

*Save this form for subsequent updating and approval.

DD FORM 1498 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 448A, 1 NOV 65 AND 448-1 MAY BE USED UNTIL EXHAUSTED. ALL OTHER EDITIONS OF THIS FORM ARE OBSOLETE.

* U.S. GPO 1974-540-843/8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 027: Arenavirus-Rodent Models for Use in Cross-Protection, Antiviral Chemotherapy and Vaccine Studies

Background:

The arenaviruses, Lassa, Junin and Machupo are highly pathogenic for humans thereby requiring P-4 containment. They are endemic in specific areas of the world where military personnel are or may be deployed, and are therefore of military importance in order to develop means of protecting personnel. Fortunately, other arenaviruses are either not known to infect humans or to produce only subclinical infections demonstrated by sero-conversion. When this work unit was initiated, lethal, adult-rodent models did not exist for those arenaviruses not known to infect humans. We therefore attempted to develop an adult, small rodent model of lethal infection for Tacaribe virus for use in cross-protection, antiviral chemotherapy, immunopathologic, and vaccine studies.

Progress:

Development of the Tacaribe (TCR) virus animal model was described previously (1, 2) as were initial studies concerned with cross-protection by live viruses, protection with inactivated viruses and studies on the immunopathologic nature of the TCR virus infection. Studies involved with developing the model, have been published (3). Only additional data will be presented.

Protection by inactivated viruses. A protection study using formalin-inactivated TCR virus has been completed. Basically a 60% suckling mouse brain (SMB) preparation containing 3.7×10^7 PFU/ml and a 60% suspension of normal mouse brain were used to treat 21-23-day-old mice. Freund's complete adjuvant and the immuno-suppressant Cytoxan were used to enhance or suppress the hosts response. Single and 2-dose treatment schedules (14-day interval) were used and IC challenge was 21 days after the last day of treatment. Both schedules included immunization with live virus as a control. The data indicate that for the single dose schedule: (a) lethal challenge is effective (61% mortality at 42 days); (b) live Tacaribe virus is protective (85% survival $P < 0.005$, Chi-square); (c) cytoxan is ineffective in modifying the response of the killed vaccine; and (d) killed vaccine offers no greater protection than normal mouse brain (NMB) when used in a single dose schedule. Considering only the 2-dose schedule: (a) it is apparent that IC challenge is lethal (no survivors in untreated controls); (b) live virus is completely effective (no deaths, $P < 0.005$);

(c) NMB + FCA controls negative (no survival); (d) NMB control negative (20% survivors); (e) killed vaccine effective (60% survival, $P < 0.005$); and (f) killed vaccine plus FCA had an increased effectiveness (80% survival, $P < 0.005$).

There is a problem in assessing the results due to a number of deaths caused by the immunizing (large volumes required) and challenge (anaphylatic) doses. We can reduce the size of the immunizing doses by additional concentration procedures. The reaction caused by the challenge dose has never occurred before and may be due to the serum used as a virus stabilizer. Use of normal mouse serum as the stabilizer will alleviate this. To wit, we prepared 3 vaccine lots of 100-300 ml each of 10^6-10^7 PFU/ml. Each has been concentrated on sucrose:renographin discontinuous gradients and purified on linear, rate-zonal renographin gradients. Final PFU determinations on the purified virus indicate a possible loss of infectivity during purification. However, total particle counts as determined by electron microscopy are to be the final basis for determining immunization protocols. Since TCR virus is a rather labile virus, a loss in total PFU was anticipated. We expect that the total particle concentration has remained constant. It is anticipated that the testing of these killed TCR virus vaccine preparations will be the final area of investigation of this work unit.

Protection by live viruses. Results of cross-protection immunization studies have generally been disappointing (1, 2). It was therefore suggested that such studies with live viruses be attempted in which both the immunizing and challenge doses be inoculated into the brain. Female mice (Flow, ICR, 6-weeks old) were immunized according to the following schedule. Mice received 0.03 ml of virus (TCR or Tamiami, TAM) at 10,000 $SMICLD_{50}$, Parana (PAR or Pichinde, PIC) at 100,000 $SMICLD_{50}$) or diluent by the IC route at 14-day intervals. Mice (18 from the respective virus group and 10 from the diluent control group) were removed from each group prior to administration of the 2nd and 3rd immunizations to determine: (a) antibody level as a result of the immunizing dose (3 mice), (b) histological examination for virus-produced lesions (3 mice), and (c) lethal IC challenge (15 immunized, 10 controls). Challenge was with $\sim$300 $SMICLD_{50}$ of TCR virus, population $\#7_4$-5 given IC as a 0.03-ml dose 21 days after the last immunization, i.e., for the 1-dose schedule challenge was at day 21, 2-dose, challenge was at day 36, and for 3-dose, challenge at day 49. All mice were observed for 21 days, deaths recorded, and percent protection calculated by the Fisher's exact test. Results are given in Table 1. As expected, any dose schedule of live TCR virus affords total protection to challenge. PAR virus affords good protection with a single dose which decreased as the number of doses increased. The results with TAM and PIC viruses were inconclusive. Antibody levels have not been determined to date nor have the results of the histological examination been received.

TABLE I. IC CROSS PROTECTION STUDIES IN MICE

VIRUS	GROUP	DOSES	DEAD/TOTAL	P[a]
TCR	Immunized	1	0/15	< 0.001
	Control	1	10/10	
	Immunized	2	0/15	< 0.001
	Control	2	6/10	
	Immunized	3	1/14	< 0.001
	Control	3	5/ 9	
TAM	Immunized	1	3/ 9	< 0.02
	Control	1	9/10	
	Immunized	2	9/15	0.12
	Control	2	9/10	
	Immunized	3	3/15	< 0.05
	Control	3	7/12	
PAR	Immunized	1	5/15	< 0.001
	Control	1	10/10	
	Immunized	2	2/13	< 0.001
	Control	2	9/10	
	Immunized	3	2/15	< 0.05
	Control	3	8/10	
PIC	Immunized	1	10/15	0.20
	Control	1	9/10	
	Immunized	2	9/15	0.27
	Control	2	8/10	
	Immunized	3	5/15	0.08
	Control	3	7/10	

[a]Fisher's exact one-tailed.

Nude mouse study. Initial studies on nude mice (2) challenged with lethal TCR virus indicated no deaths or lesions attributable to challenge, suggesting the involvement of T cells in the immunopathology observed in normal mice. The current availability of homozygous nude mice (nu/nu) and heterzygous (nu/+) litter mates made possible a study to determine:

(a) the possible protective effect of the passive administration of high-titered TCR virus antibody for nu/+ mice, and (b) the possible potential of lethality of immunopathology in nu/nu mice by the same high-titered homologous antibodies. All nu/nu and nu/+ mice (24/group) received 0.03 ml of lethal TCR virus ($\sim$300 $SMICLD_{50}$, IC) at day 0. Five days later nu/nu and nu/+ mice were divided into 4 groups of 6 mice each. Group 1 received 0.3 ml and Group 2, 1.0 ml IP of normal mouse serum. Group 3 and 4 received 0.3 and 1.0 ml of TCR virus antibody that titered 1:640 by an 80% PRN test. Results indicate (a) all nu/nu mice survived challenge in all 4 groups, and pathological lesions were totally absent indicating that antibody and probably B cells do not play a role in the lesions observed in normal mice. All nu/+ mice died (exception, 2 survived at normal serum, 0.3-ml dose) indicating that the passive administration of high-titered homologeous antibody neither protected nor altered the lesions observed at the time intervals and dosages used.

This work unit was officially terminated in May 1978 when the principal investigator was assigned another work unit (A841 00 063) of higher priority. All studies with Tacaribe virus were stopped in January 1978 with the exception of plans to test the efficacy of a killed homologous vaccine. The data on protection and cross-protection with live arenaviruses and nude mouse studies will be assembled for possible publication. Cooperative antiviral chemotherapy studies are reported under work unit A841 00 027.

Presentations:

1. Rosato, R. R. and A. V. Vorndam. Proposed nomenclature for structural proteins of Bunyaviridae. Presented, Subcommittee on Applied Molecular Virology Meeting, Las Vegas, NV, 14-18 May 1978.

Publication:

1. Rosato, R. R. M. R. Elwell, and G. A. Eddy. 1978. Virulence alternations of Tacaribe virus infection in adult mice: lethal model for encephalitis.

2. Gangemi, J. D., R. R. Rosato, E. V. Connel, C. M. Johnson, and G. A. Eddy. 1978. Structural Polypeptides of Machupo Virus. J. Gen. Virol. in Press. 1978.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1976. Annual Progress Report, FY 1976, pp. 361-368. Fort Detrick, Frederick, MD.

2. U. S. Army Medical Research Institute of Infectious Diseases. 1 October 1977. Annual Progress Report, FY 1977, pp. 219-227. Fort Detrick, Frederick, MD.

3. Rosato, R. R. M. R. Elwell, and G. A. Eddy. 1978. Virulence laterations of Tacaribe virus infection in adult mice: lethal model for encephalitis. Arch Virol. 57: In Press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6427	78 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
78 04 21	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	62776A	3M162776A841	00	029
b. CONTRIBUTING				
c. ~~1111111~~	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code) (U) Physiologically directed therapy for flavivirus 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
76 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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	0.4	70.0
FISCAL YEAR CURRENT 79	0.5	105.9

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ASSOCIATE INVESTIGATORS
NAME: Hadick, C. L.
NAME: POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Body fluids, (U) Tissue water; (U) Electrolytes; (U) Leukocytic endogenous mediator; (U) Monkeys

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

23 (U) Evaluate physiologically directed therapy with physical and nutritional means or with compounds of human antiviral activity in subhuman primates against selected flavivirus infections of military and BW importance. Research is aimed at improving treatment and prevention of these infectious diseases in susceptible military personnel when assigned to areas of the world where these diseases are endemic or where there is a BW situation.

24 (U) Measure various physiological and biochemical changes in monkeys during selected virus infections. Evaluate candidate antiviral compounds for their ability to prevent or modify the adverse virus-induced changes. Use hemoperfusion with an activated charcoal column to treat yellow fever (YF).

25 (U) 77 10 - 78 09 - Monkeys with YF at the terminal stage showed increases in plasma volume and blood volume. Extracellular Na, K and water, and cholesterol and phosphate levels in certain tissues were also elevated 5 days postinoculation compared to controls. However, red blood cell volume, hematocrit and tissue total K, intracellular water and Na, triglyceride and phospholipids decreased. Liver revealed the most changes, while lung, heart, skeletal muscle, kidney, diaphragn and the central nervous system were also affected. Hemoperfusion with an activated charcoal column was initiated for the treatment of YF in monkeys.

Publications: Kidney Int. 12:528, 1977.
Fed. Proc. 37:504, 1978.
J. Infect. Dis. 138:42-48, 1978.
Am. J. Vet. Res. 39: in press, 1978.

*Available to contributors upon originator's approval

DD FORM 1498 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. AMS 1498-1 1 MAR 68 FOR AHMM USE ARE OBSOLETE.

* U.S. GPO 1974-540-843 8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 029: Physiologically Directed Therapy for Flavivirus Infections of Unique Military Importance

Background:

Part I. Yellow fever

The liver has been considered the main organ affected by yellow fever (YF) virus (1); pathological and functional changes were also demonstrated in other organs including the central nervous system, lung, and kidney. The objectives for the present study were 2-fold: (a) to identify disease-producing mechanisms of YF and cause of death; and (b) to develop techniques for effective therapy of YF-infected monkeys. Since hemoperfusion through an activated charcoal column was used successfully to remove circulatory toxins in treatment of fulminant hepatic failure (2), and the parenteral feeding of adequate calories and amino acids prevented the marked body wasting associated with septicemia in monkeys (3), hemoperfusion or hyperalimentation, or the combination of the 2 therapeutic approaches were designed to apply to monkeys with YF.

Part II. Cardiovascular effects of leukocytic endogenous mediator (LEM).

LEM has been proposed as a mediator of host metabolic responses to infectious diseases (4). Fever, leukocytosis, hyperglucagonemia, hyperinsulinemia, plasma Zn and Fe depression, and increase in hepatic amino acid transport in rats and rhesus monkeys occur following IV or IP administration of LEM (5). The present study was designed to determine cardiovascular responses and liver function as measured by the disappearance rate of cardiogreen dye at various intervals for 24 hr after IV infusion (10 min) of a crude rabbit LEM preparation (10 ml/kg).

Progress:

Part I. Yellow fever

Body fluids, tissue water, and tissue electrolyte distribution in rhesus monkeys with YF.

The work on measurements of body fluid compartments and distribution of tissue water and electrolytes 5 days after SC inoculation of 10 PFU of YF virus has been completed. Six control and 10 infected monkeys were used for these studies. Tables I and II summarize changes observed in the terminal stages of YF-infected monkeys compared to controls.

The data suggest that there was an expansion of plasma and blood volumes in monkeys with yellow fever. Although the liver is a major target organ for YF virus, the central nervous system, heart, lung, kidney and diaphragm were also affected. In contrast with Rocky Mountain spotted

fever (RMSF) which produced intracellular overhydration in the medulla oblongata, YF virus produced intracellular dehydration of medulla oblongata, cerebellum, and spinal cord. Cellular fluid loss in the brain and spinal cord may partially contribute to death as a result of central cardiopulmonary depression in the YF virus-infected monkey.

TABLE I. SUMMARY OF CHANGES IN TOTAL AND EXTRA- AND INTRACELLULAR WATER AND ELECTROLYTES RELATIVE TO CONTROLS IN YF-INFECTED MONKEYS.

	CHANGE[a]								
	H_2O			Na^+			K^+		
TISSUE	T	E	I	T	E	I	T	E	I
Liver	↑	–[b]	–	↑	–	↑	↓	↑	–
Lung	–	–	–	–	–	–	↓	–	–
Heart	–	–	–	–	↑	↓	–	↑	–
Renal Cortex	–	↑	↓	–	↑	–	–	↑	–
Diaphragm/muscle	–	–	–	–	–	–	–	↑	–
Cerebral cortex	–	–	–	–	–	–	–	↑	–
Cerebellum	–	↑	↓	–	↑	–	↓	↑	–
Thalamus/ hypothalamus	–	↑	–	–	↑	–	–	↑	–
Medulla oblongata	–	↑	↓	–	↑	↓	–	↑	–
Spinal cord	–	↑	↓	–	↑	–	–	↑	–

[a] T=total, E=extracellular, I=intracellular.
[b] No change.

TABLE II. CHANGES IN BODY FLUIDS IN MONKEYS WITH YELLOW FEVER

PARAMETER	CONTROL (N=6)	YELLOW FEVER (N=10)
Plasma volume (ml/kg)	44.3 ± 1.8	60.3 ± 2.9*
RBC volume (ml/kg)	24.0 ± 1.5	19.8 ± 1.0*
Circulatory blood volume (ml/kg)	68.2 ± 3.4	80.2 ± 2.6*
Circulatory hematocrit, %	34.8 ± 1.1	26.3 ± 1.8*

*$P < 0.05$

Plasma and tissue lipid changes. Total cholesterol concentrations of plasma and skin increased in monkeys with YF compared to controls. Although the liver was the only organ with increased levels of total lipids postinoculation, triglyceride levels decreased significantly in the lung, liver, cerebral cortex and spinal cord of infected monkeys. Furthermore, the thalamus-hypothalamus complex showed higher free cholesterol levels and the cerebellum revealed decreased concentrations of phospholipids with YF virus infection compared to noninfected monkeys. These disturbances of lipid metabolism with YF imply that not only the liver was damaged during this disease, the lung and certain parts of the central nervous system were also affected by the virus or by the infectious processes.

Changes in tissue phosphate. During terminal YF infection in rhesus monkeys, liver, heart, and muscle showed decreased concentrations of inorganic acid-soluble phosphorus (IAS), while the skin revealed an increase in IAS. Organic acid-soluble (OAS) phosphorus levels were increased in the heart, muscle and medulla oblongata. The alterations of tissue phosphate content, whether in an organic or inorganic form, suggest that energy metabolism or cellular metabolism in general is disturbed, since phosphorus is the key element for the synthesis of ATP. For example, OAS phosphorus levels were also increased in the lungs of rhesus monkeys during staphylococcal enterotoxin B toxemia (Toxicon, in press, 1978).

Measurements of regional blood flow and vascular resistance. To develop techniques for measuring regional blood flow and vascular resistance by using ^{85}Sr-labeled microspheres, baseline values of these variables for 43 different tissues were obtained from 1 rhesus monkey. The data are in good agreement with published values (6), and much new information on additional tissues is added to the existing literature. Once techniques are established, regional blood flow and hemodynamic changes of selected organs can be determined at various intervals after infection, vaccination, intoxication, or their combinations when different tracer microspheres are serially injected into the left ventricle.

Treatment of YF. Studies were initiated to evaluate potential therapeutic techniques for YF. The therapeutic approaches include the separate and combined techniques of hemoperfusion through activated charcoal and hyperalimentation. It is believed that hemoperfusion may aid liver function, greatly impaired by YF, by removal of toxins resulting from tissue breakdown and that hyperalimentation may provide required nutrients for physiologic maintenance. Should the antiviral drug ribavirin show any effectiveness against YF, it will be combined with the above techniques.

Separation of plasma or tissue lipids. Techniques are being established for separation of plasma or tissue lipids into various fractions with a silicic acid column and an automatic fraction collector. Infection-induced changes in lipid metabolism, including free fatty acids,

cholesterol, cholesterol ester, triglycerides and phospholipids will be studied with particular emphasis on a specific phospholipid (lysolecithin) and its role in intravascular hemolysis.

Part II. Progress

Cardiohepatic responses to IV infusions of leukocytic endogenous mediator (LEM) in rhesus monkeys. With the collaboration of LTC Sobocinski, Physical Sciences Division, cardiohepatic responses to IV infusion (10 min) of a crude rabbit LEM (10 ml/kg) were studied. Ten monkeys were used along with 6 controls. It was found that mean arterial blood pressure decreased to a minimum of 80 mmHg from 107 mmHg during the first hour and remained below preinfusion levels for 24 hr. Although cardiac output increased slightly compared to controls, heart rate increased significantly during the epxerimental period after LEM infusion; decreases in stroke volume, total peripheral resistance, cardiac work, and mean cardiac power occurred within 30 min and gradually returned to baseline levels at various lengths of time. Liver function was also altered as indicated by increased values for half-life and slower disappearance rate of cardiogreen dye compared t controls (Table III). Since high fever did not develop until 4-6 hr after infusion of LEM, earlier cardiovascular changes were not directly related to hyperthermia, but rather to toxicity or the presence of prostaglandins in the crude LEM extract.

Presentations:

1. Liu, C. T., M. J. Griffin, and E. L. Stephen. Body fluid compartments and distribution of tissue water and electrolytes in monkeys with yellow fever. Presented, 10th Ann. Meeting, Am. Society Nephrology, Nov. 1977 (Proc. of the Meeting, p. 476a, 1977; Kidney Int. 12:528, 1977).

2. Liu, C. T., R. P. Sanders, C. L. Hadick, Jr., and P. Z. Sobocinski. Cardiohepatic responses to intravenous infusion of leukocytic endogenous mediator in conscious rhesus monkeys. Atlantic City, NJ, 9-14 Apr. 1978. (Fed. Proc. 37:504, 1978).

Publications:

1. Liu, C. T., D. E. Hilmas, M. J. Griffin, C. E. Pedersen, Jr., C. L. Hadick, Jr., and W. R. Beisel. 1978. Alterations of body fluid compartments and distribution of tissue water and electrolytes in rhesus monkeys with Rocky Mountain spotted fever. J. Infect. Dis. 138:42-48.

2. Liu, C. T., and M. J. Griffin. 1978. Distribution of tissue water and electrolytes in normal rhesus macaques. Am. J. Vet. Res. 39: 1692-1694, 1978.

TABLE III. CARDIOVASCULAR AND HEPATIC RESPONSES TO INTRAVENOUS INFUSION OF LEUKOCYTIC ENDOGENOUS MEDIATOR IN RHESUS MONKEYS (N=10)

VARIABLE	BASELINE	MEAN ± SE BY TIME POSTINFUSION (hr) 0.5	1	3	5	6	24
Cardiovascular							
Mean blood pressure (mm Hg)	107.30 ±4.49	79.10 ±5.99*	80.60 ±7.92	91.90 ±4.518*	93.70 ±4.50*	93.30 ±3.89*	91.60 ±4.48
Cardiac output (L/min)	1.07 ±0.06	1.08 ±0.05	1.19 ±0.05	1.28 ±0.06	1.28 ±0.06	1.42 ±0.07	1.46 ±0.14
Stroke volume (ml/beat)	1.15 ±0.08	0.88 ±0.05*	0.95 ±0.06	1.05 ±0.06	1.19 ±0.05	1.33 ±0.04*	1.55 ±0.15
Cardiac work (Joule/beat)	0.09 ±0.009	0.05 ±0.004*	0.06 ±0.007*	0.07 ±0.005	0.08 ±0.007	0.09 ±0.007	0.10 ±0.012
Mean cardiac power (Joule/sec)	0.25 ±0.016	0.19 ±0.016*	0.21 ±0.024	0.26 ±0.017	0.27 ±0.018	0.29 ±0.016	0.30 ±0.035
Total peripheral resistance ($dyne.sec/cm^5$)	8347 ±728	6021 ±544*	5445 ±535*	5855 ±362*	5896 ±305*	5377 ±358*	5467 ±513
Heart rate (beats/min)	174 ±8	227 ±4*	232 ±4*	224 ±9*	199 ±8*	196 ±6*	179 ±11
Hepatic							
Disappearance rate (%/min)	46.7 ±8.8	28.4 ±4.0*	37.0 ±6.0	27.8 ±2.5	28.8 ±2.9	32.1 ±3.7	25.9 ±2.1*
Half-life (min)	2.54 ± 0.47	2.79 ±0.34	2.67 ±0.42	3.09 ±0.35	3.05 ±0.38	2.89 ±0.42	3.13 ±0.25*

$P < 0.05$

LITERATURE CITED

1. Soper, F. L., 1942. Treatment of yellow fever. J. Am. Med. Assoc. 118:374-378.

2. Gazzard, B. G., M. J. Weston, I. M. Murray-Lyon, H. Flax, C. O. Record, B. Portmann, P. G. Langley, E. H. Dunlop, P. J. Mellon, M. B. Ward, and R. Williams. 1974. Charcoal haemoperfusion in the treatment of fulminant hepatic failure. Lancet 1:1301-1307.

3. Wannemacher, R. W., M. V. Kaminski, Jr., H. A. Newfeld, R. E. Dinterman, K. A. Bostian and C. L. Hadick. 1978. Protein sparing therapy during pneumococcal sepsis in rhesus monkeys. J. Parent. Enteral. Nutr. (Submitted)

4. Beisel, W. R., 1975. Metabolic responses to infection. Annu. Rev. Med. 26:9-20.

5. George, D. T., F. B. Abeles, C. A. Mapes, P. Z. Sobocinski, T. V. Zenser, and M. C. Powanda. 1977. Effect of leukocytic endogenous mediators on endocrine pancreas secretary responses. Am. J. Physiol. 233:E240-E245.

6. Forsyth, R. P., A. S. Nies, F. Wyler, J. Neutze, and K. L. Melmon. 1968. Normal distribution of cardiac output in the un-anesthetized, restrained rhesus monkey. J. Appl. Physiol. 25:736-741.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6420	78 10 01	CSCRD-R(U)36

3. CATEGORY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISPOSITION	8B. PUBLIC DATA / CONTRACTOR ACCESS	9. LEVEL OF SUM
78 04 21	D. CHANGE	U	U	NA	NL	YES / NO	A. WORK UNIT

10. NO. YRS	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	030
b. OTHER (ING)				
IIIIIII	STOG 78-7.2.1, 3, 6			

11. TITLE (Include Security Classification Code)*

(U) Physiologically directed treatment of SEB-induced intoxication

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMING METHOD
73 08	CONT	DA	

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*
c. TYPE: NA d. AMOUNT:
e. AGENCY/NO OF AWARD f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN-YEARS	b. FUNDS ($ Thousands)
PRECEDING FISCAL YEAR 78	0.5	220.0
CURRENT FISCAL YEAR 79	0.5	224.2

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS
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NAME:

POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Gastrointestinal function; (U) Staphylococcal enterotoxin B (SEB); (U) Toxins; (U) Therapy

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

23 (U) Evaluate treatment of SEB-induced toxemia by measuring gastrointestinal motility, absorption, secretion, splanchnic circulation, and liver functions in animal models. This information is essential for understanding mechanisms of SEB-induced gastrointestinal syndromes (vomiting and diarrhea) and for finding the most effective means for treatment and prevention. Staphylococcal enterotoxemia presents formidable problems in military facilities and in BW defense.

24 (U) Examine whether SEB is absorbed from the intestine, then study gastrointestinal responses to the administered SEB. Determine optimal therapy for food poisoning and SEB toxemia.

25 (U) 77 10 - 78 09 - SEB-induced diarrhea in anesthetized rabbits is caused by increased intestinal mobility and rapid loss of Na, K and water. The efflux rate of water from plasma to the intestinal lumen is also increased. In a short-term study, IV injection of cholera or Shigella toxin at very low dose levels is lethal for the rhesus monkey. Aerosolization of Shigella toxin had no effect in monkeys while intratracheal inoculation of extremely high doses of cholera toxin produced delayed death. Techniques for measuring regional blood flow have been established. A hemoperfusion system with an activated charcoal column for rhesus monkeys was developed. Volume for filling the column dead space is 17-20 ml and the normal heparinized monkey can tolerate the system. A lethal dose of SEB (50 or 100 µg/kg) was given IV to monkeys. Hemoperfusion was started 15 or 60 min after SEB inoculation and continued for 6 hr. Two monkeys receiving a lower dose of SEB survived indefinitely and 3 monkeys receiving the higher dose died.

Publications: Physiologist 20:57, 1977; Am. J. Vet. Res. 38:1843-1848, 1849-1854, 1977, 39:279-286, 1213-1217, 1978; Fed. Proc. 37:504, 697, 1978; Toxicon, in press; Radiat. Res. 76: in press, 1978.

*Explanation of entries on reverse of this form.

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 030: Physiologically Directed Treatment of SEB-Induced Intoxication

Background:

Part I. Treatment of SEB toxemia

Administration of SEB (25 μg/kg) IV to rhesus monkeys produces death (1). Although physiological responses to SEB toxemia have been studied extensively and several means f treatment have been applied, challenged monkeys still die (2, 3).

Previous methods for treatment of SEB toxemia were broad in nature and included fluid therapy, blood transfusion and administration of plasma albumin, bicarbonate, a cardiostimulant (isoproterenol), a vasodilator (dibenzyline), adrenocortical hormone (dexamethasone), a vasoconstrictor (epinephrine), and continuous positive pressure breathing (CPPB). SEB antiserum reverses clinical toxicity in monkeys if given within 8-12 hours of a lethal IV dose. CPPB occasionally saved lives, while other non-immunological approaches only prolonged survival by 1-2 days. These results suggest that once SEB is introduced into the circulatory system, it is difficult to reverse the deteriorating processes which ensue. It appears that the only way to diminish SEB toxicity is to remove the toxin from the circulation. We have demonstrated both in vitro and in vivo that hemoperfusion through an activated charcoal column results in adsorption of the SEB to the charcoal and its removal from the circulation.

Part II. Effects of cholera and Shigella toxins

Effects of oral cholera or Shigella toxin in producing vomiting and diarrhea have been well documented (4). Due to rapid loss of electrolytes and water via the gastrointestinal tract, severe dehydration, renal failure, and circulatory shock may result followed by death. Since there is limited evidence for intestinal absorption of these toxins and their subsequent systemic action, few investigations have been carried out using IV injections. The purposes of this short-term study were to determine the survival and cardiohepatic responses to the administration of cholera and/or Shigella toxin using the IV or pulmonary route.

Progress:

Part I. Treatment of toxemia

Kinetics of plasma water in normal and SEB-exposed rabbits. Following a single IV injection of ^3H_2O (2 μCi), kinetics of plasma H_2O were studied with and without perfusion of an isotonic Earle's solution via a segment of cannulated small intestine. Data revealed that without intestinal

perfusion, H_2O levels in plasma remained unchanged over a period of 8 hr. When the cannulated small intestine was perfused continuously with isotonic Earle's solution (300 mOsm) at a rate of 2.4 ml/min, plasma levels of H_2O decreased gradually. Addition of SEB to the intestinal perfusate (25 µg/min) did not significantly alter the rate of ^3H_2O disappearance from plasma.

With constant IV infusion of ^3H_2O (0.4 µCi/hr) and constant intestinal perfusion of isotonic Earle's solution, the appearance rate and percent of the injected dose found in the intestinal effluent showed no difference between control and SEB-exposed rabbits. However, when the same amount of SEB was added to a slightly hyperosmolal solution (350 mOsm) for intestinal perfusion, the appearance rate of the H_2O in the intestinal effluent was faster than controls (Table I). These findings indicate that a slight increase in osmolality of intestinal perfusate potentiates the effects of SEB in producing diarrhea.

TABLE I. EFFECTS OF SEB IN DIFFERENT OSMOLALITIES OF SOLUTIONS ON INTESTINAL FLUX RATES OF ^3H_2O FOR 3 HOURS IN DUTCH RABBITS

OSMOLALITY (mOsm)	GROUP	INFLUX RATE DPM/min/gm ($x10^3$)	EFLUX RATE DPM/min/gm ($x10^3$)
300	Control	2.42 ± 0.18 (n=4)	1.49 ± 0.16 (n=7)
	SEB	2.60 ± 0.20 (n=7)	1.55 ± 0.10 (n=8)
350	Control	2.63 ± 0.18 (n=4)	1.34 ± 0.10 (n=5)
	SEB	1.46 ± 0.04* (n=4)	2.40 ± 0.14* (n=5)

*$P < 0.01$

Effect of SEB in different osmolal solutions on intestine and intestinal transport. Studies were performed in Dutch rabbits with mean arterial blood pressure maintained above 70 mm Hg throughout the experimental period. When a segment of rabbit jejuno-ileum (~ 80 cm) was perfused in vivo with isotonic Earle's solution (300 mOsm) containing SEB (10 µg/ml) for 6 hr, there were decreases in rates of intestinal absorption of Na^+, K^+ and water 3 hr after initiation of SEB infusion compared to controls. By increasing NaCl content in the Earle's solution, the intestinal perfusion of slightly hyperosmolal solution (350 mOsm) containing SEB (10 µg/ml) resulted in increases in intestinal excretion of Na^+, K^+ and water, and efflux rate of ^3H_2O from plasma to the intestinal lumen. The influx rate of the H_2O (from the intestinal lumen to plasma), Na^+, K^+ and water content in the intestinal tissue, and intestinal absorption rate of glucose, Ca^{++}m and Zn^{++} decreased 3 hr post-SEB perfusion via the intestine. The SEB-exposed rabbits survived for 3-4 hr. Since the fluid loss during experiments was compensated by an IV infusion of isotonic saline (10 ml/hr), plasma osmolality was not significantly altered. The cause of death may be due to the intestinal absorption of perfused SEB, producing systemic effects leading to circulatory shock and death.

In contrast, a hyperosmolal (600 mOsm)-induced diarrhea without SEB produced rapid intestinal losses of Na^+, K^+ and water with an increased influx rate of ^3H_2O. A decrease in water content of intestine was also observed. Deaths occurred 2-3 hr after the beginning of intestinal perfusion. The rationale for comparing mechanisms of diarrhea induced by SEB and extreme hyperosomolality (600 mOsm) was to prove that the loss of intestinal water and electrolytes may be associated with different flux rates or different directions of ^3H_2O fluxes. Furthermore, the responses of intestinal cells to SEB and extreme hyperosmolal solution are also different, with SEB resulting in far less loss of intestinal tissue water.

Induction of leukopenia. Radiation-induced leukopenia appears to play a key role in the survival of Dutch rabbits exposed to lethal doses of SEB (5). Attempts to induce leukopenia by other means have met with variable success. The technique of using glass beads was not satisfactory. Hemoperfusion through a column of glass beads continuously for 4-5 hr in rabbits produced a 40-50% decrease in leukocytes from baseline levels; however, leukocytosis occurred 6 hr after hemoperfusion. WBC counts increased above baseline values and persisted despite additional hemoperfusion the following day.

Cytoxan (20 mg/kg) was used to induce leukopenia by daily IV injection through a chronically implanted cannula in rabbits. Results varied, complicated by an infection at the implantation site. Intramuscular injection of Cytoxan was ineffective in decreasing WBC counts. When a stabilized leukopenia (70% decrease from baseline values) can be established in rabbits, a lethal IV dose of SEB (100 μg/kg) will be injected to determine the survival time.

Hemoperfusion therapy with activated charcoal. Response in normal monkeys. Based upon preliminary in vitro studies showing SEB adsorption by activated charcoal, a system for hemoperfusion through an activated charcoal column was developed for the treatment of SEB toxemia in rhesus monkeys. Normal monkeys were first used to obtain basic information concerning effects of hemoperfusion on blood pressure and blood changes in the conscious monkey. An activated charcoal column designed for human use was reduced to 1/10th of the original capacity and insulated to prevent heat loss. Hemoperfusion was controlled by a pump with a flow rate of 15-20 ml/min, and the duration of perfusion was 10 hr. To prevent clots, the monkey was heparinized (4000 U); 10 min later 20 ml of concentrated heparin (1000 U/ml) were continuously infused into the hemoperfusion system for the entire experimental period. An air trap was also connected to prevent air bubbles from entering the circulation. Results are tabulated in Table II. The data show that all measured variables returned to pre-infusion levels and that no apparently serious physiological and biochemical changes resulted from the procedure. These findings support the idea that an activated charcoal column is safe to use in the monkey. Several further modifications of the system made it even more suitable for the 4-7-kg monkey: (1) column size was reduced to hold only 20-21 gm of charcoal instead of 300 gm. This small column is available commercially and made for chromatographic purposes. (2) The total dead-space volume

of the entire system was decreased to 17-20 ml. (3) The column was maintained at 37°C by circulating water around the column jacket from a constant temperature bath, (4) The animal and the system were well heparinized with 4,000 U of heparin as an initial dose and maintained with constant infusion at 1,500-2,000 U/hr.

TABLE II. HEMOPERFUSION WITH ACTIVATED CHARCOAL COLUMN FOR 10 HR IN A NORMAL RHESUS MONKEY

PARAMETER[a]	HEMOPERFUSION AT VARIOUS HR		
	3	6-10	24-48
Arterial blood pressure	↓	0	0
Hematocrit	↓	0	0
Protein	↓	0	0
P_{50}[b]	↑	↓	0
WBC	↓	↑	0
Lymphocyte	0	↓	0
Rectal temperature	0	↓	0
Hemolysis	0	↑	0
Glucose	0	↓	0

[a]No changes were observed in the following: blood pH, P_{O_2}, P_{CO_2}, HCO_3^-, total CO_2, and O_2, neutrophils, plasma osmolality, Na^+, Cl^-, K^+ and monocytes.

[b]P_{50} = P_{O_2} at which hemoglobin is (50%) saturated with O_2.

SEB-toxemic monkeys. Of 5 monkeys challenged with SEB and hemoperfused, 2 survived. Unscccessful treatment of 3 monkeys might be a result of the following: (a) the IV dose of SEB in one monkey was too high (100 µg/kg); if the charcoal only removed 50% of the dose, the remaining toxin (50 µg/kg) was still lethal and (b) formation of blood clots and blockage of blood flow in the system of hemoperfusion occurred in 2 monkeys. It appears that the 25-gm activated charcoal column has a maximal capacity for removing SEB from blood and/or a certain amount of SEB is distributed to the tissues where it cannot be removed by the hemoperfusion technique. It is almost certain that monkeys receiving 50 µg/kg of SEB IV can be treated successfully with our presently established hemoperfusion technique. The maximum time interval permitted between SEB inoculation and successful hemoperfusion will be further established.

Part II. Effect of cholera and Shigella toxins

Clinical observations and cardiohepatic responses to IV cholera or Shigella toxin. Studies were initiated to determine the cardiovascular and pulmonary changes induced by aerosol administration of cholera and Shigella toxins. Our first step was to determine survival time, and study cardiovascular and hepatic responses to a single IV injection of either toxin. Clinical signs and symptoms were observed and autopsies were performed. Results from preliminary studies are summarized in Tables II - VI. Neither cholera nor Shigella toxin produced gastrointestinal disturbances (vomiting and diarrhea) after IV inoculation. It appears that cholera toxin induces death from increased cellular concentrations of cyclic AMP through activation of adenyl cyclase in the cell membranes, while Shigella toxin operates through hypoglycemic and neurotoxic mechanisms, and both endotoxin and neurotoxin are present in the crude preparation.

TABLE III. CLINICAL OBSERVATIONS AND SURVIVAL TIME OF RHESUS MONKEYS INJECTED IV WITH CHOLERA TOXIN

MONKEY	DOSE (μg/kg)	SURVIVAL TIME (hr)	CLINICAL OBSERVATIONS AND GROSS PATHOLOGY
T-234 (chaired)	50	18.0	Loss of appetite, running nose and bleeding from nose, wheezing and coughing, cloudy eyes, pale-to-yellow skin, sores at corner of mouth, bleeding from gums, respiratory rate decreased, petechiae throughout heart muscle (all layers), much fluid in pericardial sac, edges of liver enlarged.
T-262 (chaired)	50	12.5	
T-264 (caged)	10	120.0	

TABLE IV. GENERAL CARDIOHEPATIC AND BIOCHEMICAL RESPONSES OF RHESUS MONKEYS (T-234 AND T-262) WITHIN 8 HOURS OF IV INJECTION OF CHOLERA TOXIN

INCREASED	DECREASED
Heart rate	Rate of disappearance of cardiogreen dye (impairment of liver function).
Cardiac output	Mean blood pressure (systolic and diastolic).
Pulse pressure	Total peripheral resistance
Mean blood pressure (within 15 min)	Blood P_{CO_2}, HCO_3^-, total CO_2, viscosity
Stroke volume	Hematocrit
Cardiac contractility (peak reached at 1 hr, then decreased below control values)	Plasma protein
Plasma osmolarity, Na^+, Cl^-, glucose.	

TABLE V. CLINICAL OBSERVATIONS AND SURVIVAL TIME OF RHESUS MONKEYS TO IV INJECTION OF *SHIGELLA* TOXIN[a]

MONKEY	DOSE (ml/kg)	SURVIVAL TIME (hr)	CLINICAL OBSERVATIONS AND GROSS PATHOLOGY
C-59	0.1	1.5	Convulsive, wheezing, respiratory rate decreased, clear yellow fluid coming from nasal orifices, bloody and mucous discharges from bowels, comatose state, swollen spleen.
C-78	0.01	42.0	
T-235	0.04	17.5	

[a]Protein concentration in *Shigella* toxin = 2100 µg/ml; IV lethal dose of *Shigella* toxin = 84 µg/kg.

TABLE VI. GENERAL CARDIOHEPATIC AND BIOCHEMICAL RESPONSES OF RHESUS MONKEYS WITHIN 8 HOURS OF IV INJECTION OF *SHIGELLA* TOXIN

INCREASED	DECREASED
Heart rate	Rate of disappearance of cardiogreen dye (impairment of liver function)
Plasma	Mean blood pressure (systolic and diastolic)
	Cardiac output
	Cardiac work
	Mean cardiac power
	Total peripheral resistance
	Blood HCO_3
	Blood total CO_2
	Hematocrit
	Plasma protein
	Blood O_2 content
	WBC
	Rectal temperature
	Plasma glucose

Aerosolization of Shigella toxin. Two rhesus monkeys were subjected to aerosols of *Shigella* toxin according to techniques described by Berendt (Aerobiology Division). The concentration of toxin was 2.1 mg/ml, a minimum volume of 10 ml was used. The time for administration of aerosol was 17 min and 52 min, respectively. The presented dose of *Shigella* toxin was 33.4 ug/kg for one monkey, and 15.0 µg/kg for the

longer period. It was apparent that some difficulties were encountered for producing aerosols from the Shigella toxin solution. The fraction of the presented dose transported into the circulation is unknown. Neither monkey showed any clinical signs of toxicity within a week, suggesting that aerosol dissemination of Shigella toxin does not pose a significant hazard.

Intratracheal inoculation of cholera toxin. To determine if cholera toxin was toxic by the pulmonary route, 1.6 mg of cholera toxin (0.4 mg/4 ml) was inoculated intratracheally into a rhesus monkey. The intoxicated animal showed the following clinical signs starting at 2 days: vomiting, refusal to eat and drink, muscular weakness, rapid, shallow and terminally labored breathing. Death occurred 4 days after inoculation. Pathological examination revealed the following changes: fluid accumulation in the adbominal cavity, pleural cavity, and pericardium; hemorrhages of the lung, pancreatic islets, and heart; myocardial necrosis, edema and inflammation; and inflammation of the cervical connective tissue. Changes in the heart were similar to those seen with IV injection of cholera toxin.

Previous studies in our laboratory indicated that a lethal IV dose of cholera toxin for rhesus monkeys was 50 µg/kg and that death occurred between 12 and 18 hr. When 1.6 mg of this toxin was placed into the lung directly via the trachea, the monkey did not show any clinical signs during the first 24 hours after inoculation, indicating that the toxin was not readily absorbed from the lung into the circulation. Aerosol administration would require much larger quantities to produce the same effect as intratracheal or IV administration.

Presentations:

1. Liu, C. T., and D. R. Dufault. Effects of intestinal infusion of staphylococcal enterotoxin B (SEB) on water and electrolyte fluxes; possible mechanisms of diarrhea. Presented, 28th Annual Fall Meeting, American Physiological Society, Hollywood, FL, 10-14 Oct 1977 (Physiologist 20:57, 1977).

2. Dufault, D. R. and C. T. Liu. Effects of intestinal infusion of solutions with different tonicities in control and staphylococcal enterotoxin B (SEB)-exposed rabbits. Presented, Annual Meeting, FASEB, Atlantic City, NJ, 9-14 Apr 1978 (Fed. Proc. 37:697, 1978).

3. Liu, C. T., R. P. Sanders, C. L. Hadick, Jr., and P. Z. Sobocinski. Cardiohepatic responses to infusion of leukocytic endogenous mediator in conscious rhesus monkeys. Presented, Ann. Meeting, FASEB, Atlantic City, NJ, 9-14 Apr 1978 (Fed. Proc. 37:504, 1978)

Publications:

1. Liu, C. T., and R. D. DeLauter. 1977. Pulmonary functions in conscious and anesthetized rhesus macaques. Am. J. Vet. Res. 38:1843-1848.

2. Liu, C. T., R. D. Delauter and R. T. Faulkner. 1977. Cardiovascular and hepatic responses of rhesus macaques to staphylococcal enterotoxin B. Am. J. Vet. Res. 38:1849-1854.

3. Liu, C. T., R. D. Delauter, M. J. Griffin, and R. T. Faulkner. 1978. Effect of staphylococcal enterotoxin B on cardiorenal functions in rhesus macaques. Am. J. Vet. Res. 39:279-286.

4. Liu, C. T., M. J. Griffin, and D. E. Hilmas. 1978. Effect of staphylococcal enterotoxin B on cardiorenal functions and survival in x-irradiated rhesus macaques. Am. J. Vet. Res. 39:1213-1217.

5. Liu, C. T., R. D. DeLauter, M. J. Griffin and C. L. Hadick. 1978. Effects of staphylococcal enterotoxin B on functional and biochemical changes on the lung of rhesus monkeys. Toxicon 16:543-550, 1978.

6. Liu, C. T. and D. E. Hilmas. 1978. Effect of x-irradiation on survival in rabbits with staphylococcal B enterotoxemia. Radiat. Res. 76:402-409, 1978.

LITERATURE CITED

1. Crawley, G. J., J. N. Black, I. Gray and J. W. Blanchard. 1966. Clinical chemistry of staphylococcal enterotoxin poisoning in monkeys. Appl. Microbiol. 14:455-450.

2. Beisel, W. R. 1972. Pathophysiology of staphylococcal enterotoxin type B (SEB) toxemia after intravenous administration to monkeys. Toxicon 10:433-440.

3. Liu, C. T., R. D. DeLauter and R. T. Faulkner. 1977. Cardiovascular and hepatic responses to rhesus macaques to staphylococcal enterotoxin B. Am. J. Vet. Res. 38:1849-1854.

4. Beisel, W. R. 1976. Enterotoxin-mediated diseases, pp. 1-66. In Trace Substances and Health, Part I (P. M. Newberne, ed.). Marcel Dekker, Inc., New York.

5. Liu, C. T. and D. E. Hilmas. 1978. Effect of x-irradiation on survival in rabbits with staphylococcal B enterotoxemia. Radiat. Res. 76:402-409, 1978.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0C6411	78 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
78 04 21	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	62776A	3M162776A841	00	031
b. CONTRIBUTING				
c. //////////	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)*

(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	CONT	DA	C. In-house

17. CONTRACT GRANT

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

18. RESOURCES ESTIMATE

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING 78	0.9	110.0
CURRENT 79	1.0	76.3

19. RESPONSIBLE DOD ORGANIZATION

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

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (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 text of each with Security Classification Code.)

23 (U) Develop and apply techniques for utilization of computers, biostatistics, mathematics, and biomedical engineering to process and interpret biomedical data in a research program for medical defense against BW agents, emphasizing diagnostic, therapeutic and immunoprophylactic studies.

24 (U) Theories and disciplines of numerical analysis, differential equations, statistical tests of hypotheses, experimental design, information storage and retrieval, and pattern recognition are utilized to analyze and interpret data gathered by investigators.

25 (U) 77 10 - 78 09 - Appendix I to AR-18-1 in regard to Automatic Data Processing has been prepared and approved. Purchase of the approved automatic data processing equipment will significantly upgrade the computer support available to investigators.

Research efforts have been concentrated in the areas of biomedical indices of infection, data-base design and maintenance, kinetic modeling of physiological systems, and in experimental design, statistical and computational consultation.

*Available to contractors upon originator's approval

DD FORM 1498 MAR 68 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. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 031: Mathematical and Computer Applications in Medical BW Defense Research

Background:

The research mission of the Computer Science Office is to develop and apply computer, biostatistical, mathematical, and biomedical engineering techniques to process and interpret biomedical data generated within the Institute. This work has been pursued in the areas of biomedical indices of infection, data-base design and maintenance, kinetic modeling of physiological systems, and in experimental design, statistical and computational consultation.

The automatic data processing (ADP) plan for USAMRIID identifies 3 general goals for support: (a) provide ADP equipment that will best fill the needs of investigators; (b) assure that ADP equipment is compatible with the long-term ADP plan (hardware and software compatibility); and (c) obtain the most cost-effective ADP systems.

The long-term solution to the ADP 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, terminals, and programmable calculators, but it is essential that compatibility between all ADP equipment be preserved as much as possible. Coordination of all such purchases with the Computer Science Office will help preserve the desired compatibility.

Appendix I to AR18-1 in regard to ADP has been prepared and approved. Purchase of the approved ADP equipment will significantly upgrade the computer support available to researchers at USAMRIID. Immediate plans include: (a) the purchase of a laboratory minicomputer system for real-time data acquisition and interactive data analysis; (b) an upgrade of low-speed communication terminal systems in 4 divisions; and (c) a shift of many remote job entry programs from WRAIR and the National Bureau of Standards (NBS) to the computer at Management Information Systems Directorate (MISD), Fort Detrick. MISD plans to have remote communications capabilities by the fall of 1978; NBS plans to discontinue prime-time services to non-NBS agencies as early as Oct 1978.

Progress:

Early diagnosis of infection. In an effort to obtain the required number of normal control samples for valid statistical analysis, a volunteer study on normal controls (Work Unit BS03 00 007) was conducted. Short clinical histories and 83 serum chemistry values for each of the

130 male and 80 female volunteer normals have been entered on a computer file at NBS.

Over the past few years, data for this project has been collected and stored on computer files at WRAIR in a variety of formats. A revised format has now been set so that any future data can be recorded on standardized forms and will be compatible. All of the Klainer and Woodward data on file at WRAIR has been re-formated and transferred to NBS so that now all of the indices of infection data-base is in the same format and on the same computer system.

Brief analysis of the USAMRIID normals has shown the following:

1. Few of the serum chemistry parameters have a Gaussian distribution.

2. Thirty-seven of the 83 biochemical parameters showed significant differences between male and female populations (Kolmogorov-Smirnov 2-sample nonparametric test).

3. Nonparametric box plots were made for each parameter in an effort to define normal ranges. Such plots define the spread of the data in terms of ranking and quartile distances. Box plots gave a better description of physiologically normal ranges than did the mean $\pm$ 2 SD because the distributions of most values were non-Gaussian. One disadvantage of these plots is that confidence intervals of only 50% and approximately 95% are defined.

4. For a number of parameters, USAMRIID volunteer normals, controls from West Virginia University (Klainer), and controls from the University of Maryland (Woodward) differed significantly from each other. Because of the larger sample size and more representative normal population, USAMRIID controls will be used to define physiologically normal biochemical ranges.

5. Paired blood samples were analyzed at WRAIR on SMAC and at USAMRIID for albumin, glucose, and calcium. Significant differences ($P < 0.001$) were found between results from the 2 laboratories. These differences resulted from different analytical procedures.

Work is now in progress to identify those biochemical parameters in the USAMRIID controls which demonstrate age-dependence. The major effort in the near future will be to define statistically the control population for each parameter, and then compare these values for each ill subject with the control populations to see if patterns exist in the biochemical values for specific illnesses.

Immunization system. A significant effort has been made to develop an operational computerized scheduling system for the Special Immunization Program (USAMRIID Memo 40-24). The computer programs have been written in FORTRAN, use 2 file management systems at MISD, and are now implemented on the IBM 360/50 at MISD. The system of programs generates

monthly lists of individuals due to receive shots and bleedings before and after these special immunizations. Computer-printed procedure forms for the recording of shot or titer information are prepared monthly. A monthly computer-generated list of individuals who are delinquent in required immunizations, and monthly, short immunization histories for people scheduled to receive booster shots (shot and titer history for each specific agent for which an individual is being immunized) are now routinely produced.

Ten programs have been written to query the immunization data-base to summarize shot and titer information. Some of this information will be used by the Institute's Immunization Committee to evaluate the effectiveness of current EEE and WEE vaccination procedures. A study comparing the paired titers determined by plaque reduction neutralization test and a recently developed radioimmunoassay (RIA) technique is in progress.

Efforts on the immunization system will now be directed to 3 projects: (a) writing programs to print out the complete immunization history of selected individuals or of all people receiving immunizations; (b) documentation of the computer programs; and (c) coding of past immunization records onto the system.

Other projects. Projects recently completed or in progress include: the development of a kinetic model of the changes in water distribution in the intestines, plasma, organs, and eluent as a result of constant SEB intestinal perfusion; programs for the analysis of RIA data, in which the slopes and intercepts of 2 curves are compared for log and logit plots of the data and the overall comparison of the 2 dose-response curves is done by analysis of covariance; the designing and writing of COBOL programs for the Pathology Information System for accessions; and programs to maintain a data-base and generate periodic inventories of stored infectious biological materials (seed stocks).

Consultation. Biostatistical computer consultations were held with investigators of all divisions of the Institute to develop solutions to experimental design, statistical, and computational problems. A large amount of time and resources was spent in meeting these data analysis problems.

Presentation:

Higbee, G. A. [illegible] of ARBOVIRUS Laboratory, Personnel, Equipment and Applications. [illegible] Symposium, WRAIR, Washington, 31 Jan 1980.

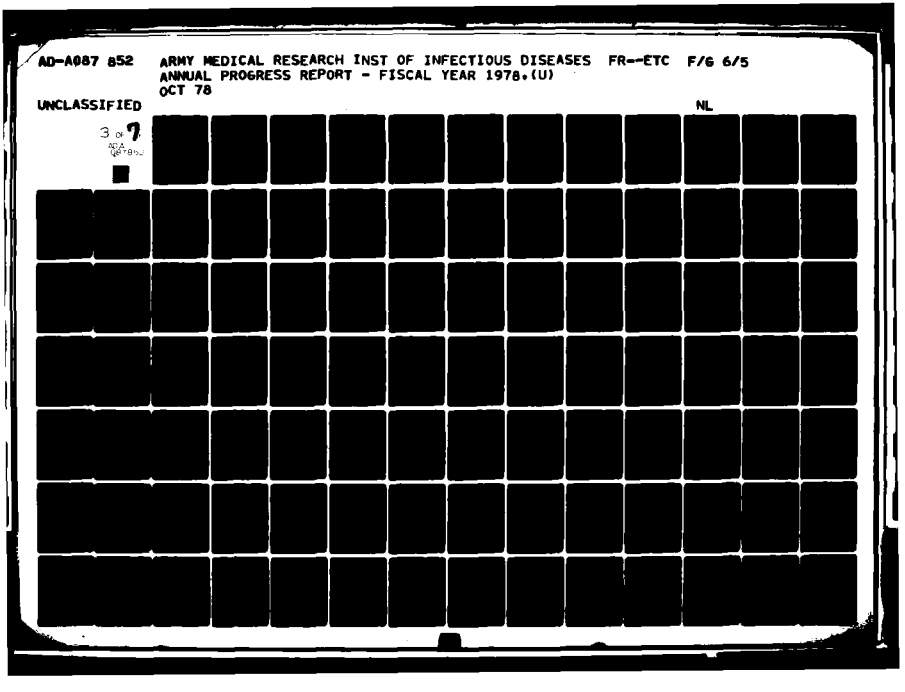
Publications:

None.

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ANNUAL PROGRESS REPORT - FISCAL YEAR 1978.(U)
OCT 78

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3 OF 7
AD A087852



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OA6413	78 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
77 10 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	036
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)* (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	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		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	0.9	15.0
	CURRENT 79	1.0	118.9

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) Laboratory animals; (U) Spontaneous diseases

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

23 (U) To evaluate and monitor the health status of laboratory animals on arrival to 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) 77 10 - 78 09 - Quality control monitoring of rodents entering the Institute during this reporting period revealed that most animals supplied to USAMRIID were of acceptable quality although not disease-free. The principal endemic diseases in incoming animals with greatest potential for disrupting or invalidating experimentation were chronic murine pneumoniae in rats and mice and encephalitozoonosis in guinea pigs. Disease surveillance of Institute colony animals revealed sporadic deaths in the rodent population which were primarily bacterial. Thirty-nine subhuman primates were lost to a variety of natural diseases. The most common cause of death among macaques was acute gastric dilatation (6 cases). The cause of this sporadic syndrome is not known.

Publications: Vet. Pathol. 14:591-605, 1977; 15:18-30, 31-39, 1978.
J. Am. Vet. Med. Ass., in press, 1978.

*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

☆ U.S. GPO: 1974—540-843/8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 036: Spontaneous Diseases in Laboratory Animals Used for 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 on experiment must be investigated and their impact on the ongoing studies evaluated.

Progress:

Quality control monitoring of rodents entering the Institute during this period revealed that most animals supplied to USAMRIID were of acceptable quality, although not disease free. The principal endemic diseases encountered were: chronic murine pneumonia (rats and mice), encephalitozoonosis (guinea pigs), intestinal nematodiasis (rats and mice), renal coccidiosis (guinea pigs) and balantidiasis (guinea pigs).

Early lesions of chronic murine pneumonia (CMP) were present in the lungs of almost all monitored rats and in approximately 10-15% of incoming mice. Caused by Mycoplasma pulmonis, CMP is a progressive disease associated with high morbidity but low mortality. Disease progression is enhanced by poor management practices and intercurrent diseases. Its principal impact is on utilization of rats and mice for pulmonary studies and chronic long-term experiments. As most experiments at USAMRIID are of an acute nature employing relatively young animals the level of observed pulmonary involvement makes these incoming animals acceptable.

Encephalitozoonosis, a clinically silent disease characterized by multifocal granulomatous encephalitis, was noted in a number of guinea pigs. The incidence of this protozoan disease caused by Encephalitozoan caniculi generally can be linked to the quality of colony management. The more unsatisfactory the management practices the higher the incidence of this enzootic disease. Encephalitozoonosis poses a particularly severe problem to the investigator who unsuspectingly uses infected animals. Stress of experimentation can precipitate clinical disease and death, giving rise to confusing experimental results and erroneous conclusions.

Disease surveillance within the animal colony during this reporting period revealed that sporadic deaths in the rodent population were primarily the result of bacterial diseases. Thirty-nine colony subhuman primates (26 rhesus, 7 squirrel, 2 African green, 2 capuchins, 1 baboon and 1 marmoset) were lost to natural diseases. Causes of primate death included: bacterial pneumonia, inhalation pneumonia, gram-negative septicemia, suppurative meningoencephalitis, bacterial enteritis, parasitic peritonitis and acute gastric dilatation. The last was the single most common cause of death (6 animals). The cause of this syndrome, which is confined to macaques, is not known. Typically, animals are found dead with no clinical signs associated with the condition. Death is the result of shock caused by intraabdominal and thoracic pressure generated by the expanding tympanic stomach. One hypothesis is that rapid overeating may precipitate the condition if feeding has been irregular over a holiday period. Investigation continues.

Presentation:

DePaoli, A. Pathology of gastrointestinal diseases of subhuman primates. Presented, Pathology of Laboratory Animals course, Armed Forces Institute of Pathology, Washington, DC, Sep 77.

Publications:

1. Baskin, G. B., and A. DePaoli. 1977. Primary renal neoplasms of the dog. Vet. Pathol. 14:591-605.

2. DePaoli, A., and D. O. Johnsen. 1978. Fatal strongyloidiasis in the gibbon (Hylobates lar). Vet. Pathol. 15:31-39.

3. Confer, A. W., and A. DePaoli. 1978. Primary neoplasms of the nasal cavity, paranasal sinuses and nasopharynx in the dog. A report of 16 cases from the files of the AFIP. Vet. Pathol. 15:18-30.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6429	78 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
78 04 21	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	62776A	3M162776A841	00	040
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code): (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	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	0.9	70.0
FISCAL YEAR CURRENT 79	1.0	280.4

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SOCIAL SECURITY ACCOUNT NUMBER:

21. GENERAL USE
Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME: Miller, J. G.
NAME: Hall, W. C.
POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Parasites; (U) Laboratory animals; (U) Microbiology; (U) Infectious diseases; (U) BW defense

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish 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) 77 10 - 78 09 - Significant findings this year included salmonellosis in rodents chronic respiratory disease in rodents and rabbits, Strongyloides in newly received primates, a tuberculosis-suspect African green monkey, and unusual central nervous system symptoms in a burro caused by the aberrant migration of nematode larvae. Cardiac abnormalities were present in an adult Macaca fasicularis and arose spontaneously in an infant Macaca mulatta and are being investigated further. No cases of herpesvirus or measles were encountered in nonhuman primates. After detecting the tuberculosis in a monkey, a quarantine was imposed and all other primates were skin-tested. After 2 consecutive negative skin tests, the quarantine was lifted.

Publication: J. Am. Vet. Med. Ass. 153: in press, 1978.

*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

* U.S. GPO: 1974—540-843/8691

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 040: Hazards and Variables Associated with Research Animals Used in Medical Defense Against BW

Background:

Even in well-managed, efficient research institutes, the constant hazard of contamination of normal conventional animals with undesired unusual disease entities exists. This is especially a problem for an infectious disease research institute, as the invading condition may be masked by the effects of the conditions under 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 agents. They are often latent carriers of disease when received. 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 established last year on newly received animals is continuing. Techniques for parasitologic and bacteriologic examination have been established and become routine. Increased emphasis is now being placed on establishing normal hematologic parameters for the "USAMRIID rodent" which can then be used as a standard of comparison for newly arrived animals. During the year, 403 animals from 8 different commercial sources were examined and data gathered. No enteric parasites were detected in rodents.

Infection with salmonellosis was detected in guinea pigs from one supplier and the animals were diverted from use in research. Salmonellosis is a frequently reported disease of guinea pigs, resulting in a variety of symptoms ranging from mild anorexia to acute death. Control and treatment of Salmonella-infected guinea pigs is virtually impossible, since both vaccination and antibiotic therapy are helpful but do not eradicate the infection. It presents a zoonotic threat, as several serotypes can infect both humans and animals. Future guinea pig orders were diverted to avoid purchasing from suppliers with potential disease problems.

Chronic respiratory disease (CRD) is found in differing degrees of severity in virtually all conventional rats; it was found in 87% of the rodents surveyed during the year. Thirty percent fell in the mild category; 57% were classified as minimal lesions histopathologically. No severe lesions of CRD were observed.

One group of newly received squirrel monkeys (Saimiri sciureus) was infested with Strongyloides sp. This common intestinal nematode of primates responded well to antihelminthic therapy and was eliminated before the animals were used in research. One African Green monkey (Cercopithicus aethiops) was suspect for tuberculosis on both paplebral and subsequent intradermal thoracic and abdominal skin testing. Because of the severe zoonotic implications, the animal was euthanatized and all animals in the immediate area placed under quarantine. No Mycobacterium sp. organisms were recovered upon culturing and complete gross and microscopic pathologic examination revealed no tuberculosis lesions. All other primates in the area had two skin tests for tuberculosis; when both were negative on all animals, the quarantine was lifted. Of the 1661 tuberculosis skin tests performed on nonhuman primates over the last year no other positive reactors or suspects were observed. No cases of herpesvirus or measles were encountered in nonhuman primates.

One burro exhibited CNS symptoms of disorientation and ataxia followed by death within 24 hr in spite of supportive therapy. Death was attributed to aberrant nematode migration to the CNS. Complete histopathologic evaluation is currently in progress.

Routine electrocardiographic (EKG) and radiologic examination of primates revealed one cynomolgus monkey (Macaca fasicularis) with a left anterior fascicular block accompanied by cardiac hypertrophy and gross cardiac enlargement. Similar EKG abnormalities were detected in an infant rhesus (Macaca mulatta) monkey with no gross cardiac changes. Subsequent examination revealed early cardiac changes in the infant with advancing age. This condition is being followed and will be further evaluated as it progresses.

Presentation:

1. Rozmiarek, H. Laboratory animal medicine support to a class 3 and 4 biomedical containment facility. Presented, Annual Session, Texas Branch, American Association for Laboratory Animal Science, Austin, TX, 17-19 May 1978.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OF6420	78 06 27	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISS'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 04 10	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	042
b. CONTRIBUTING				
c. ~~////////~~	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code) (U) Pathophysiological guide to therapy of Klebsiella pneumoniae superimposed on whole-body Co-60 irradiation

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	78 06	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 78	1.0	90.0
	CURRENT 79	0	0

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21. GENERAL USE
Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME:
NAME:

POC:DA

22. KEYWORDS (Precede with Security Classification Code) (U) Irradiation; (U) Military medicine; (U) BW defense; (U) Klebsiella (U) Respiratory infections; (U) Pathophysiology; (U) Influenza; (U) Chemotherapy

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

23 (U) Incapacitation resulting from respiratory infections and time required to regain normal pulmonary functions represent a very significant loss of military time and manpower. This work unit will define physiological mechanisms of pulmonary functional changes in selected diseases of medical importance in defense against BW agents, so that treatment regimens can be found which will reduce both lost time and losses of military manpower.

24 (U) Expose laboratory animals by the respiratory route to influenza virus or Klebsiella pneumoniae. Measure pulmonary functions using available, or adapt known, techniques. Determine therapeutic methods to reverse these defects.

25 (U) 77 10 - 78 06 - When CO-60 irradiation (200-700 rads) was superimposed on K. pneumoniae infection of rats, there was a significantly increased mortality. This may be explained by the multiorganism bacteremia observed which added to the stress of pneumonia and to decreased immune responses. Another possible contribution is the potential toxin-producing capacity of Klebsiella.

The investigator has separated from the Army. The work unit is terminated.

Publication: Fed. Proc. 37:715, 1978.
Proc. Soc. Exp. Biol. Med. 158: in press.

*Available to contractors upon requestor's approval

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 042: Pathophysiological Guides to Therapy in Respiratory Infections Acquired via an Aerosol

Background:

An animal model for respiratory infection was developed in the Dunning-Fisher rat infected with Klebsiella pneumoniae. Details on the pathogenesis of the infection with emphasis on respiratory parameters and blood gasses were described in the last annual report.

The K. pneumoniae rat model was further utilized to examine the combined stress of infection and exposure to ionizing radiation. Such a combination of radiation injury and respiratory infection has the potential of producing significant loss of manpower in a battlefield environment.

Radiation exposure either preceding or following bacterial infection can markedly increase the pathogenesis of both the infection and the radiation injury.

Progress:

Combined radiation infection studies. Preliminary studies were conducted to determine the physiological effect of 200 R total-body-^{60}Co irradiation on rats. Blood gasses, respiratory parameters, body weights, and rectal temperatures of control rats were compared over a 21-day period to those rats exposed to 200 R of total-body irradiation (50 R/min). A significant ($P < 0.001$) leukopenia (primarily lymphopenia) existed for 14 days after irradiation, and a decreased rate of growth was observed. Blood platelets were also reduced ($P < 0.01$) 7 and 9 days postirradiation. No other significant changes were noted.

Intratracheal inoculation of 10^3 K. pneumoniae organisms in 100-gm Dunning-Fisher rats produced moderately severe bronchial pneumonia without mortality. When nonlethal (200 rads) whole-body ^{60}Co irradiation was given 48 hr prior to intratracheal inoculation of 10^3 K. pneumoniae, mortality exceeded 50%. The following parameters were compared among infected, irradiated, irradiated-infected, and noninfected-nonirradiated groups; respiratory tidal volume, minute volume, and frequency, arterial blood pH, oxygen and carbon dioxide tensions, bicarbonate, lung lesion scores, luekocyte and differential counts, and qualitative and quantitative bacteremias.

Leukopenia, primarily lymphopenia, developed in both irradiated groups, while a trend towards leukocytosis developed in the infected group. Comparable lung lesion scores were present in both infected groups. The minor trend changes in arterial pH, P_{O_2}, P_{CO_2}, respiratory

tidal volume, minute volume, and frequency were not of significant magnitude to explain the differences in mortality observed.

Bacteremia 3 days postirradiation in the irradiated-infected and 1-day postinfection in the infected group were approximately 10^5 and 10^4 organisms/ml, respectively. Counts decreased linearly until day 14, when negative cultures were obtained. Only Klebsiella was isolated from the infected group, while Klebsiella, Escherichia coli, and Pseudomonas were isolated from the irradiated-infected group. Both irradiated and control groups were negative for circulating bacteria throughout the experiment. Increased mortality in the irradiated-infected group may be explained by the radiation-induced multiorganism bacteremia superimposed upon the stress of pneumonia and decreased immune responses.

There appears to be 2 explanations, or a combination of both for increased mortality by the radiation-induced multiorganism bacteremias. As 200 rads of total-body irradiation alone do not cause significant intestinal epithelial damage, the elevation in bacteria counts from non-Klebsiella organisms may be due to the decreased phagocytic ability of irradiated cells or the inability of phagocytes to completely dispose of ingested endogenous bacteria. The increased mortality in the irradiated-infected group may also be partially explained by the potential toxin-producing capacity of Pseudomonas.

Therapy of combined radiation-infection injury. Equally sized groups of rats were exposed to varying doses of ^{60}Co irradiation (25-700 rads) and then reallocated into 3 groups receiving 10^1, 10^3, or 10^5 K. pneumoniae organisms 48 hr postirradiation. The radioprotectant amino-propylamino-ethyl-phosphorothioate (WR 2721, 250 mg/kg) was given IP 30 min prior to irradiation, or an antibiotic (gentamycin sulfate, 10 mg/kg, IM) was given 72 hr postirradiation b.i.d. for the first day, then once a day for 6 days. A third group was given both WR 2721 and gentamycin in the same therapeutic regimen. Mortality results are shown in Table I. With one exception, the treatment with gentamycin 24 hr postinfection prevented mortality. In the one group where death occurred, the mortality rate was significantly reduced when compared with the nontreated control group. The use of WR 2721 30 min prior to irradiation was less effective than gentamycin in preventing mortality. However, it reduced mortality when compared with nontreated controls, and also appeared to be more effective at high challenge doses. The combined treatment with an antibiotic and radioprotectant was also very effective in reducing mortality. It appears reasonable to assume that the efficiency of this therapeutic regimen is largely due to gentamycin sulfate and not WR 2721.

The above findings confirm the fact that the mortality observed in Klebsiella-infected rats previously exposed to ^{60}Co irradiation is due to bacterial sepsis from endogenous bacteria or their toxins, and the use of a broad-spectrum antibiotic supports the suppressed immune system by preventing overpopulation of pathogenic bacteria.

The principal investigator for this work unit separated from the Army and the work unit has been terminated.

TABLE I. EFFECT OF A RADIOPROTECTANT AND/OR AN ANTIBIOTIC ON MORTALITY OF KLEBSIELLA-INFECTED RATS PREVIOUSLY EXPOSED TO ^{60}CO IRRADIATION.

K. PNEUMONIAE	% MORTALITY BY RADIATION DOSE (RADS)							
TREATMENT GROUPS	50	100	200	300	400	500	600	700
10^1								
Control	0	0	0	17	17	83	83	ND*
Antibiotic	0	0	0	0	0	0	0	ND
Radioprotectant	0	16	33	17	33	67	67	ND
Antibiotic + radioprotectant	0	0	0	0	0	0	0	ND
10^3								
Control	17	33	67	83	100	100	100	100
Antibiotic	ND	0	0	0	0	0	0	0
Radioprotectant	ND	17	0	17	50	83	67	67
Antibiotic + radioprotectant	ND	0	0	0	0	0	0	0
10^5								
Control	83	100	100	100	100	100	100	ND
Antibiotic	0	0	0	33	ND	ND	ND	ND
Radioprotectant	ND	17	17	50	ND	ND	ND	ND
Antibiotic + radioprotectant	0	0	0	33	ND	ND	ND	ND

*ND = Not done.

Presentations:

Tschorn, R. R., and P. S. Loizeaux. Effect of Klebsiella pneumonia superimposed on whole-body ^{60}Co-irradiated rats. Presented, FASEB, 14-19 Apr 1978, Atlantic City, NJ (Fed. Proc. 37:715, 1978).

Publications:

Tschorn, R. R., J. B. Arensman, and D. E. Hilmas. 1978. Effect of small particle aerosols of rimantadine and ribavirin on pathophysiologic changes associated with A/NJ influenza in mice. Proc. Soc. Exp. Biol. Med. 158: in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0E6411	78 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
77 10 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	043
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)* (U) Respiratory disease mechanisms, pathogenesis and therapy of airborne infections

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
73 02	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	0.9	225.0
FISCAL YEAR CURRENT 79	2.0	254.7

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) Prophylaxis; (U) Viral disease; (U) Aerosols; (U) Respiratory infections; (U) Pathogenesis

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

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) 77 10 - 78 09 - Japanese B encephalitis (JE) virus was highly stable at relative humidities between 30 and 80%. CNS tissues are primary sites for virus replication and histopathological changes in mice after aerosol exposure to this virus. Direct virus transport across the cribriform plate is apparently the primary mechanism for CNS infection, but hematogenous spread may occur. Upper respiratory tract challenges produced lethal infections similar to those seen with deep pulmonary challenges. Mice surviving initial infections were solidly protected against rechallenge but killed virus vaccination and immune serum administration failed to protect. Aerosol challenges with JE virus produced lethality in both hamsters and squirrel monkeys; nonlethal infections were produced in guinea pigs and rats.

*Available to contractors upon originator's approval

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* U.S. GPO: 1974-540-843/8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No: A841 00 043: Respiratory Disease Mechanisms, Pathogenesis and Therapy of Airborne Infections

Background:

Japanese B encephalitis (JE) virus must be considered a potential threat against U.S. populations, both military and civilian, in a BW environment. Even in areas of the world where the virus is endemic and high proportions of the human populations circulate neutralizing antibodies, widespread epidemics of clinical encephalitis occur with mortality rates approaching 30% (1, 2). Laboratory studies have demonstrated that the virus is easily produced in high concentrations by methods amenable to reasonable scale-up, and the virus can be stored as a frozen concentrate for extended periods of time without serious losses of virus due to inactivation (2).

In conducting medical defensive studies, we previously reported on suckling mouse brain (SMB) harvests of the virus which demonstrated that JE infections were lethal for mice at relatively low doses by respiratory challenge. High virus titers were measured in tissues of the upper respiratory tract and brain of these mice during infection, but virus was recovered infrequently from lungs; viremias could not be demonstrated by plaque assay methods (3). Histopathological changes were confined mainly to tissues of the CNS; mice died with signs typical of encephalitis. Attempts at antiviral therapy failed to modify the JE infections (3).

Continuing research was directed to: (a) investigations on the stability of JE virus in aerosol; (b) further examination of the pathogenesis of respiratory infections in mice; (c) studies of the protective immunity afforded the animal host as a consequence of prior infection on immunization with killed antigens; and (d) determinations of the susceptibility of laboratory animal species other than the mouse to airborne infection with JE virus.

Progress:

Stability of JE virus aerosols. Four aerosol trials were completed at each of 3 relative humidity conditions (80, 55 and 30%) in one of the 6200 L static aerosol chambers. The aerosols were maintained under environmentally controlled (75^{o}F) conditions for 60 min after dissemination in each trial. Aerosol samples were collected at period midpoints of 4, 32 and 60 min.

Table I summarizes the results obtained and presents the mean $\log_{10}$ PFU/L of aerosol at each sampling period with each relative humidity condition. Also shown are total decay rates expressed as percent decay per minute.

TABLE I. EFFECT OF RELATIVE HUMIDITY ON AEROSOL CONCENTRATIONS AND DECAY RATES OF AIRBORNE JE VIRUS

AEROSOL AGE (min)	LOG_{10} PFU/L AEROSOL BY RELATIVE HUMIDITY 80%	55%	30%
4	4.70	4.78	4.90
32	4.33	4.47	4.56
60	3.73	3.98	4.26
Decay Rate (%/min)	4.00	3.29	2.62

The total decay rates were at levels suggesting a high degree of aerosol stability with evidence that stability improved as relative humidity was decreased. Calibration studies have shown that the physical component of decay for particles $\leq$ 5μm in diameter in these static aerosol chambers is approximately 1.5%/min. Therefore, the biological decay rates (losses due to virus inactivation) were in the range of $\sim$ 1.1-2.5%/min; the decay rate of the lower is equivalent to a half-life of approximately 65 min, while the decay rate of 2.5%/min represents a half-life of approximately 56 min.

Aerosol concentration measures indicated that essentially all the virus subjected to aerosolization was accounted for as aerosol at the 4-min sampling period. In fact, in many of the trials, the recoveries at 4 min exceeded 100%, suggesting that in the process of dissemination there was some disaggregation of the virus-containing particles in suspension. These results indicated that the aerosol stability properties of JE virus could be expected to meet and exceed the minimum characteristics that might be required in a BW environment.

Pathogenesis of JE virus infections in mice. We previously reported that following small-particle aerosol (SPA) challenge of mice with JE virus, the virus concentrations in lungs were minimal and transient. Virus was recovered first, at about 3 days after

challenge, from tissues of the upper respiratory tract and brain; high concentrations of virus persisted in these tissues throughout the course of infection. Mouse spleens and livers were consistently negative for virus, and there was no evidence of viremia when blood was assayed by plaque procedures (3).

In continuing studies, mice were challenged with $\sim$ 25 respiratory LD_{50} of virus as SPA. Brains from groups of 3 mice were assayed periodically between 1 and 14 days. Two tissue samples, including a specimen which was exclusively nasal turbinate and one that included the cribriform plate, were taken from the upper respiratory tract at each period. In addition, blood samples were reexamined for evidence of viremia with the assay based on intracerebral (IC) injection of weanling mice as opposed to plaque assays.

Table II summarizes the results of this study. Virus recoveries from the brain, while showing inconsistencies among sampling periods, reflected a pattern similar to that routinely observed. Virus concentrations in the specimen that included the cribriform plate were similar to those seen in the brain, while virus concentrations in the turbinate specimens were generally lower. These results confirmed earlier indications that proliferation of virus in upper respiratory tissue is minimal and that CNS tissues must represent the sites of primary replication. The weanling mouse assay of blood samples was successful in detecting low levels of viremia. Virus was present in the blood only early (days 1-3) after challenge and at low levels. Nonetheless, these results suggested a potential for hematogenous transport of the virus between the respiratory tract and CNS tissues.

TABLE II. CONCENTRATIONS OF JE VIRUS IN MOUSE TISSUE SPECIMENS AS A FUNCTION OF TIME AFTER SPA CHALLENGE WITH $10^{4.4}$ PFU VIRUS

POSTCHALLENGE	MEAN LOG_{10} PFU/WHOLE SPECIMEN[a]			BLOOD[b]
	Brain	Cribriform plate	Turbinate	(No. dead/6)
1	0	0	0	0,2,3
2	0	0	0	5,6,6
3	0	3.0(1)	2.5(1)	6,6,1
4	4.0(2)	4.0	2.7(2)	0,1,1
7	7.4	7.2	4.4(2)	0,0,0
8	0	0	0	0,0,0
9	7.7(1)	6.8(1)	0	0,0,0
10	0	0	0	0,0,0
11	7.6(2)	6.6(2)	3.7(2)	0,0,0
14	7.8(1)	6.1(1)	2.5(1)	0,0,0

[a] ( ) = No. mice positive when less than 3; virus concentrations are means of positive mice only.

[b] Suckling mice inoculated IC with blood of test mice.

In view of the accumulated evidence that JE virus may be transported directly across the cribriform plate in mice after a respiratory exposure and that the lung plays a minor role in pathogenesis, it was reasoned that deposition of the virus only in the upper respiratory tract could be expected to produce infections similar to those seen after an SPA challenge. To test this, mice were challenged by the IN route with a low-volume inoculum (0.001 ml) designed to deposit virus exclusively in the upper respiratory tract. Groups of 20 mice were challenged with from $10^{1.0}$-$10^{5.0}$ PFU of JE virus in 10-fold increments, and observed for lethality for 21 days. Additional mice were given the high dose of $10^{5.0}$ PFU and sacrificed in groups of 3 at 1-4 and 7 days after challenge. Specimens of nasal turbinates, cribriform

plate, and brains were collected for virus assay. Samples of blood were collected on days 1-4 and assayed for virus by IC inoculation in suckling mice.

The LD_{50} by this low-volume IN challenge was estimated at $10^{3.0}$ PFU with a mean time-to-death (MTD) of 9.3 days, suggesting that upper respiratory tract challenge yielded lethal dose response characteristics nearly identical to those seen with SPA exposure. Virus population profiles were similar also to those observed previously. An early, transient, low-level viremia was observed when the assay was performed in suckling mice. High concentrations of virus were seen in specimens of the cribriform plate with somewhat lower concentrations in nasal turbinates. Table III summarizes the virus concentration observations.

TABLE III. CONCENTRATIONS OF JE VIRUS IN MOUSE TISSUE SPECIMENS AS A FUNCTION OF TIME AFTER LOW-VOLUME (0.001 ml) IN CHALLENGE

	MEAN LOG_{10} PFU/WHOLE SPECIMEN[a]			
POSTCHALLENGE	Brain	Cribriform Plate	Turbinate	BLOOD[b] (No. dead/6)
1	Neg	Neg	Neg	0,0,0
2	Neg	Neg	Neg	1,3,6
3	2.67(1)	3.89(1)	1.30(1)	6,6,0
4	5.20(2)	5.26(2)	2.53(2)	2,1,1
7	9.16	8.31	5.84	- - -

[a] ( ) = No. mice positive when less than 3; virus concentrations are means of positive mice only.

[b] Suckling mice inoculated IC with blood of test mice.

Studies of protective immunity. All of the studies on pathogenesis of respiratory JE infections in mice employed the Peking strain of virus. Ancillary observations revealed that mice which survived the SPA challenges developed high HI serum antibody titers against the homologous virus and were solidly protected against a lethal SPA rechallenge. Accordingly, studies were undertaken to compare initial challenges by the IP and aerosol routes on the basis of protective properties against a lethal SPA rechallenge and to determine the cross-protective characteristics between 2 strains of virus, the standard Peking and Nakayama strains. The latter is a potential parent strain for vaccine development.

Four groups of mice were initially challenged by either the IP or SPA route. Challenge doses calculated to approximately 1 LD_{50}/mouse were employed. At 21 days, 5 surviving mice from each group were exsanguinated for determination of serum HI antibodies against the primary Peking strain. The remaining survivors were challenged with ∿ 40 respiratory LD_{50} of Nakayama or Peking strain virus aerosols. Groups of 20 mice not previously infected were also exposed and served as controls for virus virulence.

Table IV presents the HI titers at 21 days after initial challenge and summarizes the survival characteristics following lethal respiratory rechallenge; all the sera from surviving mice were positive. Within virus strains, the titers of mice challenged by the IP route were similar to those challenged by SPA; somewhat lower titers were seen with mice challenged with Nakayama virus than those challenged with the Peking strain. Compared with controls, a high degree of protection was afforded to all mice surviving initial infection regardless of the strain of virus or route of administration. Although not significant with the few mice employed, somewhat better protection appeared to be offered to mice initially challenged with Peking strain.

TABLE IV. MOUSE SURVIVAL FOLLOWING LETHAL RESPIRATORY RECHALLENGE WITH JE VIRUS 21 DAYS AFTER SUBLETHAL EXPOSURE

INITIAL CHALLENGE			SPA RECHALLENGE SURVIVORS/TOTAL (%)	
Virus Strain	Route	HAI Titer (21 day, n=5)	Nakayama	Peking
Nakayama	IP	15	9/12(75)	7/13(54)
	SPA	60	11/12(92)	10/12(83)
Peking	IP	160	4/ 4(100)	3/ 4(75)
	SPA	211	10/10(100)	10/10(100)
None	None	0	5/20(25)	3/20(15)

The potential for passive immune protection of mice against inhalation infections with JE virus was examined in a subsequent study. A pool of guinea pig immune serum with an HI antibody titer

of 1:320 was tested for passive protection. Protection with this material could not be demonstrated in mice challenged with ∿ 40 respiratory LD_{50}. Fourteen of 20 (70%) mice treated at 4 hr before challenge with 0.2 ml of the immune serum died, while 13 of 20 (65%) and 18 of 20 (90%) died when treated at 4 and 24 hr, respectively, after exposure to virus. These results are to be compared with 17 deaths in 20 (85%) untreated control mice exposed to the virulent virus aerosol.

We were similarly unsuccessful in demonstrating immunoprotection in a preliminary study to test the potential of killed antigen vaccination. A 2-passage LLC-MK_2 tissue culture harvest of JE virus, containing $10^{7.4}$ PFU/ml prior to inactivation, was formalin-inactivated (0.05%) and used to vaccinate groups of 50 mice by aerosol exposure and by IP inoculation of either 1:3 or 1:100 dilutions of the antigen (0.5 ml/mouse). Bloods from 10 mice of each group were negative for serum HI antibodies at 20 days. Death patterns among the immunized mice following exposure to lethal SPA aerosols were similar to those observed among unimmunized controls.

Challenges of other animal species. Table V summarizes the results of studies completed to determine the susceptibility of animal species other than the mouse to lethal infections after aerosol exposure to JE virus.

TABLE V. LETHAL DOSE RESPONSES AND GEOMETRIC MEAN TIMES TO DEATH AMONG ANIMAL SPECIES CHALLENGED WITH JE VIRUS AEROSOLS

SPECIES	SPA DOSE (LOG_{10} PFU)	NO. DEAD/TOTAL	GEOM. MTD (days)
Hamster	5.5	13/14	10
	4.4	6/6	18
	3.4	5/6	15
Guinea Pig	5.8	0/8	-
Rat	5.4	0.8	-
Squirrel Monkey	6.0	3/4	13
	4.9	0/4	-
	2.8	0/4	-

Hamsters were particularly susceptible to lethal infection with 5 deaths in 6 animals exposed to the lowest dose. Both guinea pigs and rats were infected as evidenced by seroconversions in all of the animals exposed to a high dose of more than 10^5 PFU of virus. On the other hand, none of the animals succumbed, and none showed signs of illness during the course of infection. Only the high dose of 10^6 PFU produced deaths among squirrel monkeys. Evidence of infection was present at the lower exposure doses, however, where 3 of 4 monkeys in each group demonstrated seroconversion based on the presence of HI antibodies.

Presentations:

1. Larson, E. W. Experimental aerobiology and the transmission of respiratory infections. Presented, Department of Microbiology, University of Maryland Dental School, Baltimore, MD, 17 Feb 1978.

2. Larson, E. W. Hazard potential from laboratory activities: aerosol generation. Presented Short Course, Biohazard and Injury Control in the Biomedical Laboratory. State University of New York, Stonybrook, NY, 12 Jun 1978.

Publication:

None

LITERATURE CITED

1. Clarke, D. H., and J. Casals. 1965. Japanese encephalitis virus, pp. 626-631. In Viral and Rickettsial Infections of Man. 4th ed. (F. L. Horsfall and I. Tamm, eds). J. B. Lippincott, Philadelphia.

2. MacCallum, F. O. 1961. Japanese B encephalitis, pp. 326-328. In Virus and Rickettsial Diseases of Man. (A. W. Downie, F. O. MacCallum and C. H. Stuart-Harris, eds. Williams and Wilkins, Baltimore.

3. U. S. Army Medical Research Institute of Infectious Diseases. 1 Oct 1977. Annual Progress Report, FY 1977, pp. 321-326. USAMRIID, Fort Detrick, MD.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6420	78 06 27	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
77 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	044
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(U) Pathogenesis of Bolivian hemorrhagic fever

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
72 10	78 06	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 78	0.8	18.9
	CURRENT 79	0	0

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21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Pathology; (U) Hemorrhagic fever; (U) Machupo virus; (U) Arenaviruses; (U) Macaque monkeys

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 pathogenesis of arenaviruses in appropriate animal models. Understanding the sequence of events occurring in arenavirus infections pathogenic for man, their pattern of development, and effects on the host, is necessary for the formulation of an effective means of treatment and protection.

24 (U) Development and characterization of a hemorrhagic fever model using an arenavirus that is nonpathogenic for man is necessary for complete understanding of this disease process. Animals dying of the disease following its natural course will be compared with those which have been vaccinated, immunosuppressed, and passively protected to determine the role of the immune response in the pathogenesis of arenavirus infection.

25 (U) 77 10 - 78 06 - Pichinde virus infection was characterized. The 8th spleen passage in guinea pigs produced strain GP8 lethal (4.3 logs, SC) for 70% of Hartley strain guinea pigs. The infection caused roughened hair coat, lassitude and 30% weight loss by days 12-16. At necrospy, abdominal fat stores were depleted. Liver was pale tan with pinpoint yellow necrotic foci; 1-cm patchy necrotic areas were seen. Microscopically, lesions were seen in liver and spleen. Lesions of lethal Tacaribe (TCR) virus in adult mice were characterized. Athymic homozygous nude mice challenged with TCR did not die; heterozygous nude mice had lesions. Lethal encephalitis caused by TCR virus is immune-mediated and dependent on intact T-cells.

The investigator has transferred from the Institute. The work unit is terminated.

*Available to contractors upon originator's approval

DD FORM 1498 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

* U.S. GPO 1974-543-843 PE91

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BODY OF REPORT

Project No. 3M762776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 044: Pathogenesis of Bolivian Hemorrhagic Fever

Background:

The arena virus group consists of 10 member viruses of which 4 are known to be pathogenic for man. One member, Machupo virus, is the etiologic agent for Bolivian hemorrhagic fever (BHF), a highly fatal disease in man (1). The rhesus monkey (Macaca mulatta) has been shown to be a good model for the study of BHF (2). Little is known of the pathogenesis of this disease in man or the rhesus monkey. Emphasis is presently directed toward developing a laboratory animal model that is more practical than the primate for BHF pathogenesis studies.

In conjunction with this work other arenavirus infections are being examined in various animal models. These experiments are designed to further study the disease processes occurring in arenavirus infections and to develop a hemorrhagic fever model produced by an arenavirus that is not pathogenic for man.

Progress:

Pichinde virus infection was characterized. The 8th spleen passage in guinea pigs produced strain GP8 lethal (4.3 logs, SC) for 70% of Hartley strain guinea pigs. The infection caused roughened hair coat, lassitude and 30% weight loss by days 12-16. At necrospy, abdominal fat stores were depleted. Liver was pale tan with pinpoint yellow necrotic foci; 1-cm patchy necrotic areas were seen. Microscopically, lesions were seen in liver and spleen.

Lesions of lethal Tacaribe (TCR) virus in adult mice were characterized. Athymic homozygous nude mice challenged with TCR did not die; heterozygous nude mice had lesions. Lethal encephalitis caused by TCR virus is immune-mediated and dependent on intact T-cells.

The investigator has transferred from the Institute. The work unit is terminated.

LITERATURE CITED

1. Child, P. L., R. B. MacKenzie, L. R. Valverde, and K. M. Johnson. 1967. Bolivian hemorrhagic fever. A pathologic description. Arch. Pathol. 83:434-445.

2. Terrell, T. G., J. L. Stookey, G. A. Eddy, and M. D. Kastello. 1973. Pathology of Bolivian hemorrhagic fever in the rhesus monkey. Am. J. Pathol. 73:477-494.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6412	78 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
77 10 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	045
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code) (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	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	115.0
FISCAL YEAR CURRENT 79	1.0	220.9

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POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH 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 (Furnish individual paragraphs identified by number. Precede text 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 breeding colonies of other laboratory animals as needs arise for specific entities dealt with by USAMRIID investigators.

25 (U) 77 10 - 78 09 - A new card system of record keeping and single-mate pairing was begun in all 3 rodent colonies to assist in the establishment of a minimum in-breeding system. The Calomys colony experienced reduced reproduction due to several factors, ambient relative humidity, mite infestation and seasonal breeding fluctuations were considered as possible etiologies. Correction of these conditions resulted in a return to high level breeding performance. One abortion, 9 live births and 2 adult deaths occurred in the rhesus colony. A new schedule of data collection was established for the rhesus colony; data collected now includes radiographs of the hand, wrist and forearm showing centers of ossification which may be used to determine the age of wild-caught rhesus monkeys.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 045: Animal Models and Animal Resources for Medical Defense 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 these facts are known, 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 have been identified as models for diseases under investigation at the Institute, but are not available commercially. A Calomys callosis (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. A small colony of Microtus montanus (vole) is also colonized and maintained while being evaluated as a potential animal model for the study of rickettsial and arboviral agents.

Macaca mulatta (rhesus monkey) has long been the favorite nonhuman primate for a variety of research endeavors, and continues as such at the Institute. Increasingly stringent export restrictions by the Indian Government over the past several years culminated early in 1978 with a total ban on its export from that country. 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. In an effort to reduce the coefficient of inbreeding in outbred colonies of each of the rodent species, new record keeping and mating methods were employed. Monogamous mating was used exclusively this year in all 3 colonies and has significantly decreased the incidence of fight-induced trauma. A single 3 x 5" card now serves as the permanent record for each breeding unit, with information contained thereon enabling the investigator to quickly ascertain reproductive history and current status.

Establishment of definitive numbers of each species required by the Institute allowed stabilization of colony sizes, with Sigmodon and Calomys colonies each consisting of 50 breeding pairs. The Microtus colony had continuing difficulty after an initial breeding surge when new stock from USDA was introduced; after one or two litters from each of the new pairs, conception ceased and over a period of several months many of the males died of unknown causes; 24 weanlings were issued to the only anticipated user of Microtus for a feasibility study, the results of which will determine the future of this colony.

The Sigmodon colony continues to thrive with very little difficulty. The unpleasant disposition of this species leads to occasional fighting and cannabalism, but reproduction is at a level where replacement of unsuccessful pairings is readily accomplished.

The Calomys colony was the source of the greatest consternation, but also the most gratification. Early in this year, production came to a virtual standstill, even with many new young pairs being added to the breeding stock. Identification and elimination of several undesirable conditions seem to have resolved the problem. Low ambient relative humidity was discovered when a condition known as "ring-tail" was noted in many of the animals. Diagnosis of acariasis caused by Demodex sp. mites in the colony was also viewed as a possible contributing factor. The possibility that the Calomys are somewhat seasonal breeders was considered in the search for a cause of the reduced fertility and/or fecundity. With an increase in room humidity, therapy for the mites, and the arrival of spring, reproduction increased greatly. The colony is now producing sufficient offspring to meet investigator needs and to replenish breeding stock.

Rhesus colony. Collection of growth and hematologic data on colony infants was reevaluated early this year and major changes instituted. The new schedule calls for collection of blood samples and body measurements every 2 weeks for animals from birth to 3 mon and monthly for those 3-18 mon old. Radiography of the hand, wrist and forearm was begun on the same schedule to study the processes of growth and skeletal maturation. Radiographic appearance and union of secondary centers of ossification will provide a meaningful measure of maturation which can be used to determine with some accuracy the age of wild-caught animals.

Two deaths occurred in breeding females during the year, both being attributable to gastric dilatation. The possibility of females being denied access to food by the males while breeding, then overeating when removed to separate cages is considered to be the most likely etiology of this condition; close monitoring of food intake has been initiated. One additional male was added to the colony this year making the total numbers of animals involved: 5 males, 21 females, and 19 offspring.

Nine live rhesus births were recorded this year; all had weaned by the end of the year. One spontaneous mid-gestational abortion occurred; 3 females are confirmed pregnant at the close of this fiscal year.

Introduction of new breeding-age females and laparoscopic capabilities are anticipated for the near future to enhance reproductive capabilities of this colony.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6422	78 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
78 04 21	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	62776A	3M162776A841	00	046
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(U) Role of bacterial exotoxins in disease pathogenesis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 02600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
75 07	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		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.0	90.0
	CURRENT 79	1.0	122.2

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Exotoxins; (U) Pseudomonas aeruginosa; (U) Burn infections; (U) Laboratory animals

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

23 (U) Characterize certain potent bacterial exotoxins and identify their role in infection. The exotoxin (PE) used as a model, Pseudomonas aeruginosa, is believed to be responsible for the high frequency and severity of Pseudomonas infections of burn wounds. Both the bacterium and its exotoxin are potential BW agents. Characterization of this exotoxin will improve our ability to prevent, diagnose, and treat bacterial infections or intoxications, and deal with novel uses of these and other pathogenic agents.

24 (U) After characterizing and developing an assay for exotoxins in mice, examine the biological factors involved in toxin synthesis and pathogenesis.

25 (U) 77 10 - 78 09 - Modifications of the large scale PE production method were made which increased the toxicity of the product. PE was supplied to investigators in the U.S. and abroad. Variants of P. aeruginosa were generated which produced slightly elevated levels of PE. The glutaraldehyde toxoid of PE was tested in burned rats and mice; a small protective effect was found in mice, but not in rats. A protein closely related to PE, denoted toxin S or exoenzyme S, was partially purified and evidence for a subunit was obtained. Close similarities in the interaction with cells of radiolabeled diphtheria toxin and low density lipoprotein strongly suggested that toxins may enter cells by adsorptive endocytosis. Conjugates of toxins and antitoxins with ferritin and colloidal gold were made to visualize toxin receptors by electron microscopy. A method for radiolabeling PE was developed which maintains the toxin's potency.

Publications: Abstracts - 1978, ASM, p. 29.
Infect. Immun. 19:839-845, 1978.
Biochem. Biophys. Res. Comm. 81:532-538, 1978.

*Available to contractors upon originator's approval

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* U.S. GPO: 1974-540-843/8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 046: Role of Bacterial Exotoxins in Disease Pathogenesis

Background:

Bacterial exotoxins are responsible for the pathogenesis seen in certain infections (diphtheria, cholera, tetanus, anthrax) and suspected of playing a role in others. Characterization of a number of these pathogenic 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. 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.

As part of a broad ranging study of the Pseudomonas toxin we are examining how the protein enters cells and becomes enzymatically active. Recent evidence suggests that some toxins enter cells by absorptive endocytosis and may be activated in lysosomes. This hypothesis is a novel one which, if confirmed, would lead to an important reorientation of studies on the mode of action of toxins.

Progress:

We have continued to purify PE from 50-L cultures of P. aeruginosa strain PA103, both to fulfill our own needs and to have toxin stocks to supply others. During this period we have provided toxin and/or antitoxin to 5 investigators outside USAMRIID. As reported last year, some batches of PE produced in FY 1977 had lowered cytotoxicity; no reason for this difference had been evident. However, during attempts to radioiodinate PE, the toxin was shown to be very sensitive to oxidation. This prompted inclusion of a reducing agent in our recent purifications. In addition, more care was taken to avoid foaming during $(NH_4)_2SO_4$ precipitations. Apparently one or both of these modifications was important, since all recent preparations have typical, high cytotoxicity.

Although exotoxin A can be obtained in amounts (100 mg) adequate for many purposes, further studies of the toxin's structure would be facilitated by selection of high toxin-producing strains. Following the example of work with S. aureus (Work Unit BS03 00 018) we have

produced mutants expected to have modified membranes. Mutants sensitive to acidic media (pH 5.7), to elevated levels of salt (0.33 M NaCl), or resistant to polymyxin have been obtained after mutagenesis. A rocket immunoelectrophoresis technique was developed for quantitative measurement of toxin concentration in culture supernatants. Preliminary results suggest that some low-pH sensitive mutants may make elevated quantities of toxin. In an alternate approach to selection of high producers, mutants deficient in production of Fe-chelating compounds were sought, since Fe-deficient cells would not experience the normal Fe-dependent repression of toxin synthesis. Because chelators are synthesized by most bacteria from an intermediate in the aromatic amino acid pathway, chorismic acid, mutants unable to synthesize chorismic acid were sought. Such mutants are easily recognized as ones simultaneously requiring the amino acids, Tyr, Try and PHE. A number of the desired mutants were obtained after mutagenesis and penicillin enrichment. Initial tests showed that toxin production by these mutants is not elevated above that of the parent strain in the presence or absence of Fe. It has subsequently been learned through discussions at a recent national meeting that P. aeruginosa has 2 Fe chelators, and that these may be synthesized via rather novel routes. The selection of high producers will remain difficult until a simple method for screening large numbers of colonies becomes available.

If PE is a significant virulence factor in Pseudomonas infection, then the glutaraldehyde toxoid we have developed should protect animals. Tests were conducted in well-established burned-rat and-mouse infection models in collaboration with investigators in other DOD laboratories. Studies in the burned-rat model were performed at the Institute of Surgical Research (USAISR) with Drs. H. Walker, C. McLeod and A. Mason. Groups of rats were immunized with either (a) small doses of live organisms, (b) serum albumin absorbed on aluminum phosphate-protamine, or (c) toxoid on the same adjuvant. Investigators at USAISR immunized the rats at weekly intervals since their prior experience showed that live organisms given at this interval induced resistance to challenge. When rats were burned and challenged, only those animals immunized with live organisms survived; toxoid did not confer protection. This experiment was repeated with the same result. Sera drawn from a few animals prior to challenge were sent to USAMRIID. Assays for neutralizing antibodies showed that the rats had titers somewhat below those of rats immunized with toxoid here at USAMRIID. These lower titers may have resulted from use of a shorter immunization schedule. While it therefore appears that PE is not a major virulence factor in burned rats, it remains possible that higher titers of antibody might confer some protection. It should also be pointed out that rats are significantly less sensitive to PE than certain other animals, such as mice.

Extensive tests of the ability of the glutaraldehyde toxoid to protect burned mice from fatal infection are being conducted with Dr. O. R. Pavlovskis of the Naval Medical Research Institute, Bethesda, MD. All recent tests have employed the $AlPO_4$ protamine adjuvant since it is not expected to induce nonspecific bacterial immunity as was observed with complete Freund's adjuvant (CFA). In typical experiments mice received

2 doses of 10 μg of toxoid at an interval of 20 days. At various times after the 2nd immunization the mice were subjected to an alcohol burn and injected with an amount of a bacterial culture previously determined to be lethal. In initial tests where the interval from booster to challenge was 10 days, no protection was seen against strain PA103. When the interval after the booster was extended to 21 days some protection was observed. This is consistent with the results of immunization using CFA, where it was found that the resistance acquired by mice continued to increase for at least 4 weeks after the booster. Further experiments are underway to measure whether resistance is also obtained against the challenge strains PA86 and PA220, which are more virulent than PA103.

A toxoid designed to protect against Pseudomonas infection should include all major variants and serotypes of exotoxin. Dr. Iglewski recently identified several isolates of P. aeruginosa whose culture supernatants have very high ADPR-transferase activity (1). Although the reaction is very similar to that catalyzed by exotoxin A, the acceptor of ADPR is not elongation factor 2, but is instead some other, as yet unidentified, protein in eukaryotic cytoplasm. This enzymatic activity was first denoted "exotoxin S," but the relatively low toxicity to mice and cultured cells led to its redesignation as "exoenzyme S." Since we suspected that this enzymatic activity might reflect the presence of an activated form or peptide fragment analogous to that of exotoxin A, we began an effort to purify and characterize this material. Using strain 388 provided by Dr. Iglewski we first studied the media constituents which promote toxin S production. We confirmed that nitrilotriacetic acid (NTA) is an essential media constituent. NTA is believed to act either by repressing protease activity through chelation of divalent ions or by chelating Fe, a known inhibitor of exotoxin A production. Monosodium glutamate (MSG) also promotes toxin S synthesis. Several entirely synthetic media were found to support toxin S production when supplemented by appropriate amounts of NTA and MSG.

Preliminary tests to discover methods for purifying toxin S showed that it precipitates completely at 50% saturated $(NH_4)_2SO_4$, adsorbs to DEAE-cellulose at pH 5-9, survives boiling and SDS treatment, and has an apparent isoelectric point of 7-8. The activity seems more stable at low pH, so that many operations are now done at pH 5.0. Attempts to purify and characterize toxin S on gel filtration columns revealed a behavior reminiscent of Escherichia coli enterotoxin. The toxin behaved as a very high molecular weight (MW) species, excluded by Sepharose 4B (4% agarose) at pH 5 or 7. However, when SDS-treated samples were run on columns of Sephadex G-150 or BioGel A1.5 (8% agarose) in SDS the activity eluted between albumin and staphylococcal enterotoxin B (SEB) markers, suggesting a MW of 50,000-60,000. Electrophoresis on polyacrylamide gels also showed that the toxin migrated between albumin and SEB. Apparently toxin S has high affinity for some polymeric material (lipopolysaccharide?) present in culture supernatants. Subsequently, we found that 0.1% Triton X-100 prevents the association of toxin S to the polymeric material. When analyzed by gel filtration in Triton, the toxin moves as a single peak well ahead of albumin, suggesting a MW of 100,000-200,000. In view of

the lower MW found in denatured samples, this result suggests that the toxin may contain several subunits.

Rational design of measures to protect animals from bacterial toxins can best be achieved when the mode of action of each toxin is fully understood. For Pseudomonas exotoxin A, where the intracellular mode of action is known, the principal uncertainty is the means by which the protein enters cells and becomes enzymatically activated. Basically the same uncertainty exists for diphtheria toxin. The mode of entry of these toxins into cells has remained unknown, in part because few precedents exists for the internalization of intact proteins by cells.

The entry of diphtheria toxin (as well as other toxins) into cells is a major subject of work by Dr. Middlebrook, Pathology Division (Work Unit BS03 00 019), in collaboration with the work described here. This research has recently led to the first unambiguous demonstration of a membrane receptor for diphtheria toxin. In reviewing the binding of radiolabeled diphtheria toxin to receptors on monkey kidney cells, a strong resemblance was noted to the behavior of several other proteins, notably epidermal growth factor (EGF) and low density lipoprotein (LDL). The processing of LDL in particular has been well characterized (2); it has been shown that uptake is via absorptive endocytosis through regions of the fibroblast membrane described as "coated pits." LDL bound to receptors localized in coated pits is taken into "coated" vesicles which later fuse with lysosomes. The uptake of LDL provides an attractive model for the uptake of bacterial toxins and has already proven to have great predictive value. Thus a method used in the study of LDL (2) was tested in collaboration with Dr. Middlebrook and found applicable to diphtheria toxin. This method employs TCA precipitation of tissue culture supernatants to measure the amount of toxin degraded by the cells, a measure of the toxin internalized and processed through lysosomes. It has already allowed us to measure the rates at which diphtheria toxin bound to receptor is internalized and degraded. The technique has been used to show that some of the drugs which protect cells from toxin do so by blocking lysosomal processing.

While biochemical evidence is rapidly accumulating, which implicates absorptive endocytosis, the involvement of particular cellular structures (coated pits, vesicles, lysosomes) can only be demonstrated by electron microscopic methods. Therefore, a number of approaches have been used to visualize toxin bound on cells. Initial attempts employed conjugates of diphtheria toxin with the electron-opaque marker ferritin. A 2-step glutaraldehyde procedure led to coupling of 5% of the toxin to ferritin, but the conjugate did not compete with ^{125}I-toxin binding to cells. A carbodiimide-activated coupling procedure led to 10% toxin incorporation, and the resulting conjugate, purified by agarose gel filtration, had some ability to block ^{125}I-toxin binding. This conjugate was incubated with cells, and the samples examined by transmission electron microscopy (Dr. White); no ferritin was observed. Other methods were used to attach both diphtheria and Pseudomonas toxin to ferritin. Again a yield of 5-10% was obtained, but the conjugates were not effective blockers of ^{125}I-toxin-binding. It appears therefore that attachment of a large protein (ferritin MW = 400,000) prevents effective interaction with

receptor.

As an alternative method we have begun use of gold colloid conjugates. Procedures were developed for preparation of toxin and antitoxin conjugates using 15-20-nm gold particles. An encouraging preliminary test showed that the gold anti-Pseudomonas toxin conjugate bound to the surface of L929 cells previously treated with the toxin at 4°C. There appeared to be some localization over "coated pits." Further tests are underway.

Our success in characterizing the diphtheria toxin receptor has encouraged continued efforts to demonstrate a Pseudomonas exotoxin A receptor. Progress was for a considerable period prevented by difficulties in radiolabeling the toxin. A systematic study showed that exotoxin A was rendered nontoxic by exposure to very low concentrations of chloramine T, the oxidizing agent used in chemical iodinations. Subsequent work has identified an effective lactoperoxidase procedure which does not decrease the toxin's cytotoxic activity. Preliminary studies show that the radiolabeled toxin does bind specifically to certain sensitive cell lines. Rapid progress in characterizing the interaction of exotoxin A with cells should now be possible.

Presentation:

Leppla, S. H., O. C. Martin, and O. R. Pavlovskis. Development of an efficacious glutaraldehyde toxoid of Pseudomonas exotoxin A. Presented, American Society of Microbiology, Las Vegas, NV, 14-19 May 1978 (Abstracts, p. 29).

Publications:

1. Snell, K., I. A. Holder, S. H. Leppla, and C. B. Saelinger. 1978. Role of exotoxin and protease as possible virulence factors in experimental infections with Pseudomonas aeruginosa. Infect. Immun. 19:839-845.

2. Leppla, S. H., O. C. Martin, and L. A. Muehl. 1978. The exotoxin of P. aeruginosa: proenzyme having an unusual mode of activation. Biochem. Biophys. Res. Commun. 81:532-538.

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2. Goldstein, J. L., and M. S. Brown. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. Annu. Rev. Biochem. 46:897-930.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OD6421	78 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
77 10 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	047
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede 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	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		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.0	125.0
	CURRENT 79	1.0	76.3

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 (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Vaccines; (U) Q fever; (U) Rickettsia

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified 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) 77 10 - 78 09 - Initial results of tests to determine the effect of enzyme treatment on the biological properties of the soluble phase I antigen of C. burnetii indicate that changes in antigenicity can be induced by treatment with proteinase, lipase or lysozyme. Lysozyme was also found to produce a reduction in reactogenicity. Modification by enzymatic action may represent another approach to the development of improved vaccines and may provide basic information on the relationship of specific components of vaccines to the expression of antigenicity, immunogenicity and reactogenicity.

Evaluation by Pathology Division revealed that there were no qualitative differences in skin reactions produced by the particulate Q fever vaccine (NDBR-105) and the soluble phase I antigen; however, the soluble antigen was 100 times less reactogenic than the NDBR vaccine.

Publication: Infect. Immun. 22: in press, 1978.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 047: Physicochemical and Biological Characterization of Components of Coxiella burnetii

Background:

One goal of this work unit is to evaluate the potential of the soluble, phase I antigen of Coxiella burnetii for use as a vaccine. This antigen, which has been tested successfully as a vaccine for man in Czechoslovakia (1) and Romania (2), is far less reactogenic than either cell-wall or intact preparations of this organism (3, 4). We have demonstrated, and reported earlier, that (a) the immunogenicity of the soluble antigen can be enhanced by a complex of polyriboinosinic-polyribocytidylic acid, poly-l-lysine and carboxymethylcellulose [poly(ICLC)]; (b) that the antigen has the capacity to trigger a cellular immune response; (c) that it is stable to lyophilization and can be stored at 4°C in either a dry or liquid state without loss or antigenicity; and (d) that it is about 100 X less skin-reactive than the Merrell-National Laboratories particulate, phase I vaccine (NDBR-105). We also found that preparations of the soluble antigen generally employed in the past [dialyzed trichloroacetic acid (TCA) extracts of phase I C. burnetii] can be separated by filtration through Sephadex G-200 columns into 2 major components, only one of which is antigenic.

The unique, soluble, phase I antigen of C. burnetii is composed of carbohydrate protein, and lipid (4, 5). We are investigating the relationship of these components to the biological properties of antigenicity, immunogenicity and reactogenicity by treating the antigen with enzymes or other reagents. In addition to increasing our knowledge of the chemical composition of the antigen, this approach has, as a rationale, the idea that an enzymatically or chemically modified antigen might possess enhanced immunogenicity or be free of reactogenicity. Also, information obtained with this antigen might be applicable to other rickettsial or bacterial antigens.

Progress:

Modification of the antigenicity/immunogenicity of the soluble phase I antigen of C. burnetii by enzyme treatment. Guinea pig protection and skin reactivity tests were initiated using antigen treated with Proteinase K (Beckman), wheat germ lipase (37°C/18 hr, acid phosphatase, Worthington) or lysozyme (37°C/18 hr, egg white, Difco or Sigma). In exploratory tests, treatment with Proteinase K (53°C/24 hr) caused no change in the elution profile of the antigen from Sephadex G-200 gel columns other than an increase in optical density, and no change in antigenicity (CF titer, phase I). However, results of an initial

guinea pig protection test indicated that the immunogenicity of the antigen was destroyed, or markedly reduced, suggesting that protein is an integral part of the fully active antigen.

In a more comprehensive experiment to determine the effect of the above enzymes on the immunogenicity of the phase I antigen, guinea pigs (8/group) were inoculated with 5 µg protein of treated or untreated antigen suspended in Freund's incomplete adjuvant. Serum samples were taken 14 and 26 days postvaccination. Booxter doses (3 µg protein) of the same type of preparations were administered 10 weeks after the initial dose. Guinea pigs were bled 14 days after the booster and were challenged 1 week later with 5×10^5 ID_{50} of the 4th yolk-sac passage of phase I C. burnetii (Henzerling strain). Temperatures were recorded daily for 10 days; guinea pigs with temperatures > 40.0°C for $\geq$ 2 consecutive days were considered unprotected. Serum samples were obtained 17 days postchallenge. Postvaccine and postbooster sera were assayed for phase I and II agglutinating antibodies and phase II CF antibody. (Phase I CF antibody is not formed in response to the phase I antigen or to inactivated particulate C. burnetii vaccines, but only to the live rickettsia.)

At 14 days postvaccine only phase II agglutinating (MA-II) antibody was detected and this was present in low titer in only a few samples. However, at 26 days all 3 antibody types were present; Table I lists the percentage of sera positive and the geometric mean titers of each group. No phase II CF (CF-II) antibody was found in sera from animals that received Proteinase K-treated antigen and, inexplicably, no MA-II antibody, in sera from those receiving untreated antigen. Antigen treated with lysozyme or lipase had enhanced production of both phase I and II agglutinating antibody; lipase-treated antigen also elicited a CF-II antibody response 5 X higher than that from untreated antigen.

TABLE I. EFFECT OF ENZYME TREATMENT OF PHASE I ANTIGENS OF C. BURNETII ON ANTIBODY RESPONSE IN GUINEA PIGS AT 26 DAYS

	CF-II		MA-I		MA-II	
TREATMENT	%+	Titer[a]	%+	Titer	%+	Titer
None	67	4.0	80	3.0	0	-
Proteinase-K	0	-	100	2.5	34	1.3
Lysozyme	38	1.5	100	6.7	88	2.6
Lipase	86	20.0	100	3.6	63	5.4

[a]Geometric mean titer

Table II lists the antibody levels present 14 days after the booster dose. Again, guinea pigs that received Proteinase K-treated antigen possessed no CF antibody but did have MA-I and MA-II antibodies at the same levels as animals that received untreated antigen. Lysozyme-treated antigen evoked a much lower CF-II response but a somewhat higher agglutinating antibody response than did untreated antigen. Lipase-treated antigen produced CF-II, MA-I and MA-II titers $\leq$ 3 X higher than those produced by untreated antigen.

TABLE II. EFFECT OF ENZYME TREATMENT OF PHASE I ANTIGEN OF C. BURNETII ON ANTIBODY RESPONSE IN GUINEA PIGS FOLLOWING BOOSTER DOSE

	CF		MA-I		MA-II	
TREATMENT	%+	Titer[a]	%+	Titer	%+	Titer
None	80	12	80	5.4	80	7.0
Proteinase-K	0	0	86	8.8	86	4.5
Lysozyme	29	1.8	100	18.0	100	4.4
Lipase	80	37	80	12.0	100	18.0
Saline controls	0	0	0	0	0	0

[a]Geometric mean titer

As mentioned, a challenge dose of phaseI C. burnetii was administered 3 weeks after the booster dose of antigen. Data in Table III illustrates the degree of protection against challenge afforded guinea pigs by treated and untreated phase I antigen. Control animals exhibited a more severe

TABLE III. EFFECT OF ENZYME TREATMENT ON THE IMMUNOGENICITY FOR GUINEA PIGS OF THE PHASE I ANTIGEN OF C. BURNETII

TREATMENT	ANIMALS WITH FEVER/TOTAL	FEVER DAYS	FEVER DAYS/ ANIMAL
None	0/4	1	0.25
Proteinase K	1/7	10	1.4
Lysozyme	0/6	4	0.67
Lipase	0/5	1	0.20
Saline controls	6/6	35	5.8

febrile response than had been seen in earlier tests. In spite of this strong challenge, complete protection was present in those groups that received untreated, lysozyme-treated and lipase-treated antigen, with very few fever days. Even Proteinase-treated antigen conferred nearly complete protection; in an earlier test it appeared that Proteinase-K either destroyed or sharply reduced the immunogenicity of the phase I antigen. It seems likely that the high degree of protection observed in this experiment might be related to the use of Freund's incomplete adjuvant with the antigen.

Postchallenge sera were assayed for CF and MA antibodies. Geometric mean titers for each group of guinea pigs are listed in Table IV. Eighty percent of the sera, including those of the saline-control animals, had CF-II titers of 1:512 to 1:4096. CF-I antibody levels were much lower and were completely absent in saline-control sera. Guinea pigs that received Proteinase-treated antigen had very high CF-II and MA-II antibody titers compared to relatively low phase I levels. Overall, the highest antibody levels were present in animals that had been immunized with lysozyme-treated antigen, the CF antibody being 3.5 - 5.5 X higher than that from untreated antigen. Sera from animals that received lipase-treated antigen had titers the same as, or lower than, those that received untreated antigen.

TABLE V. EFFECT OF ENZYME TREATMENT OF PHASE I ANTIGEN OF C. BURNETII ON POSTCHALLENGE ANTIBODY LEVELS IN GUINEA PIGS

TREATMENT	RECIPROCAL GEOMETRIC MEAN TITERS			
	CF-I	CF-II	MA-I	MA-II
None	45	304	861	432
Proteinase-K	32	1630	313	1250
Lysozyme	161	1630	1290	576
Lipase	45	304	513	76
Saline controls	0	1290	40	204

Skin reactogenicity of the phase I antigen of C. burnetii. The soluble phase I antigen of C. burnetii has been found in our tests and by other investigators (3, 4) to be about 1/100 as active as the intact rickettsiae, e.g., particulate Q fever vaccines, in eliciting skin lesions in sensitized guinea pigs or rabbits. We were interested

in knowing whether the skin reactions produced by the soluble antigen and particulate vaccines differed in nature. Therefore, with the cooperation of Pathology Division, a test was conducted to compare the phase I antigen with the Merrell-National Laboratory NDBR-105 vaccine. A second test was designed to determine the reactogenicity of antigenic-positive (CF^+) and antigenic-negative (CF^-) fractions obtained by Sephadex gel filtration of preparations of the phase I antigen.

Sensitized guinea pigs employed in these tests had received injections of the phase I antigen followed by challenge with phase I C. burnetii. Graded concentrations were compared by ID inoculation of 0.1-ml volumes along the shaved flank of the same guinea pig. Reactions were assessed at 24, 48, and 72 hr postinjection by measuring the diameter and intensity of erythema and the increase in skin thickness using skin calipers (Schnelltaser A02T, Kroplin).

The NDBR vaccine was compared at protein levels of 2.0, 0.2 and 0.02 µg in 2 guinea pigs (Table V). Since individual animals vary markedly in response, it is obviously advisable to employ larger numbers of those shown here. A positive skin reaction has been defined (3) as an erythematous area at least 5 mm in diameter present 72 hr after injection. On this basis negative readings were observed in guinea pig No. 1 for even 10 µg of the soluble antigen and induration values were low. Guinea pig No. 2 was more sensitive to the soluble antigen but the areas of erythema produced by the antigen were diffuse and faint as compared to the red areas produced by 2.0 and 0.2-µg samples of the NDBR vaccine. The least lesion-producing dose for the NDBR vaccine was 0.02 µg and for the antigen 2.5 µg.

TABLE V. COMPARISON OF REACTOGENICITY OF NDBR VACCINE AND PHASE I ANTIGEN OF C. BURNETII 72 HR AFTER INJECTION

INOCULUM (µg protein)	GUINEA PIG NO. 1 Induration (mm)	Erythema (mm)	Pathology score	GUINEA PIG NO. 2 Induration (mm)	Erythema (mm)	Pathology score
NDBR Vaccine						
2.0	14	8 x 8	+	12	11 x 12	+++
0.2	2	4 x 7	+	6	9 x 11	+
0.02	4	3 x 7	+	2	4 x 5	+
Antigen						
10.0	5	3 x 4	+	9	15 x 17	++
2.5	2	4 x 5	+	3	6 x 10	+
0.5	2	3 x 4	+	3	4 x 5	+
Saline	0	0	+	0	0	0

The summary of the pathological examination of injection sites (sampled at 72 hr) was as follows: "The inflammatory reaction was limited to the subcutaneous adipose and connective tissue in all except injection site 7 (saline) where there was a minimal folliculitis within the epidermis. No inflammatory reaction was noticed in the epidermis or dermis in 13 out of 14 injection sites. In guinea pig No. 1 there was no significant difference in the reaction precipitated by any of the materials. In guinea pig No. 2, a more severe reaction was elicited by the 2-µg dose of the NDBR vaccine. Qualitative differences were not observed between the complete organism (NDBR vaccine) and the soluble antigen (TCA extract)."

In the second skin test evaluated by Pathology Division 10 and 2.5 µg protein quantities of the CF^+ and CF^- fractions from Sephadex gel filtration were compared to the same concentrations of the TCA extract applied to the Sephadex columns. As shown in Table VI, the CF^- component possessed no significant reactogenicity. Reactions given by guinea pig No. 1 indicated that the CF^+ component was less reactive than the TCA extract applied to the Sephadex which suggests that some reactogenicity may have been removed by the gel-filtration step. Although this difference was not seen with guinea pig No. 2 in this animal, even the saline control produced a 1+ pathology score. The results of the pathology evaluation of the skin reactions are as follows:

"In guinea pig No. 1 three injection sites were essentially normal (sites 4, 6, and 7) and site No. 5 had only a minimal inflammatory response. Both TCA extracted material (high and low dose) and the high dose positive peak material elicited similar reactions. There was no ulceration of the skin produced at injection site No. 1. The major reaction at sites 2 and 3 was within the subcutaneous tissue. In guinea pig No. 2 the inflammatory reactions were present in the subcutaneous connective or adipose tissue along with inflammatory cells present in the superficial dermis. The severity of reaction did not appear different regardless of the material injected."

TABLE VI. REACTOGENICITY OF COMPONENTS OF TCA EXTRACT OF PHASE I ANTIGEN OF C. BURNETII 72 HR POSTINJECTION

INOCULUM (µg protein)	GUINEA PIG NO. 1 Induration (mm)	Erythema (mm)	Pathology score	GUINEA PIG NO. 2 Induration (mm)	Erythema (mm)	Pathology score
TCA Extract						
10.0	21	6 x 12	+++	17	5 x 12	++
2.5	9	5 x 10	+++	4	5 x 8	++
CF+						
10.0	8	6 x 7	+++	11	7 x 8	++
2.5	2	2 x 3	0	6	7 x 13	++
CF^-						
10.0	0	0	0	0	0	+
2.5	0	0	0	0	0	+
Saline	0	0	0	0	0	+

In additional skin tests being conducted at this time, further comparison of the reactogenicity of the phase I antigen and the NDBR vaccine is being obtained. The main purpose of these tests, however, is to confirm our observation that the reactogenicity of the antigen, and possibly of the NDBR vaccine, can be reduced by treatment with lysozyme.

Publications:

Wachter, R. F., G. P. Briggs, and C. E. Pedersen, Jr. 1978. Enhancement of the immunogenicity of phase I antigen of Coxiella burnetii. Infect. Immun. 22:in press.

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1. Brezina, R., S. Schramek, J. Kazár, and J. Urvölgyi. 1974. Q-fever chemovaccine for human use. Acta Virol. 18:269.

2. Cracea, E., S. Dumitrescu, D. Botez, E. Toma, C. Banda, L. Ioanid, and N. Chirescu. 1973. Immunization in man with a soluble Q fever vaccine. Arch. Roum. Pathol. Exp. Microbiol. 32:45-51.

3. 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.

4. Ormsbee, R. A., E. J. Bell, and D. B. Lackman. 1962. Antigens of Coxiella burnetii. I. Extraction of antigens with nonaqueous organic solvents. J. Immunol. 88:741-749.

5. Anacker, R. L., W. T. Haskins, D. B. Lackman, E. Ribi, and E. G. Pickens. 1963. Conversion of the phase I antigen of Coxiella burnetii to hapten by phenol treatment. J. Bacteriol. 85:1165-1170.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6412	78 10 01	DD-DR&E/AR(A)36

3. DATE PREV SUMRY	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
77 10 01	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NUMBERS	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	049
b. CONTRIBUTING				
c. //////////	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(U) Pathogenesis of hemorrhagic vascular lesions induced by nonindigenous rickettsiae

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
76 03	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

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING 78	1.0	60.0
CURRENT 79	1.0	120.0

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: DePaoli, A.

NAME: Kenyon, R.

21. GENERAL USE

Foreign intelligence considered

POC: DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Nonindigenous rickettsiae; (U) Vascular permeability; (U) Laboratory animals; (U) Immunity

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

23 (U) Investigate the pathogenesis of the vascular lesions induced by dangerous nonindigenous rickettsiae. Knowledge of the pathogenesis of such lesions is essential to the understanding of the pathophysiology and planning of rational therapy for the soldier infected by any one of a number of these potential agents. Such adjunctive therapy, to be utilized along with antibiotics in treating infected soldiers, will reduce hospitalization time and enable the individual to return to a duty status more rapidly.

24 (U) Using an appropriate rodent or primate model system, study morphologic sequence and location of vascular changes and the influence of immune processes, kinins, etc. on them. Use this knowledge to improve therapy.

25 (U) 77 10 - 78 09 - Using a chick embryo model for rickettsial infection, it was found that lesions could be induced by Rickettsia conorii in vessels of the chorioallantoic membrane as well as in other vessels of the chick embryo. Organisms were always associated with vessels containing lesions but were also found in vessels without lesions. Ontogenetically, lesions appear before complement and coagulation proteins are fully developed, and before the embryo is fully competent immunologically thus indicating a direct relationship between rickettsiae and the development of vascular lesions. This model should enhance our knowledge of the pathogenesis of these rickettsial diseases.

Publications: Antimicrob. Agents Chemother. 12:660-664, 1977.
J. Clin. Microbiol. 8: 242-245, 1978

*Available to contractors upon originator's approval

DD FORM 1498 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1494, 1 NOV 65 AND 1495, 1 MAR 68, FOR ARMY USE ARE OBSOLETE. — * U.S. GPO 1974-540 843 8-91

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 049: Pathogenesis of Hemorrhagic Vascular Lesions Induced by Nonindigenous Rickettsiae

Background:

Vasculitis is characteristic of the lesions found in rickettsial spotted fevers. The mechanism of induction of vascular changes has not been elucidated. Lesions are characterized by infiltration of a segment of an affected vessel by a variety of inflammatory cells, predominately mononuclear. In later stages of the disease, fibrinoid degeneration is observed in some vessels and thromboses are frequent. The pathogenesis of these events is not known. Wolbach (1) observed rickettsiae within damaged vessels and postulated a direct causative effect. In later stages of the disease, it was shown that intravascular coagulation and immune mechanisms play a role in lesion severity and development (2, 3). Whether the vascular damage is caused directly by the rickettsiae or their products or indirectly by host defense mechanisms remains to be elucidated.

Progress:

Rickettsia conorii infection in chick embryos. Several experiments were conducted in chicken embryos by IV inoculation of Rickettsia conorii. As reported last year, chick embryos appear to be a good model for pathogenetic studies of rickettsial spotted fevers for the following reasons: (a) infection is easily established by IV inoculation, (b) segmental vascular lesions develop which are similar to those observed in mammals with spotted fever, and (c) host-defense mechanisms can be controlled by varying the age of the embryos being inoculated.

In order to characterize the vascular inflammatory reaction microscopically, embryos were inoculated IV with 250 or 1000 R. conorii and killed at various intervals thereafter. The earliest lesions were seen at 48 hr in those given 1000 and at 72 hr in embryos given 250 organisms. The initial lesion is similar in both and is characterized primarily by heterophils partially filling some allantoic vessels and by margination of leukocytes in others. Small inflammatory nodules were also found at these times which appeared to be contiguous with small vessels. The early inflammatory infiltrate was predominately heterophils. Subsequently, there was an increase in mononuclear cells and a reduction of heterophils so that in lesions 5 days after inoculation, the inflammatory infiltrate consisted almost entirely of macrophage-like cells. Endothelial cells appeared hypertrophic in areas of inflammation and mitotic figures were occasionally found in them.

When lesions were examined ultrastructurally, rickettsiae were found in the cytoplasm of endothelial cells both within lesions and also within endothelium, which appeared normal. Some of the cells had vesiculated cytoplasm but similar changes were found in inoculated controls. Therefore, no significance can be attributed to this vesiculation. The next event to occur was inflammatory cell infiltration; affected vessels were not permeable to colloidal carbon. The appearance of inflammatory cells as an initial morphological event in rickettsial vasculitis indicates that the inflammatory cells may contribute to vessel damage. The rickettsiae themselves may be chemotactic.

Chemotactic studies. Several attempts have been made to establish a reliable chemotactic assay for studying the role of various rickettsiae to attract leukocytes. The technique being tried is that described by Nelson et al. (4). A variety of methods for harvesting guinea pig leukocytes was tried. Shellfish glycogen was inoculated IP into guinea pigs and the exudate was harvested 5 hr later. The exudate contained a large number of polymorphonuclear leukocytes but it was believed that a better yield could be obtained from peripheral blood. Guinea pig blood was anticoagulated with EDTA and cells were separated by 2 methods. One involved separation of leukocytes in dextran followed by Ficoll hypaque. Contaminating leukocytes were removed by osmotic lysis. The resulting cell suspension was inadequate because few intact neutrophils could be found. The other technique involved lysis of erythrocytes by 0.87% NH_4Cl and washing the remaining cell suspension with Hank's medium. Large numbers of leukocytes were obtained with viable neutrophils.

In performing the Nelson technique (4) of chemotaxis, agarose is prepared in Petri dishes and semihardened in a refrigerator; 3 holes are punched in it. The central well contains the leukocyte suspension. Chemotactic agent and control are each placed in 1 of the other 2 wells. Chemotaxis is measured by the distance moved toward the chemotactic agent minus that toward the control. Our initial successful studies have shown chemotaxis of leukocytes toward Escherichia coli culture filtrate as positive control. Problems are still being encountered, as it appears that leukocytes are being lysed subsequent to fixation. Rickettsial suspensions have not yet been utilized.

Studies with Rickettsia akari. In collaboration with Dr. Kenyon, an experiment was performed to determine the usefulness of R. akari, the agent of rickettsial pox, in studies of rickettsial disease pathogenesis. Mice were inoculated IP with 1000 MLD_{50} of yolk sac grown R. akari and killed at days 0, 1, 3, 5 and 7. The major lesion observed in mice was necrotizing hepatitis at 5 and 7 days. In addition, there was mild hypertrophy of endothelial cells of vessels of the brain of infected mice 7 days after inoculation. However, similar hepatic lesions were found in sham controls, thus making results questionable.

Q Fever Pathogenesis in Nude Mice. In collaboration with LTC Kishimoto, Aerobiology Division, a study was undertaken to determine

pathogenicity of Q fever in athymic nude mice. Athymic and euthymic mice were infected with 10^4 Coxiella burnetii by small-particle aerosol. Essentially, all mice lived for 60 days. However, only euthymic mice cleared the infection while athymic mice remained infected for the duration of the experiment. Lesions were found in lung, liver, spleen, kidney and adrenal glands and were characterized by a diffuse pyogranulomatous inflammation consisting of macrophages and neutrophils. Lesions essentially resolved in euthymic mice after day 14. An abundance of rickettsiae were found in reticuloendothelial cells of all tissues of athymic mice but not in euthymic mice on day 60. Thus, it appears that phagocytic cells are capable of endocytosing the organisms but their ability to digest them is impaired.

Presentations:

1. Hall, W. C. Respiratory diseases of nonhuman primates. Presented, Pathology of Laboratory Animals Course, Armed Forces Institute of Pathology, Washington, DC, Sep 1977.

2. Hall, W. C. Rickettsial diseases pathogenesis studies utilizing the chick embryo. Presented, American College of Veterinary Pathologists, Toronto, Canada, Nov 1977.

Publications:

1. Hall, W. C., J. D. White, and G. H. Scott. 1977. Use of a photodensitometric technique to quantify microscopic lesions in mice: antiviral activity against swine influenza virus. Antimicrob. Agents Chemother. 12:660-664.

2. Hall, W. C., and L. R. Bagley. 1978. Demonstration of Rickettsia rickettsii in formalin-fixed, paraffin-embedded tissues by immunofluorescence. J. Clin. Microbiol. 8:242-245.

LITERATURE CITED

1. Wolbach, S. B. 1919. Studies in Rocky Mountain spotted fever. J. Med. Res. 41:1-198.

2. deBrito, T., S. Hoshino-Shimizu, M. O. Pereira, and N. Rigolon. 1973. The pathogenesis of the vascular lesions in experimental rickettsial disease of the guinea pig (Rocky Mountain spotted fever group). A light, immunofluorescent and electron microscopic study. Virchows Arch. (Pathol. Anat.) 358:205-214.

3. Moe, J. B., D. F. Mosher, R. H. Kenyon, J. D. White, J. L. Stookey, L. R. Bagley, and D. P. Fine. 1976. Functional and morphologic changes during experimental Rocky Mountain spotted fever in guinea pigs. Lab. Invest. 25:235-245.

4. Nelson, R. D., P. G. Quie, and R. L. Simmons. 1975. Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J. Immunol. 115:1650-1656.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6416	78 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
78 04 21	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	62776A	3M162776A841	00	050
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(U) Therapy of respiratory bacterial infections transmitted via aerosols

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	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 78	1.0	215.0
FISCAL YEAR — CURRENT 79	1.0	178.4

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 (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Respiratory diseases; (U) Antibiotic therapy; (U) Bacterial diseases; (U) Respiratory disease models

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede 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) 77 10 - 78 09 - Aerosol administration of kanamycin was compared with IM administration for the treatment of respiratory Klebsiella pneumoniae infection in squirrel monkeys. In contrast to previous observations with mice in which aerosol treatment was more effective than IM, the 2 modes of therapy were equally effective with regard to mortality, clinical illness and changes in certain hemoproteins. Since K. pneumoniae causes lobar pneumonia in monkeys and bronchopneumonia in mice, the improved efficacy of the IM route in monkeys compared to mice may be due to differences in pathogenesis. In contrast, aerosols of kanamycin were much more effective than IM injection in both mice and monkeys when used prophylactically against K. pneumoniae.

Studies of the respiratory infectivity of Legionnaires' disease organisms have been initiated in guinea pigs and monkeys.

Publications: J. Infect. Dis. 136(Suppl.):S712-S717, 1977;
Clin. Res. 26:403A, 1978;
Infect. Immun. 20:581-583, in press, 1978;

*Available to contractors upon originator's approval

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* U.S. GPO 1974-540-843/8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 050: Therapy of Respiratory Bacterial Infections Transmitted Via Aerosols

Background:

Termination of the swine influenza project (1) has made possible resumption of aerosol therapy experiments. Because several squirrel monkeys survived the influenza studies, they could be used for therapy studies. Such experiments first required development of a model for respiratory Klebsiella pneumoniae infection which were followed by replicate comparisons of IM and aerosol kanamycin therapy in the infected host. During the course of this investigation, a previously reported difference in host response as a consequence of route of challenge was again observed (1) and was investigated in greater detail. Another outgrowth of therapy experiments was investigation of the possible use of single-dose aerosols of antibiotic for prophylaxis or therapy. This possibility was investigated in mice and monkeys.

Finally, preliminary studies with Legionnaires' disease organism are described.

Progress:

A model for the study of respiratory K. pneumoniae infection was developed in squirrel monkeys. Intratracheal (IT) inoculation of 700 organisms suspended in 1.5 ml of media caused clinically apparent disease at 24 hr. Illness was characterized by fever ($\sim 104^{\circ}F$), tachypnea, anorexia, lethargy and dyspnea. Bacteria were isolated from blood and pharynx for 24-72 hr. Death occurred in about half the monkeys in 40-72 hr. Monkeys that survived had leukocytosis during the course of disease, whereas those that died had marked leukopenia. Also, surviving monkeys had smaller numbers of bacteria in the blood and lower serum lysozyme levels than those that ultimately died.

To determine the pathogenesis of K. pneumoniae in squirrel monkeys, 16 were infected and serially killed in groups of 4 at 6, 24, 30 and 48 hr for histopathological examination and determination of viable organism concentration in tissues.

Tissues tested, in collaboration with CPT Knutsen, Pathology Division, included lungs, liver, spleen, kidney, heart, trachea, blood and oropharynx. In general, the concentration of organisms increased with time in all tissues; the highest concentrations ($>10^6$/gm at 48 hr) were observed in the lungs, the lowest

($\sim 10^2$/ml at 48 hr), in the blood. The histopathological change was that of a steadily developing lobar pneumonia that rarely involved the walls of the airways. The finding of lobar pneumonia in the monkey was in distinct contrast to the Klebsiella broncho-pneumonia seen in rodent models (2, 3) and resembled the form of the acute disease described in humans (4).

The primate model was employed in a comparison of the efficacy of IM kanamycin therapy with aerosol administration of the same antibiotic. Dosages in every study were 15 mg/kg/day; half was administered in the morning, the remainder in the afternoon. Only a single experiment could be carried out over a 12-month period with appropriate controls included to ensure that the virulence of the cultures remained constant throughout. Challenge was by IT instillation of ~700 K. pneumoniae. Treatment was initiated either when illness was first apparent (24 hr) or well established (30 hr). A description of the various treatment groups, the number of monkeys and the mortality in each are given in Table I. Two-thirds of the infected-untreated monkeys died. Only one treated monkey died (IM group first treated at 30 hr) indicating that the 2 modes of therapy were equally effective in preventing death.

TABLE I. EFFECT OF KANAMYCIN ON MORTALITY FOLLOWING K. PNEUMONIAE INFECTION IN SQUIRREL MONKEYS

GROUP	TREATMENT TIME	N	DEAD	%
Controls				
Kanamycin controls. Aerosol		7	0	0
IM		15	0	0
Sham infected-sham treated		15	0	0
Infected				
K. pneumoniae infected-sham treated		21	14	67
IM treatment	24 hour	15	0	0
	30 hour	14	1	7.1
Aerosol treatment	24 hour	8	0	0
	30 hour	7	0	0

Since both modes of kanamycin treatment were equally effective in their effect on mortality, various clinical parameters were analyzed to determine whether the clinical courses of disease were different with one kind of therapy than with the other. The effect of kanamycin therapy on the presence of K. pneumoniae in blood and oropharynx is shown in Table II. Therapy, regardless of route of administration, had little effect on bacteremia except for the IM therapy initiated early. IM treatment beginning at 30 hr had the least effect on presence of organisms in the oropharynx.

TABLE II. EFFECT OF KANAMYCIN ON DURATION OF BACTEREMIA AND PHARYNGEAL SHEDDING IN SQUIRREL MONKEYS

GROUP	TREATMENT TIME	DURATION OF BACTERIAL DETECTION (DAYS)	
		Blood	Pharynx
Untreated Klebsiella control		6	5
IM therapy	24 hr	3	2
	30 hr	6	6
Aerosol therapy	24 hr	5	2
	30 hr	4	2

The effect of treatment on respiratory rate and temperature is shown in Table III. All modes of treatment reduced the duration of fever, but only early IM therapy effectively inhibited the increase in respiratory rate. It was interesting to note that aerosol therapy actually increased the duration of tachypnea, suggesting that the deposition of aerosol particles of antibiotic in the lungs of infected monkeys had an adverse effect. Uninfected aerosol-treated monkeys did not show this increase.

The effect of therapy on food intake and body weight is shown in Table IV. With one exception (IM treatment started at 30 hr), treatment seemed to have little effect on appetite or weight loss. These animals ate less and lost more weight than the untreated controls, indicating that IM administration may have had an adverse effect on appetite.

TABLE III. DURATION OF TACHYPNEA AND FEVER IN KANAMYCIN-TREATED SQUIRREL MONKEYS

GROUP	TREATMENT TIME	DURATION (DAYS) Tachypnea[a]	Fever[b]
Untreated Controls		4	7
IM therapy	24 hr	1	2
	30 hr	3	1
Aerosol therapy	24 hr	7	3
	30 hr	7	2

[a]Arbitrarily defined as an increase of ±30% over baseline respiratory rate.

[b]Arbitrarily defined as an increase of ±1.5°F over baseline rectal temperature.

TABLE IV. EFFECT OF KANAMYCIN THERAPY ON FOOD CONSUMPTION AND BODY WEIGHT IN K. PNEUMONIAE-INFECTED MONKEYS

GROUP	TREATMENT TIME	MEAN % CHANGE FROM BASELINE VALUE Food Intake (day)	Body Weight (day)
Untreated-Klebsiella control		-25(4)*	-5.5(7)
IM therapy	24 hr	-15(4)	-2.5(3)
	30 hr	-75(2)	-7.0(6)
Aerosol therapy	24 hr	-25(1)	-2.5(4-6)
	30 hr	-25(2)	-5.0(6-7)

*Numbers in parentheses are day of maximum occurrence.

In addition to the clinical parameters, the concentration or activity was measured of 4 plasma proteins, α_1-antitrypsin, haptoglobin, lysozyme and ceruloplasmin. Their concentrations increased during infection, but the effect of therapy upon them was

variable. Both aerosol and IM administration of kanamycin reduced α_1-antitrypsin levels; aerosol treatment also inhibited an increase in lysozyme activity. The results of all other determinations were equivocal. Possible use of the 4 proteins as prognostic indicators will require more extensive investigation.

Aerosol antibiotic therapy did not offer a significant advantage over the IM route in the monkey, although we have previously reported that an advantage did exist in the mouse (2). It is possible that the difference in the effect of therapy on the 2 species could be attributed to the kinds of pneumonia produced; this hypothesis is difficult to prove because we have not been able to produce the 2 disease processes in one host species with the same organism.

Another possible advantage of aerosols of antibiotics lies in their use as prophylactic agents, or in the treatment of at-risk patients between the time of exposure and onset of clinical disease. The advantage in this case is the result of the persistence of aminoglycoside antibiotics in lung tissue (2, 5). Such prophylaxis was tested in both mice and monkeys.

Mice were given a single dose of 15 mg/kg of kanamycin by the aerosol or IM route. Control mice were exposed to the citrate-sulphate buffer solvent for the antibiotic. At selected times thereafter, mice were challenged by aerosol with approximately 10 respiratory LD_{50} of K. pneumoniae. Prophylactic aerosols of kanamycin offer a distinct advantage over IM in preventing mortality (Table V); this difference was most marked when antibiotic was administered from 24-72 hr prior to challenge. These data, incidentally, serve to illustrate that the kanamycin bound in the lungs persisted there in an active form.

TABLE V. EFFECT OF PROPHYLACTICALLY-ADMINISTERED KANAMYCIN ON SUBSEQUENT RESPONSE OF MICE TO K. PNEUMONIAE CHALLENGE

HR BETWEEN PROPHYLAXIS AND CHALLENGE	CITRATE-SULFATE CONTROL	NO. DEAD/TOTAL (%)	
		Aerosol	IM
0.5	0/30 (0)	40/40(100)	33/40(83)
4.0	0/30 (0)	40/40(100)	23/40(83)
24.0	0/30 (0)	16/30(53)	0/30(0)
48.0	0/30 (0)	18/40(45)	1/40(3)
72.0	0/30 (0)	18/40(45)	1/40(3)

A test of prophylactic efficacy of aerosols of kanamycin was also carried out in monkeys; IM administration was compared with aerosol administration (15 mg/kg) 6 hr prior to challenge with K. pneumoniae. The value of a single dose of kanamycin given therapeutically 30 hr after challenge was also determined. The data (Table VI) suggest that the aerosol route was more effective than the IM, but single doses were ineffective therapeutically. This experiment will be repeated in order to provide statistical support for the assumption of superior efficacy of prophylactic aerosols.

We have previously reported (1) that striking differences existed in the response of mice to aerosol and IN challenge and treatment. The LD_{50} values for mice by both aerosol and IN routes have been determined (Table VII) and shown that dosage required for IN is far lower than the aerosol. The failure of large-particle aerosols (LPA) and small-volume (0.001 ml) IN inoculation to produce mortality rules out upper respiratory tract infection as an explanation of observed differences. In a subsequent

TABLE VI. MORTALITY OF KLEBSIELLA-INFECTED SQUIRREL MONKEYS AS A FUNCTION OF TIME AND ROUTE OF ADMINISTRATION OF KANAMYCIN

TREATMENT TIME	ROUTE	NO DEAD (n=4)
6 hr prior to challenge[a]	Aerosol	0
	IM	3
30 hr postchallenge	Aerosol	3
	IM	4
None		4

[a]Intratracheal challenge with ∿ 10 respiratory LD_{50}.

TABLE VII. VIRULENCE OF K. PNEUMONIAE FOR MICE AFTER AEROSOL OR IN CHALLENGE

PARAMETERS	VOLUME INSTILLED (ml)	MEAN PARTICLE DIAM (μm)	$LD_{50} \pm SE$[a] (cells)
Route of challenge			
IN	0.05		17.9 ± 6.2
SPA		2.2	1470 ± 506
LPA		7.0	> 2000
Effect of inoculum			
Volume (IN)	0.05		14.0
	0.001		> 5×10^6 cells

[a] 5 replicate determinations

experiment, we have shown that the LD_{50} of aerosolized K. pneumoniae collected in a sampler and then instilled was the same as that of nonaerosolized organisms when the 2 preparations were inoculated IN. This observation indicated that the difference between IN and aerosol challenge was not the result of loss of virulence due to aerosolization. Further studies have shown that the pathogenesis of disease is similar after challenge by the 2 routes but the lesions develop more slowly after aerosol than after IN challenge.

The relationship of portal of entry and inoculum volume has also been studied in squirrel monkeys (Table VIII). Aerosols of K. pneumoniae did not produce clinical disease at doses as high as 10^7 organisms, whereas the LD_{50} by IT challenge was ∿ 700 cells (in 1.5 ml). The response to IT inoculation also varied with the volume instilled, presumably because volumes larger than 1.5 ml inhibited host clearance mechanisms.

TABLE VIII. EFFECT OF DOSE AND INOCULUM VOLUME ON REACTION OF SQUIRREL MONKEYS TO INTRATRACHEAL OR AEROSOL CHALLENGE

DETERMINATION AND ROUTE OF CHALLENGE	DOSE (cells)	INOCULUM VOLUME (ml)	N	RESPONSE
Dose response				
Aerosol	2×10^2		2	None detectable
	2×10^3		2	
	3×10^4		2	
	2×10^5		2	
	3×10^6		4	
	1×10^7		6	
IT	3×10^2	0.5	2	Mild illness, 2-3 days
	3×10^4		2	Death, 30-48 hr
	3×10^6		2	Death, 30-48 hr
	3×10^8		2	Death, 30-48 hr
Effect of volume of inoculum				
IT	3×10^3	0.5	4	Mild illness, no deaths
	3×10^3	1.0	4	Death, MTD[a] = 40 hr
	3×10^3	1.5	4	Death, MTD = 40 hr
Dose response (1.5-ml volume)				
IT	7×10^1	1.5	4	Transient, mild illness
	7×10^2	1.5	4	2 dead; 2 ill, recovered
	7×10^3	1.5	4	2 dead, MTD = 40 hr

[a]MTD = Mean time to death

Finally, research has been initiated to develop a respiratory disease model for Legionnaires' disease organism. This work is being carried out in collaboration with Bacteriology Division. Preliminary experiments involve aerosol exposure of guinea pigs as well as aerosol and IT exposure of normal and immunosuppressed squirrel monkeys. In the event a model can be developed, it is anticipated that studies of immunization, chemoprophylaxis and therapy will be initiated.

Also scheduled for future investigation is a study of the efficacy of aerosol therapy of Coxiella burnetii infection in cynomolgus monkeys (in collaboration with LTC Kishimoto).

Presentation:

Powanda, M. C., G. L. Knutsen, and R. F. Berendt. Non-human primate model for respiratory Klebsiella pneumoniae. Presented, Am. Soc. Clinical Nutrition, San Francisco, CA, 28 Apr 1978 (Clin. Res. 26:403A, 1978).

Publications:

1. Berendt, R. F., and G. H. Scott. 1977. Evaluation of commercially prepared vaccines for experimentally induced type A/New Jersey/8/76 influenza virus infections in mice and squirrel monkeys. J. Infect. Dis. 136 (Suppl.):S712-S717.

2. Berendt, R. F. 1978. Relationship of method of administration to respiratory virulence of Klebsiella pneumoniae for mice and squirrel monkeys. Infect. Immun. 20:581-583.

3. Berendt, R. F., G. L. Knutsen, and M. C. Powanda. 1978. Nonhuman primate model for the study of infection induced by Klebsiella pneumoniae. Infect. Immun. 22:275-281.

LITERATURE CITED

1. U.S. Army Medical Research Institute of Infectious Diseases. 1 Oct 1977. Annual Progress Report, FY 1977, pp. 364-373. Fort Detrick, MD.

2. Berendt, R. F., G. G. Long and J. S. Walker. 1975. Treatment of respiratory Klebsiella pneumoniae infection in mice with aerosols of kanamycin. Antimicrob. Agents Chemother. 8:585-590.

3. Berendt, R. F., G. G. Long, F. B. Abeles, P. G. Canonico, M. R. Elwell and M. C. Powanda. 1977. Pathogenesis of respiratory Klebsiella pneumoniae infection in rats:bacteriological and histological findings and metabolic alterations. Infect. Immun. 15:586-593.

4. Julianelle, L. A. 1941. The pneumonia of Friedlander's bacillus. An. Intern. Med. 15:190-206.

5. Prokhorova, I. I. 1968. [Comparative study on efficacy of inhalation and subcutaneous administration of kanamycin in treatment and prophylaxis of experimental pneumonia of albino mice]. Antibiotiki 13:529-533.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6428	78 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
77 10 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3Mi62776A841	00	051
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code) (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	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		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING 78	1.0	180.0
	CURRENT 79	1.0	178.4

19. RESPONSIBLE DOD ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER

21. GENERAL USE
Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME: Shirey, F.
NAME:
POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Rickettsia; (U) Influenza; (U) Mycoplasma; (U) Ultrastructure; (U) Toxins; (U) Microscopy

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text 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) 77 10 - 78 09 - The vascular lesion of rickettsial spotted fever seen in hen egg chorioallantoic membranes is markedly similar to that seen in mammalian hosts. In addition, surface changes associated with release of rickettsiae from endothelial cells were comparable to those observed in infected cells in culture. Technical difficulties encountered in demonstrating initial ultrastructural changes involved in cellular uptake of rickettsia were described. These can be countered by using microcultures of cells on EM grids.

Publication: Am. J. Trop. Med. Hyg. 37:822-826, 1978.

*Available to contractors upon originator's approval

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

* U.S. GPO 1971-543-543 8

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 051: Analysis of Subcellular Structures in Microbial Infections of Potential BW Importance

Background:

Ultrastructural studies of infectious diseases are concerned with mechanisms by which the infectious microorganisms enter and leave cells and with the subsequent damage or changes in subcellular structure and function. Scanning electron microscopy (SEM) is used not only to obtain information about topography and surface changes on cells and organisms but also to determine spatial and orientational relationships between the microorganisms and cell surfaces. Within the limitations imposed by methodology and performance characteristics of the electron microscope (EM) we have shown that a large homomorphic virus, e.g., vesicular stomatitis virus, is easily recognized on infected cells by SEM while smaller viruses and pleomorphic viruses are difficult to identify without the use of specific markers. Swine influenza virus has been localized in respiratory tissues of experimentally infected mice using scanning immunoelectron microscopy. Larger organisms, amenable to study by SEM, such as rickettsiae have been examined. Host material is intimately associated with Rickettsia rickettsii, which is released from infected cells by focal dissolution of plasma membrane, disruption of cellular folds, and extension within cytoplasmic stalks.

Progress:

Within 3 days following IV inoculation of 12-day-old embryonated hen eggs with either Rickettsia conorii or Rickettsia sibirica, organisms were seen on both surfaces of the chorioallantoic membrane (CAM) and within endothelial cells of thin-walled vessels in the stroma. The presence of intracellular rickettsia did not appear deleterious to the ultrastructural integrity of endothelial cells; however, there was hypertrophy and vacuolization. Segmental lesions, consisting of heterophils and mononuclear cells, were established by transmural migration of these cells. The appearance of this lesion in the CAM was markedly similar to that seen in mammalian hosts. In addition, surface changes associated with the release of rickettsia from endothelial cells were comparable to those observed in infected cells in culture: namely, the formation of stalked appendages containing rickettsia and the emeocytotic release of rickettsia from the cells.

In an attempt specifically to study cell penetration of rickettsia, the CAM surface of embryonated eggs was inoculated using standard procedures and the membranes were harvested. Uninoculated eggs, with the CAM separated from the shell membrane in the same way, served as controls. Eggs were harvested as early as 1/2 and 1 hr: it was impossible to find cellular penetration by rickettsiae even though the organisms were seen on the

epithelial surface. The morphology of the "dropped-membrane" from inoculated and control eggs was compared with that of the membrane fixed either in situ after removal of the embryo or immediately after its removal and separation from the embryo. The "dropped-membrane" and the membrane thus fixed lost portions of the outer epithelium during mechanical manipulations. This exposed the contraluminal surface of some capillary endothelial cells. It would seem that this artifact in "dropped-membrane" would favor the experimental design and expose target cells to direct contact with the rickettsiae. In spite of this, there was no evidence of rickettsial interaction with these cells. This may be evidence showing that this cell surface is devoid of receptor sites, a possibility which cannot be excluded at this point. (A morphologic barrier does not exist on this surface because there is no basement membrane.)

Although we are able to discern similarities between the lesion in avian and mammalian hosts as well as morphologic correlates in rickettsial release from cells of these animal classes, it would be desirable to document the ultrastructural changes in mammalian endothelial cells. There are 2 possibilities: (a) cell cultures of endothelial cells or (b) isolated segments of blood vessels in situ or in organ culture. In this manner, small areas of susceptible tissue, i.e., endothelium, can be exposed to large numbers of rickettsia. We found that isolated segments of guinea pig aorta can be maintained for short periods of time (48 hr or less) in a culture medium with minimal change in the ultrastructural integrity of the endothelial lining. Ring-segments of aorta were aseptically removed from a guinea pig and placed in separate wells of a microtiter plate containing complete medium 199 and 10% fetal calf serum. Approximately 2×10^5 R. conorii were added and aorta segments were removed after 5 and 25 min at 37°C and fixed for EM preparation. Rickettsiae were seen by SEM on the endothelial surfaces from both time periods, and the endothelium appeared normal. By transmission EM (TEM), rickettsiae were seen on the luminal surface of endothelial cells at 5 and 25 min and within cells only at the later time. No intermediate stages in cell penetration have been found thus far.

In addition to the endothelial cells in segments of blood vessels, cells in culture provide a model system for examining ultrastructural changes in infectious processes. In order to obtain cell samples exposed to the largest numbers of organisms possible, a suspension cell culture was used. Irradiated L-929 cells were concentrated by centrifugation, resuspended in yolk sac seed of Rickettsia akari and sampled at intervals. The L-929 cell is a small cell which is profusely covered with microvilli. The irradiated cell culture contains many cells with small bizarre forms that cover the plasmalemma which in turn is convoluted with folds and distorted with blebs. As a result, it was concluded that irradiated L-929 cells were not suitable for studying the early stages of cell penetration and infection by R. akari. This experiment was modified and repeated using fetal rhesus lung cells (FRhL-2) in culture. A cell suspension, prepared from monolayer cultures, was concentrated and resuspended in yolk sac seed of R. akari to give a ratio of 10 rickettsiae

to each FRhL-2 cell. Suspensions were held at 4°C for 15 min after which time aliquots were placed at 37°C. Samples were fixed by the addition of 2% glutaraldehyde after 5 and 15 min of incubation at 37°C, and processed for TEM examination. It was learned subsequently that the FRhL-2 cells used in this experiment were from an old culture which died shortly afterwards. This was reflected in their morphology and fine structure; there were numerous dead cells and the majority of living cells contained numerous vacuoles filled with myelin figures and cellular inclusions. In spite of the large numbers of dead and altered cells, there were sufficient numbers of healthy appearing cells to determine that by 5 min at 37°C R. akari starts to enter the cell. This resembled early stages of phagocytosis in that filopodal cell processes were in contact with the rickettsiae. The rickettsiae were found inside the cell at 15 min. With one exception, intracellular rickettsiae were not within a membrane-bound vacuole but appeared to be free in the cytoplasm. One possible explanation is rickettsiae enter the cell surrounded by the plasmalemma, which is then quickly lost, leaving the organism free in the cytoplasm. These preliminary results are very encouraging and provide a valid basis for more intensive study of the very early stages in rickettsial penetration and cell pathology.

It is obvious that sampling is still the major handicap to ultrastructural studies of initial events in cell penetration. This has been pointed out by Ewing et al. (1), whose ultrastructural observations are in agreement with those presented here. It is apparent that no matter how large a ratio of microorganisms to cells is used, only a minute, randomly selected portion is eventually seen in the EM. The ultimate goal would include examination of each cell in the experiment; however, this is not only impractical but virtually impossible. The problem may become manageable by exposing small numbers of cells to large numbers of microorganisms in a microculture system which can be processed in toto through all stages of preparation and examination. We have attempted with some success to grow Vero cell cultures on EM grids prepared with a substrate film to cover the open portions of the mesh. The success was random and appeared to be correlated with the integrity of the plastic coating. There was no growth of cells on the grid or the immediate area surrounding the grid where bare metal was exposed to the culture medium and cells. This, in spite of the fact that the remainder of the plastic culture dish was covered confluently with cells. Similar results were obtained with WI-38 cells on nickel grids. As a result we tested grids of different metals as growth supports as well as conditions for seeding and attachment and other cell lines.

EM grids made of stainless steel, titanium, nickel, aluminum, or gold were tested for suitability with WI-38, FRhL-2, and Vero cell cultures. The grids were coated on one side with parlodion and carbon, sterilized in chloroform, and placed in plastic plates containing culture medium. A suitable concentration of monodispersed cells was added dropwise and the cultures placed at 37°C in a CO_2 incubator. Under these conditions only the gold grids were satisfactory for preparing minicultures. Formvar could be used instead of parlodion for the plastic support over the grid mesh without any noticeable difference. Selection between these 2 substances will depend upon whether solvent resistance or tensile strength is more important. In addition to

determining the appropriate seeding concentrations of WI-38 and FRhL-2 cells needed to obtain usable monolayers, it was found that these monolayers could be produced more consistently when the plastic and carbon-coated gold grids were conditioned in fetal calf serum or a solution of either poly-L-lysine or collagen. Although the latter substances were more efficacious than serum, only collagen can be used if the cultures are to be exposed to microorganisms or other substance with acidic residues. Poly-L-lysine, by virtue of charge interaction, binds to acidic residues and its presence promotes unwanted binding to materials to surfaces other than the cells.

These studies have been possible through the collaboration of the following individuals: MAJ W. C. Hall, R. conorii and R. sibirica; Dr. J. Osterman, R. akari; Mr. D. Leatherman, grid cultures.

Presentations:

1. White, J. D. SEM in study of infectious diseases. Presented, University of Maryland Dental School, Baltimore, MD, Feb 78.

2. White, J. D. TEM and SEM in research applications. Presented, Dept. of Biology, Western Maryland University, Westminster, MD, Apr 78.

LITERATURE CITED

Ewing, E. P., Jr., A. Takeuchi, A. Shirai, and J. V. Osterman. 1978. Experimental infection of mouse peritoneal mesothelium with scrub typhus rickettsiae: An ultrastructural study. Infect. Immun. 19:1068-1075.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0F6423	78 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
78 04 21	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	62776A	3M162776A841	00	052
b. CONTRIBUTING				
c. //////////	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code) (U) Therapeutic manipulation of metabolo-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	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	0.4	175.0
FISCAL YEAR CURRENT 79	1.0	118.9

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 (Precede EACH 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 (Furnish individual paragraphs identified by number. Precede text of each 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 undesirable 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 effect of bacterial and viral infection on insulin production and release will be measured as will the effect of manipulations of pH, bacterial and viral toxins, trace metal concentration and hormones, all of which undergo change in infection. In addition, the effect of these parameters on the binding of insulin to its receptors and thus its physiologic action will also be characterized.

25 (U) 77 10 - 78 09 - Laboratory procedures including preparation of the 125-I-insulin tracer, insulin receptor assay, IM-9 cell culture and isolated islets of Langerhans preparation have all been successfully established. Preliminary studies have demonstrated alterations in insulin binding (and thus insulin effect) in conditions in which there are changes in pH, trace metals and alterations of hormone levels occurring during infection. In addition, preliminary studies have demonstrated that infection with bacterial organisms produces an increase in insulin-receptor binding. Initial studies on the effect of VEE (TC-83) virus indicates an inability to infect the isolated islet in vitro.

*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 MAR 68, FOR ARMY USE ARE OBSOLETE

* U.S. GPO 1974-540-843/8691

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 052: Endocrine-Metabolic Controlled Responses to Infection of Unique Military Importance

Background:

Previous observations have noted an unexplained relative hyperinsulinism during infection or experimentally induced endotoxemia. During infection the liver seems unable to produce ketones, which may be a reflection of this hyperinsulinism. A vital area of concern in the clinical management of soldiers who might be BW victims, or who develop wound-related infections, is the supply of energy substrates during periods of high fever-induced metabolic needs and a reduction in the catabolic effects that prolong recovery from illness.

Insulin is the principal hormone involved in energy supply and reversal of catabolism. Previous investigations dealing with insulin response in, and effect on, infectious diseases have been confined to measurements of insulin production, circulating concentrations and target organ effects. With the radioreceptor assay we now have the capability to explore binding of insulin to its cellular receptor (necessary for the action of insulin) and thus can evaluate many facets of insulin pathophysiology during infection-associated events, e.g., changes in various proteins, pH, trace-metal concentration and other hormone systems.

In the in vivo animal, the beta cell, the major component of the islets of Langerhans, is subject to the changing concentrations of stimulants and inhibitors in the circulation as well as neurogenic effects on the endocrine pancreas. With the introduction into the laboratory of the technique for isolating islets of Langerhans, we now have available a tool for directly measuring the effects of specific hormones, chemicals, toxins and/or microorganisms on the beta cell itself without unmeasurable interference from other substances. This gives us the ability to evaluate the effect of microorganisms on the synthesis and release of insulin as well as their effects on circulating insulin and insulin binding.

Progress:

1. Insulin Receptor Assay Techniques. Refinement of the assay procedure for measuring insulin receptors and binding has continued. Improvements have been made in the preparation of the [^{125}I] insulin tracer which is important in that, unlike the standard RIA, the tracer must be: (a) monoiodinated; (b) of high specific activity (200 μCi/μg); and (c) completely active biologically. This requires a stoichiometric micro-iodination technique which has proven to be more sensitive to minor trace containments and alterations in reaction conditions than the classic methods. The IM-9 cell line of lymphoblastoid cells has been

established and further characterized with respect to its insulin-binding properties as well as its suceptibility to various bacteriologic and viral infections.

Because of the requirement for large numbers of mononuclear cells and the adaptation of the assay procedure to small laboratory animals instead of human beings, considerable work has been devoted to attempting to increase the recovery of mononuclear cells from blood taken from small animals as well as to improve the sensitivity of the assay procedures to allow smaller numbers of cells to be utilized. In addition, studies are underway to adapt our procedures to using isolated adipocytes as well as the cellular membranes from such tissues as liver and muscle. With these modifications it will be possible to study the effect of infection in a single animal, a technique which will provide a higher degree of statistical significance.

In addition, because of recent advances in insulin-receptor assay techniques our laboratory has become interested in attempts to measure receptor binding on circulating erythrocytes. Thus far our attempts have not reproduced results reported in the literature. Use of n-butyl-phthalate to isolate RBC from the incubation media of the assay has been adopted for use in our other assays which utilize mononuclear cells.

A collaborative effort with Captain Little (Bacteriology Division) has resulted in the establishment in our laboratory of an assay procedure utilizing hepatic membranes. This study is vitally important because, as noted in innumerable metabolic studies, the liver may have a responsiveness which is not characteristic of other tissues. Utilization of a hepatic-membrane insulin-receptor assay will enable us to measure changes in various body tissues, thus possibly establishing direct evidence for differences which might explain altered metabolic events which occur in the liver during infection.

A major drawback to utilization of hepatic plasma membranes has been the fact that viable hepatic membranes have a tremendous potential for the degradation of insulin, leading to significant artifacts in receptor-binding studies. In an attempt to modify the assay procedure to eliminate the artifact, degradation was determined by paper chromotography, trichloroacetic acid precipitation, and double antibody precipitation techniques. As predicted, the inverse correlation seen between the curves of binding as a function of temperature and degradation as a function of temperature is significant with a sharp rise in degradation noted at 37° C, the temperature at which the membranes normally function in the liver's role. Studies are now underway measuring specific differences between infected and control animals.

An assay has been developed which demonstrates the presence of insulin receptors on cultured mouse macrophages supplied in a collaborative effort with Major Peters (Virology Division). Studies are now being designed in which changes in insulin receptors will be compared to those of viral receptors to determine if such changes are specific or nonspecific.

As a benefit of the requirement for this laboratory to prepare ^{125}I-labeled insulin tracer, we have been able to supply the RIA laboratory with sufficient tracer for all of the insulin assay procedures utilized.

At the present time because of the complex interrelationship between insulin and glucagon metabolism in infected animals, this laboratory is working on modifications to allow radiolabeling of glucagon and measurements of its receptors.

2. Insulin Receptor Studies. Initial studies done during the first part of the year in establishing the insulin-receptor assay evaluated the effects on insulin binding of several trace metals. Neither physiologic nor supraphysiologic concentrations of Zn or Co produced significant alterations in insulin binding. Cu at supraphysiologic concentration (100 mg/100 ml) produced a significant decrease in binding which was attributable primarily to a decrease in receptor affinity but not receptor number. Additional investigations failed to demonstrate any significant change in insulin-receptor binding utilizing ranges of Cu concentrations that might be expected in vivo. LiCl, which has a significant inhibitory effect on insulin secretion, failed to produce any significant change in insulin receptor number or affinity in the cultured IM-9 cells. Although there was a significant difference in glucose tolerance in animals given chronic oral Li, there was no measurable difference between control and Li-treated animals with respect to insulin receptor number or affinity utilizing the peripheral rat monocytes.

D_2O, which is known to stabilize microtubular structures in the cell and thus inhibit secretion of insulin from the beta cell, was studied for its effect on insulin binding in cultured lymphoblastoid cells. Utilizing a 50% concentration of D_2O in the growth media of the cells, a slight increase in binding was demonstrated.

Utilizing cultured IM-9 cells, 2 chemicals known to effect binding of diphtheria toxin to its receptors were investigated in conjunction with Dr. Middlebrook (Pathology Division). Ruthenium red, known to inhibit the uptake of Ca- and Mg-dependent triphosphatases, has been shown to inhibit insulin-dependent lipogenesis in fat cells and was therefore investigated to determine if it would inhibit insulin binding: no significant inhibition of binding of insulin to insulin receptors occurred at concentrations ranging from 0.1 M to 1 M. Adenosine-5'-tetraphosphate, an analogue of ATP, was investigated to determine if its ability to inhibit binding of diphtheria toxin was specific or whether it inhibited binding of other ligands also. It did not significantly alter insulin receptor binding.

Since the effect of hypophyseal hormones on insulin and glucagon physiology during infection is not well characterized, an initial experiment has been conducted utilizing hypophysectomized rats to investigate insulin binding on mononuclear cells of infected animals compared to noninfected. Data from the first experiment did not demonstrate a significant difference in binding; however, data to determine the degree of infection of the rats is still being analyzed.

Leucine and methionine enkephalin, 2 synthetic pentopeptide, opiate-like substances, were investigated for their effect on insulin receptor binding in IM-9 cells. Enkephalins are derived naturally from the breakdown of endogenous endorphins, which are believed to be synthesized in the hypothalamus. They have been shown to increase insulin secretion from the pancreas, when injected into animals in pharmacological doses. Experiments indicate a possible inhibition of insulin binding. More detailed experiments are now being conducted. Because endogenous endorphins might contribute to the increases of insulin during stress conditions such as infection, endorphins or enkephalins as well as the agents which block them may play a significant role in studies of increases in circulating insulin during infection.

The effect of biologic agents, such as endotoxins, has also been studied in relation to insulin-receptor number and affinity. Two representative endotoxins and one exotoxin were assayed. There was no significant variation in insulin binding seen in cells incubated 18 hr with 1 or 5 mg/100 ml concentrations of endotoxin B or 2 mg/100 ml concentrations of endotoxin W, both toxins derived from Escherichia coli. The effect of an 18-hr incubation of a 10 mg/100 ml concentration of diptheria exotoxin was also not significant. Binding studies utilizing leukocytic endogenous mediator (LEM) at various concentrations also demonstrated no significant binding alterations.

The most rewarding work in insulin receptors studies has been the effect of bacterial infection on insulin binding in animals. Using Streptococcus pneumoniae as a representative bacterial organism, groups of male Wistar rats were injected with live or heat-killed organisms or saline and sacrificed at 24 or 48 hr. The infected groups had demonstrable significant increases in insulin-receptor binding compared to heat-killed and saline controls. Current studies are underway to establish whether this increased binding is due to an increase in receptor number or affinity or a combination of both. It is important to point out that in all physiological systems thus far studied, chronic increases in peripheral levels of insulin have resulted in decreased receptor number (and thus decreased binding), so that, in fact, infection seemed to produce a paradoxical effect.

This increase in insulin to binding receptors appears to be unrelated to circulatory factors. Incubation of cultured lymphoblastoid cells in media containing 36% plasma from infected, fasted rats did not produce a change in insulin binding from that seen with cells incubated in plasma from rats injected with saline or heat-killed organisms.

3. Insulin Secretion and Glucose Tolerance. Because of the importance of determining the physiologic benefits of increased insulin secretion during infection, it has become vitally important to determine controlling mechanisms of insulin secretion which might be strictly limited to beta cell function in the islets of Langerhans and not affect other physiologic hormone responses during infection. One of the most promising of these substances appears to be LiCl, which has previously

been shown to inhibit release of insulin from isolated pancreatic islets of Langerhans. Initial studies of glucose tolerance in rats given chronic LiCl were completed using intragastric administration of glucose as a challenge. Glucose values in Li-treated rats revealed a delayed peak value which rose to levels comparable to controls. The insulin values of treated rats reflected depressed basal values with no obvious response to glucose stimulus. Because of the suspected effect of Li on gut absorption of glucose as a cause of the decreased insulin response, studies were then conducted utilizing IP glucose challenge. Animals were given LiCl orally over a period of 10 days and then challenged with glucose in a concentration of 2 gm/kg body weight; glucose responses were identical. Of importance, however, the insulin values in the Li-treated rats were significantly lower. Because of the fact that the treated rats did not gain weight at the same rate as the control rats, these experiments are being repeated utilizing controlled, force-feeding diets so that weight gain in the treated and control animals will be comparable, eliminating any possible artifactual decrease in responsiveness. In addition, studies are currently underway to determine possible receptor changes that might account for the demonstrated lack of glucose intolerance despite impaired insulin release.

In vivo testing in rats of the effects of systemic acidosis on insulin utilization over a wide range of plasma pH produced by intragastric administration of NH_4Cl demonstrated that relatively mild decreases in pH resulted in diminished insulin/glucose ratios, but once pH values were significantly lower than physiologic (~7.2) the normal glucose insulin relationships were no longer proportional due to the interaction of other multiple mechanisms involved in homeostasis.

4. Isolated Islets of Langerhans. Studies of viral effects on isolated islets of Langerhans have proceeded in 3 directions.

The first study attempted to demonstrate viral replication in isolated islets of Langerhans. Growth curves of VEE and Pichinde (PIC) virus have been monitored in isolated islets of guinea pigs, hamsters and rats. VEE virus added to incubating isolated islets and then washed from the media did not demonstrate evidence of replication during an 18-hr incubation period. Growth curves for PIC virus are being analyzed. Studies are also underway in which relatively large numbers of islets, 500 - 5,000/well are being exposed to specifically labeled VEE vaccine strain (TC-83) virus to determine the amount of virus that enters the beta cells. Initial studies indicate that virus does enter the beta cell but does not replicate.

The second approach has been the measurement of glucose-stimulated insulin secretory ability of islets isolated from control and TC-83-infected guinea pigs. Animals have been studied both during the acute infection and at intervals of convalescence up to one month. Since the virus had been adapted to use in guinea pigs, no results are available on insulin secretion from guinea pig islets, since there is no commercially available anti-insulin antibody which will react with guinea pig insulin.

Our laboratory has been forced to attempt production of anti-insulin antibody in rabbits in order to measure the insulin levels. Rabbits have been immunized and tests are currently underway to determine antibody titer levels and set up an appropriate assay to measure the insulin secretion of these isolated guinea pig islets.

The third phase is concerned with recovery of viral material in islets from animals infected prior to sacrifice. This study utilizing immunofluorescent antibody tagging will differentiate between in vitro viral growth and actual viral infection demonstrating beta cell tropism.

Because of the hyperinsulinism seen with bacterial infection in rats we investigated the secretory response of islets isolated from animals infected with S. pneumoniae compared to noninfected litter mates. Using glucose challenge there was no significant difference between the 2 groups.

In an associated study, the effect of trace-metal changes on insulin secretion from isolated rat islets was evaluated. Changes in trace metals comparable to these seen during infection did not produce significant alterations in insulin response to glucose challenge. Supraphysiologic concentrations of Zn did inhibit insulin release but the physiologic implications of this observation are still being evaluated.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0F6418	78 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. DISS'N INSTR'N	8b. SPECIFIC DATA: CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	053
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with 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	CONT	DA	C. In-house

17. CONTRACT GRANT

a. DATES/EFFECTIVE: EXPIRATION:
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e. KIND OF AWARD:
f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	115.0
FISCAL YEAR CURRENT 79	1.0	118.9

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21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with 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 (Furnish individual paragraphs identified by number. Precede text of 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) 77 10 - 78 09 - Attempts were made to separate the protective antigen from suspensions of Rickettsia rickettsii or from soluble antigen obtained by ether extraction of these suspensions. Vaccines prepared from soluble antigen as well as ether extracted organisms protected guinea pigs from challenge with R. rickettsii. The protective factor was partially precipitated by 20% sodium sulfite and completely precipitated by tricholoroacetic acid. Phenol extraction destroyed the factor.

Three guinea pigs and a monkey were inoculated with Rickettsia montana, a spotted fever group rickettsia not known to cause disease in man. None of the animals developed observable illness. After challenge with virulent R. rickettsii, the monkey developed a mild illness lasting several days, but recovered promptly. Two guinea pigs succumbed to the challenge, but the third was completely refractory.
Publication: J. Clin. Microbiol. 7:389-391, 1978.

*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

* U.S. GPO: 1974-540-843/8691

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 053: Characterization of Nonindigenous Tick-borne Rickettsiae for Vaccine Development

Background:

For the preparation of a vaccine against any infectious agent, the most important component to be included is that element of the organism that elicits a protective response in man or animals. For spotted fever rickettsiae, this component is present in purified organisms and in the aqueous phase of ether-extracted rickettsiae, but does not appear to be associated with any antigens involved in serological tests for the agent. Little is known of the chemical or physical nature of this antigen, but studies with Rickettsia prowazekii (1, 2) suggested that it is a carbohydrate-protein combination that resides in the slime layer surrounding the organism or is in the cell wall. To study the nature of this antigen in spotted fever rickettsiae, an investigation was begun in which Rickettsia rickettsii was grown in quantity and fractionated by various physical or chemical procedures. Each separated component was injected into guinea pigs to determine its protective capacity by challenging the animals with virulent R. rickettsii.

A number of species of the spotted fever group of rickettsiae are known to be avirulent for guinea pigs or to cause only mild disease. Several of these organisms, Rickettsia parkeri, Rickettsia montana and Western Montana U, have not been known to cause disease in man (3). These rickettsiae are isolated from ticks or small rodents, and grow well in embryonated eggs and tissue culture. Vaccines prepared from these organisms produce a protective response in guinea pigs against challenge with R. rickettsii, although the response produced by R. montana has been described as erratic. For these reasons, these organisms could all be considered as candidates for a live spotted fever vaccine for man. In conjunction with CPT Gonder (Rickettsiology Division), a preliminary study was conducted to determine whether inoculation of a monkey and guinea pigs with live R. montana would cause a clinically observable disease in the monkey and protect both animal species from subsequent challenge with R. rickettsii.

Progress:

The experimental procedure used to test for the presence of a protective antigen in separated fractions of R. rickettsii is contained in the following outline. The R strain of R. rickettsii was grown in roller bottles in chick embryo fibroblast cells. After infection and incubation

of the cells, growth media was drained from the bottles and replaced with 10 ml of sucrose-phosphate-glutamate solution. This fluid was shell-frozen in the bottles, then thawed to disrupt the cells and free the rickettsia. Fluid from 20 roller bottles was pooled and centrifuged at 750 x g for 20 min to sediment the cellular debris; the supernatant was used as the starting material for fractionation. After suitable treatment, the separated fractions were dialyzed against distilled water and concentrated to about 2-3 ml by pervaporation. The concentrated samples were mixed with an equal volume of incomplete Freund's adjuvant and injected SC into guinea pigs. The vaccinated animals were bled 28 days later, then challenged IP with 10^4 PFU of virulent R. rickettsii. Febrile responses and other clinical and serological signs of infection were used to determine whether the animals were protected.

Two preparations were used for these studies, the first was the whole rickettsial suspension. The second was obtained from the whole rickettsial suspension by extraction with 2 vol of diethyl ether containing 10% methanol, separating the 2 phases and re-extracting the aqueous layer with an equal volume of ether. The aqueous layer was then heated to 40°C in a strong air flow to remove the dissolved ether. The suspension was centriguted at 15,000 x g for 40 min to sediment the rickettsial bodies; the supernatant, referred to as the soluble antigen, was used for further fractionation.

In the initial experiments, soluble antigen preparations and rickettsiae sedimented from them were dialyzed, pervaporated and injected into guinea pigs. Both preparations effectively immunized the animals against a virulent challenge with R. rickettsii (Table I).

The whole rickettsial suspensions from the roller bottles were pooled, the volume measured and enough sodium sulfite added to obtain a 20% solution. After standing overnight at 4°C a flocculent precipitate formed which was readily sedimented at 750 x g for 15 min. The supernatant and the sediment were treated as indicated above and injected into guinea pigs. As Table I shows, both preparations immunized the animals against challenge.

TABLE I. SEROLOGICAL AND CHALLENGE RESPONSES OF GUINEA PIGS FOLLOWING INJECTION OF FRACTIONS OF CHEMICALLY TREATED WHOLE RICKETTSIAL *SUSPENSIONS OR SOLUBLE RICKETTSIAL ANTIGEN*

TREATMENT	FRACTION	MA TITER[a]	FEVER (days)[b]
Ether[c]	Supernatant	8	0
	Sediment	32	0
Ether[d]	Supernatant	16	0
	Sediment	16	0
Sodium sulfite[c]	Supernatant	16	0
	Sediment	32	0
Sodium sulfite[d]	Supernatant	<8	0
	Sediment	8	0
Trichloroacetic Acid[c]	Supernatant	<8	4
	Sediment	16	0
Trichloroacetic Acid[d]	Supernatant	<8	7
	Sediment	8	0
Phenol[c]	Precipitate	32	7

[a]Reciprocal microagglutination titers of prechallenged guinea pigs.

[b]After challenge with 10^4 PFU of the R strain of R. rickettsii.

[c]Whole rickettsial suspension.

[d]Soluble rickettsial antigen.

A similar experiment was conducted using the soluble rickettsial antigen. The precipitate obtained was not as copious as with the whole rickettsial suspension, but the results obtained from immunizing guinea pigs were the same. As Table I indicated, the protective factor was found in both the supernanatant and the sediment.

Starting with the whole rickettsial suspension, enough trichloroacetic acid was added to make an 8% solution, and the mixture kept overnight at 4°C with stirring. A precipitate formed which was readily sedimented at 1000 x g in 15 min. The supernatant was removed and dialyzed against distilled water; the sediment resuspended in water and also dialyzed. Both samples were pervaporated to 2-3 ml, mixed with adjuvant and inoculated into guinea pigs. When the guinea pigs were challenged, the protective factor was found in the sediment, no activity was left in the supernatant.

A sample of the whole rickettsial suspension was subjected to a cold phenol extraction. Equal volumes of suspension and 83% phenol were mixed and shaken vigorously for 5 min at 24°C. The mixture was centrifuged at 1000 x g for 10 min; the upper aqueous layer was removed and its volume measured. One third of this volume of 8% potassium acetate was added to the aqueous phase along with 2 times the total volume of cold (-10°C) absolute ethanol. The mixture was placed at -10°C overnight; a gelatinous precipitate formed. Centrifugation at 1000 x g for 15 min sedimented the precipitate; the supernatant was removed and discarded. The precipitate was washed once with cold absolute ethanol and dissolved in 3 ml of saline. This solution was mixed with 3 ml of Freund's incomplete adjuvant and injected SC into a guinea pig. The animal was bled 28 days later and challenged with virulent R. rickettsii. Results of this experiment are shown in Table I, no activity was found in this phenol-extracted material.

A male cynomolgus monkey weighing 3.5 kg was inoculated IV with 1.3×10^4 egg LD_{50} of R. montana. No febrile response or other clinical or hematologic signs of illness were noted during the following 3 weeks. Four weeks after vaccination, the monkey was challenged SC with 2×10^4 egg LD_{50} of the virulent R strain of R. rickettsii. It became slightly anorectic on day 4 and remained somewhat ill for several days. It showed a low grade fever on days 4 and 5. On day 6, its total leukocyte count was slightly elevated, but returned to normal on day 9. No other signs of illness were observed and recovery was quick and uneventful.

Blood samples were obtained from the monkey during the course of both infections, and MA tests were conducted on the sera using an antigen prepared from R. rickettsii. Results of these tests are shown in Table II.

TABLE II. SEROLOGICAL RESPONSES OF A MONKEY AND 3 GUINEA PIGS TO VACCINATION WITH LIVE R. MONTANA AND CHALLENGE ON DAY 28 WITH R. RICKETTSII

ANIMAL	DAYS AFTER VACCINATION	MA TITER	CHALLENGED	DIED
Monkey	-5	2		
	7	64		
	21	64		
	28		+	-
	35	64		
Guinea Pig #1	28	16	+	+
Guinea Pig #2	28	32	+	+
Guinea Pig #3	28	16	+	-
	45	16		

Three guinea pigs were also vaccinated with R. montana using the same dose given the monkey. No febrile responses or other indications of illness were observed in these animals during a 2-week observation period. Four weeks after infection, the guinea pigs were bled by cardiac puncture and challenged with 2×10^4 egg LD_{50} of the virulent R strain of R. rickettsii. Two guinea pigs, #1 and #2, succumbed to the infection. They had 3 and 5 days of fever, respectively, and died on days 7 and 8. Guinea pig #3, however, was completely refractory to challenge, showing no febrile response or other signs of illness. Before challenge all 3 guinea pigs had low MA titers against R. rickettsii antigen and appeared to be in good health.

As monkeys become available, further studies will be conducted with R. montana, R. parkeri and if a culture can be obtained, with strains of Western Montana U.

Publication:

Johnson, J. W., and C. E. Pedersen, Jr. 1978. Plaque formation by strains of spotted fever rickettsiae in monolayer cultures of various cell types. J. Clin. Microbiol. 7:389-391.

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1. Plotz, H. 1948. The soluble substances of the rickettsiae, pp. 198-202. In Rickettsial Diseases of Man (F. R. Moulton ed.). American Association for the Advancement of Science, Washington.

2. Reiss-Gutfreund, R. J., P. Cappucinelli and G. Cavallo. 1975. The soluble antigens of Rickettsia prowazekii, R. typhi and R. canada. Investigation of their inter-relationship by various serological methods. Z. Immunitaets Forsch. 148:315-329.

3. Lackman, D. B., E. J. Bell, H. G. Stoenner, and E. G. Pickens. 1965. The Rocky Mountain spotted fever group of rickettsias. Health Lab Sci. 2:135-141.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6422	78 10 01	DD-DR&E-RDA(A)

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
77 10 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	054
b. CONTRIBUTING				
c. //////////	STOG 78-7.2.1; 3, 6			

11. TITLE (Precede with Security Classification Code): (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	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	155.0
FISCAL YEAR CURRENT 79	1.0	127.7

19. RESPONSIBLE DOD ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS
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21. GENERAL USE: Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Congo-Crimean hemorrhagic fever; (U) Korean hemorrhagic fever; (U) Fluorescent antigen; (U) Vaccines

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

23 (U) Congo-Crimean hemorrhagic fever (C-CHF) and Korean hemorrhagic fever (KHF) are 2 distinct groups of diseases widespread throughout militarily important areas of the world. Both are associated with significant morbidity and mortality in affected areas and with man's intrusion into previously undisturbed environments. Both diseases are of unique military concern, and in fact in the early 1950s KHF was only associated with military field-deployed personnel. Vaccine prophylaxis for these diseases is therefore necessarily a high priority goal. The objective is to develop the basic knowledge required to assess the potential for developing vaccines to protect against the respective diseases.

24 (U) Recently developed plaque assay systems for C-CHF viruses will be utilized to assess the growth of the viruses in vaccine-certifiable cells. High titered stock preparations of KHF will be inoculated into normal or altered laboratory animals in an attempt to reproduce disease observed in man. KHF virus will be characterized by various physiochemical means utilizing the A-549 cell assay system.

25 (U) 77 10 - 78 09 - Plaque assay systems and fluorescent antibody spot-slide technology have been established for Hazara and Congo viruses. Immunologic comparison of the 2 viruses by FAT, PRNT and cross-protection tests in mice indicate the 2 viruses are closely related and the greater in vitro growth potential and natural avirulence of Hazara virus might make it a suitable C-CHF group vaccine. An in vitro assay system for KHF virus has been established in A-549 culture. KHF induces a noncytolytic persistent infection that does not inhibit A-549 cell division. These findings open up a whole new era in the study of KHF and should make possible characterization of the virus in the near future.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 054: Characterization and Evaluation of Selected Hemorrhagic Fever Agents for Vaccine Development

Background:

Part I. Congo-Crimean hemorrhagic fever (C-CHF).

As indicated in last years annual report, work with this group of viruses has been oriented toward familiarization and characterization of 2 avirulent members of the group, Hazara (HAZ) virus and Congo (CON) virus strain IbAr 10,200. The majority of the data reported here was derived from the 11th suckling mouse brain (SMB) passage of HAZ virus (HAZ-SMB P-11) and the 11th SMB passage of CON virus (CON-SMB P-11) or the 1st Vero passage from CON-SMB P-11. The objectives for this years work were: (a) characterize these stock strains in mice; (b) develop a reliable plaque assay system for both viruses; (c) develop a fluorescent antibody test (FAT) for both viruses and produce a limited number of spot slides for routine serologic diagnosis; and (d) compare the 2 viruses for antigenic and immunologic relatedness. In addition, a limited number of growth curve evaluations were performed in certifiable cell lines.

Part II. Korean hemorrhagic fever (KHF).

In the previous annual report we indicated that a laboratory colonized rodent, Calomys callosus, was an adequate substitute for the natural rodent host of KHF, Apodemus agrarius coreae (1). Although sufficient numbers of Calomys were not available for routine studies such as virus titrations, there were adequate numbers to provide infectious material for attempts to develop an in vitro assay system in cell cultures and continue the efforts to find a laboratory animal model of disease. Accordingly then, our objectives for the year were: (a) develop an in vitro assay system for the agent of KHF; (b) begin the characterization of the agent by electron microscopy, biochemical and physical means, and (c) continue to survey laboratory rodents and subhuman primates in search of an animal model of disease.

Progress:

Part I. C-CHF.

Characterization of stock strains in mice. HAZ-SMB P-11 and CON-SMB P-11, the 3rd SMB passage in this laboratory of seeds obtained from Dr. Jordi Casals were titrated for IC lethality and infectivity in both suckling (SM) and weanling (WM) mice. Infectivity was determined by IC back-challenge

28 days later. Survivors with 100-1000 $ICLD_{50}$ were considered to have undergone an immunizing infection as a result of the original nonlethal but infectious inoculation. Results are shown in Table I.

TABLE I. MOUSE IC LETHAL AND INFECTIVE TITERS OF C-CHF STOCK VIRUSES

	HAZ SMB P-11		CON-SMB P-11	
	Titer (Log_{10}/ml)	AST[a] (days)	Titer (Log_{10}/ml)	AST[a] (days)
$SMLD_{50}$	7.4	5.7	6.8	6.6
$SMID_{50}$	7.4	-	ND	
$WMLD_{50}$	4.5	5.4	3.1	7.9
$WMID_{50}$	6.0	-	ND	

[a]Average survival time.

As reported previously the average survival time (AST) of HAZ virus-lethally infected weanling mice is slightly shorter than suckling mice. The same is not true for CON virus-infected mice in that the AST is 1.3 days longer in weanling mice. Infective but not lethal titers for CON virus in weanling or adult mice could not be determined because of inadequate lethal titers of the than available CON stocks for back-challenge. Further passages of either virus in suckling mice, variations in dose inoculated, or time of harvest failed to yield higher titered SMB preparations. Neither CON virus or HAZ virus is lethal for other than suckling mice when inoculated by peripheral routes. Both viruses are, however, infectious for older mice by peripheral routes of inoculation. In mice 7 days of age or younger the infective titer of most preparations inoculated by peripheral routes is equivalent to that determined by the IC route of inoculation.

It was of interest to determine at what age suckling mice inoculated IM with these viruses survive infection so that cross-protection studies could be performed. To accomplish this, groups of 20 mice of increasing age were inoculated IM with 1000 $SMICLD_{50}$ of either HAZ-SMB P-11 or CON-SMB P-11. The results indicated that suckling mice 4-6 days of age or younger inoculated IM with HAZ virus died. By the time mice were 8-10 days of age however, 90% of HAZ virus-infected mice survived and by 14 days of age all HAZ virus-inoculated mice remained healthy. CON virus on the other hand did not kill all newborns, and by 4-6 days of age only 50% succumbed to infection.

Development of plaque assay systems for HAZ and CON IbAR 10200 viruses. HAZ virus can be plaqued satisfactorily and to equivalent titer on BS-C-1, Vero, BHK-21 and A-549 cells. The most reliable system at the present time is on A-549 cells, although even these cells are not as sensitive as IC titrations in suckling mice. The latter normally yield $ICLD_{50}$ titers 6-10 fold higher than PFU titers determined by plaque titration. Clear plaques (2mm) are produced on A-549 cells 7 days postinoculation. Plaque titrations are performed in 6-well plates overlayed with 0.6% agarose in M-199 with 5% fetal calf serum (FCS). Neutral red is applied in a 2nd overlay at 6 days; plaques are scored 24 hr later.

CON virus, which previously has never plaqued satisfactorily on any cell line, has recently been found to plaque reproducibly on SW-13 cells. The plaque assay system is similar to that described for HAZ virus except that the 2nd overlay containing neutral red can be applied on day 3 and plaques can be counted on day 4. Unlike HAZ virus, plaque titration of CON virus yields virus titers equivalent to those determined by IC inoculation of suckling mice. Unfortunately, HAZ virus on SW-13 cells produces only pinpoint plaques which are difficult to count; similarly CON plaques are pinpoint on A-549 cells. Thus, the same cell line cannot be used for assay of both viruses.

Virus replication in various cell lines. HAZ virus and CON virus have different replication capacities and different spectra of cell lines for optimal growth. Neither virus grows well in certifiable cell types tested to date which include CEF, WI-38 and FRhL cells. HAZ virus, at low MOI, yields 5×10^7 PFU/ml on BHK-21 cells at 72 hr; however, CON virus on BHK-21 cells yields only 1% of this at its peak 24 hr later. The best yields to date of CON virus occur at 5 days, again at low MOI, on Vero cells at which time 5×10^6 PFU/ml have been achieved. In contrast, HAZ virus yield on Vero cells is only 1% of that on BHK-21 cells. Congo virus does not produce detectable levels of infective virus on CEF but low yields in the order of 10^5 PFU/ml can be achieved on WI-38 and FRhL cells. Other certifiable cell lines will be tested for their abilities to allow replication of both viruses. Virus replication, at least in the case of HAZ virus on BHK-21 cells is directly proportional to the concentration of FCS in the cell culture medium. In two separate experiments with nearly identical results, maximal HAZ virus yields varied from 1.1×10^6 per ml at 1% FCS to 3.2×10^7 PFU/ml at 10% FCS. Other serum concentrations tested for which yields were proportional included 2, 4 and 7% FCS.

Fluorescent antibody assays for C-CHF Viruses. Acetone-fixed 10-spot slides of satisfactory quality have been prepared for both HAZ virus and CON virus strain IbAr 10200. Optimal fluorescence in the indirect assay procedure is achieved for HAZ virus on BS-C-1 cells at 3 days with in imput of 3000 PFU/T-75 flask of cells. CON virus spot slides are prepared with Vero cells 5 days postinoculation with the same input multiplicity. The brightest and most distinct fluorescence occurs with both viruses at a time when too high a proportion (>50%) of the cells are fluorescent. To

Circumvent this problem infected cells are mixed with noninfected cells in a 1:1 ratio immediately before fixation. Two hundred 10-spot slides for each virus have been prepared, safety tested and turned over to the Rapid Diagnosis group for routine use in their program.

Studies on physical characteristics of CON and HAZ viruses.

Millipore filtration of HAZ virus. Filtration experiments with HAZ virus were conducted to determine what loss we could expect with these viruses and to gain perspective as to what percent of the viral population was aggregated. Millipore membranes of 0.45, 0.22, 0.1 and 0.05 μ average porosities were prepared in the usual manner with membrane-coating substance to reduce nonspecific virus adsorption to the filter. A HAZ-SMB P-11 virus was diluted 1:10 and passed successively through the membranes of decreasing porosity. A small proportion of the filtrate of each membrane was removed and frozen for later assay. The results shown in Table II indicate that a large proportion of this virus exists as aggregates. It is interesting to note, however, that 0.2% of the measurable virus passed the 0.1-μ filter. The bunyaviruses, which include the C-CHF viruses, reportedly average 95 nm (0.095 μ) in diameter.

TABLE II. FILTRATION OF HAZ VIRUS

	FILTRATE	
PORE SIZE	PFU/ml	% Residual
Control	4.4×10^6	100
0.45 μ	1.5×10^6	34
0.22 μ	1.0×10^6	23
0.10 μ	2.5×10^3	0.2
0.05 μ	$< 1 \times 10^1$	< 0.0001

Thermal and freeze-thaw stability of C-CHF viruses. Both viruses appear to be unusually heat-labile at 36°C, our routine temperature of incubation. At this temperature CON virus has a plotted half-life of 82 min and HAZ virus, 90 min in media with 10% FCS. This compares to a previous estimate of "2-3 hr" for a member of this group (2), 3.5 hr for VEE virus (3) and 5 hr for JE virus (4). On the other hand, CON virus appears to be relatively stable to the stress of freeze-thaw cycles. No change in surviving virus could be measured over the course of 3 cycles in serum concentrations of 1, 2, 5, 7 or 10% FCS. The relative thermal lability of these viruses at 36°C may account for the apparent proportion of height of virus titer achieved in replication to percent of serum in the growth medium, i.e., increasing serum concentration may be a requirement for increased thermal stability of

the virus rather than a nutritional requirement of the cells to replicate virus.

Cross-protection and serologic cross-reactions. The increased replicative capacity of HAZ virus vs. CON virus prompted the question of whether or not there were sufficient antigenic similarity between the 2 viruses to consider use of the former as a potential vaccine virus for the group. Casals has previously shown minor but readily apparent serologic cross-reactions (5). Although HAZ mouse immune sera did not neutralize CON virus IbAr 10200 in a neutralization test and cross-reactions by CF test were barely detectable, there was extensive cross-reaction by HI. FAT results (Table III) seem to parallel the reported results of CF tests. However, reciprocal PRN_{50} titers for the 2 viruses indicate that infection-induced HAZ antiserum neutralizes CON virus to the same titer as HAZ virus. This finding has been confirmed in a repeat experiment, and supported by cross-protection tests in mice. Suckling mice were immunized by peripheral inoculation of 10^3 ID_{50} of CON or HAZ virus at an age when infection does not produce a lethal infection. Stock preparations of both viruses were then titrated IC in infection-immunized and control litter mates when the mice were 26 days old. Complete cross-protection was conferred by a previous immunizing infection with either virus. Both HAZ and CON-immunized mice survived IC inoculation with $10^{4.3}$ $WMICLD_{50}$ of HAZ virus or $10^{2.7}$ $WMICLD_{50}$ of CON virus, the maximum available dose of either. Thus, it would appear that HAZ virus should not be excluded as a vaccine candidate virus for the C-CHF virus group.

TABLE III. SEROLOGIC CROSS-REACTIONS OF HAZ and CON VIRUSES

SERUM	RECIPROCAL TITER			
	HAZ		CON	
	FAT	PRN_{50}	FAT	PRN_{50}
Mouse anti-HAZ	640[a]	320	20	320
Mouse anti-CON	< 10	40	160	1280

[a] 2+ or greater fluorescence

Part II. Korean Hemorrhagic Fever

KHF studies in cell cultures. The search for in vitro assay system was continued in essentially the same manner as described previously. Modifications included the use of 24-well plates with glass 12-mm coverslips for the FA studies in place of the preparation of spot slides. Efforts during the year centered on previously untreated cell cultures which included primary and subsequent passages of C. callosus kidney and lung cultures, primary and subsequent passages of vole (Microtus montanus) embryo cell cultures, a rabbit endothelial line derived from vena cava, a human alveolar epithelial cell line derived from a carcinoma of the lung and leukocytes derived from various sources.

Limited success was achieved with primary Calomys kidney cell cultures, but not lung cell cultures or kidney cell cultures beyond the primary passage level. One possibility for the loss of FA positivity with Calomys kidney cell cultures beyond the primary level was a loss of a specific epithelial-like target cell upon passage. These cell cultures rapidly convert to primarily fibroblast-like cells on subculture. An attempt was made to circumvent this problem by passaging kidney and lung cells from Calomys in media in which D-valine replaced L-valine. Smooth muscle fibroblast-like cells do not divide in this media in that they lack the enzymes required to convert D- to L-valine. Epithelial cells have the required enzyme systems for this conversion and can be passaged and selected for in this medium. Utilizing this procedure we have obtained irregular but specific fluorescence of Calomys kidney cells out to the 4th passage. However, results are not uniform enough to utilize this system for assay; further, we have been unsuccessful in numerous attempts to passage kidney tissue beyond the 4th passage. Cultured Calomys lung cells have not yielded fluorescence from primary to 5th passage, with or without the L-valine-deficient media. Similarly, vole embryo cells have been uniformly negative. Other cell types unsuccessfully tested during the year included the rabbit endothelial cells, Calomys primary and secondary bladder cells, Calomys peritoneal and alveolar macrophages, and human peripheral leukocytes from 2 different donors. The latter studies were all accomplished with an extended examination period covering 8-24 days postinoculation with the infectious material.

Fortunately, studies with the human alveolar epithelial cell line (designated A-549) finally led to positive results. This cell line first became FAT-positive in the indirect test 12 days postinoculation with an infectious Apodemus lung suspension. The first indication of success was a single focus of < 12 cells in which fluorescence appeared as discrete pinpoint granules throughout the cytoplasm. Fluorescent foci increased in number and brightness on paired coverslips through day 18 postinfection. Subsequent passages have led to a reduction in time of the first appearance of specific fluorescence to as little as 4 days by the 5th passage in A-549 cells. Although the original isolation of the agent in A-549 cells was subsequently demonstrated to be contaminated with mycoplasma, we have since isolated the agent in A-549 cells 3 times, free of mycoplasma contamination.

Identification of the agent isolated in A-549 cells as KHF was accomplished in different experiments: (a) 2nd passage-infected cells were tested by FAT against known negative and positive sera including 4 paired acute and convalescent sera from previously identified KHF patients. Only the positive sera were positive by FAT and all paired sera clearly showed < 4-fold rises in titer; (b) 2nd passage material was inoculated IM into 4 C. callosus. At 20 days lungs were removed and examined by FAT in the usual manner; 2 of 4 had typical KHF fluorescence. This is similar to the ratio of positivity we normally observe in Calomys inoculated with KHF infectious Apodemus lung suspension; and (c) 21-day sera from 2 squirrel monkeys inoculated with the A-549 agent were tested by FAT against known positive Apodemus lung sections. The lung sections demonstrated typical fluorescence. Our conclusion is that we have isolated the agent of KHF in A-549 cells, and thus, have established an in vitro assay system.

A-549 KHF-infected cells do not demonstrate cytopathology and apparently can be maintained indefinitely. Acridine orange and May-Grunwald strains of infected coverslips at various times postinfection have not demonstrated the presence of inclusions. Infected cells undergo cell division normally and without loss of FAT positivity, and infected cells remain positive for at least 70 days. Infectivity titers of spent cell culture media from infected cells are moderate; 10^5 $TCID_{50}$ is the maximum achieved to date.

Although the data at present are still very preliminary, it appears that we have an agent system in which a noncytolytic persistent infection is induced in a genetically homogenous population of cells that are uniformly susceptible to infection. Further, it would appear that infection does not inhibit cell division and cell-to-cell transmission of infection occurs during cell division.

KHF studies in nonhuman primates. Eight species of subhuman primates have been studied to date in an attempt to establish an animal model of disease. Species tested have included squirrel monkeys (Saimiri sciureus), rhesus monkeys (Macaca mulatta), cynomologus monkeys (Macaca fascicularis), African green monkeys (Cercopithecus aethiops), capuchin monkeys (Cebus spp.), marmosets (Callithrix sp.), pigtail monkeys (Macaca nemestrina) and baboons (Papio anubis). In general, the approach has been the same, although the experiments have been conducted over a period of 18 mon. Four animals of the species under test are acclimated to the study area for a period of 2 weeks. During the 2nd week rectal temperatures are taken daily in the first 2 hr in the morning; heparinized blood is collected every other day to establish baselines for temperature, total and differential and leukocyte counts and hemotocrits. All animals are sedated with Ketamine for both procedures. Three of 4 of the animals are then inoculated IM with the most concentrated infectious preparation available. During the early phases of these experiments the inoculum was Apodemus lung suspension (10%). Later, as this material became in short supply, we utilized Calomys lung suspension; in the last experiment the pigtail monkeys and baboons were inoculated with infected A-549 cell culture media. Beginning the day after inoculation and continuing every other day through day 21, rectal temperatures were taken and heparinized blood collected for evaluation. Viremia determinations, for those animals in the more recent experiments for which we had the capability to test viremia, were tested 2 ways; (a) direct inoculation of a portion of the heparinized blood onto A-549 cell coverslips and (b) inoculation of blood leukocytes, separated from other blood constituents in Wintrobe tubes, onto A-549 cell coverslips and subsequent examination by FAT. Serum was also collected for FAT examination on days 14, 21, 28 and 35.

Results have been disappointing, although the viremia determinations have not been completed on the last experiment. Some or all animals of all species tested are susceptible to infection as determined by subsequent development of FAT antibody, but none developed frank clinical illness. The most uniform species with regard to susceptibility to infection appears to be squirrel monkeys. We have studied 16 animals to date; all inoculated with infectious material developed antibody. About 25% developed a rash on the face, chest or armpits with onset at 10-15 days. None developed fever or showed any recognizable change in their blood picture.

Although we have nearly exhausted the readily available subhuman primate potential candidate species, we plan to continue these studies. In repeat experiments, species already tested will be inoculated with higher titered infectious material on the assumption that there might be a threshold level required to induce clinical disease. The maximum infectious dose tested to date probably has not exceeded 2.5 $\log_{10}$ $TCID_{50}$.

Preliminary studies on the characterization of the KHF agent. A limited number of studies to date have been conducted in an attempt to characterize the agent of KHF. H. W. Lee in his Annual reports to USAMRDC (DAMD 17-77-G-9431) of 1977 and 1978 reported that the agent was chloroform-sensitive and passed a 0.1-μ filter. We have attempted to verify these findings with some success. Lipid solvent sensitivity was tested with ether as an alternative to chloroform. The test system was C. callosus; only a very few animals were available. The ether-treated material failed to infect any of the inoculated animals whereas there was 1 definite positive and 3 probable positives in the 4 contral animals inoculated with untreated material. This study will be repeated in the A-549 cell culture system. We have clearly demonstrated on a number of occasions that the agent of KHF passes a 0.2-μ filter. However, our studies with 0.1-μ filters is less convincing. We have gotten a positive result on only one occasion of numerous trys; this positive result is suspect because of the possibility of cross-contamination from other positive coverslips in the same 24-well plate.

Electron microscopy studies performed in conjunction with CPT David Gangemi, this Division, and Dr. John White, Pathology Division have failed to reveal any virus-like particle not also present in controls. Recent studies have shown that A-549 cells are probably latently infected with what might be an adenovirus and possibly a parvovirus that may be an adenovirus satellite virus. The relationship of these agents to the infection of A-549 cells with KHF is unclear but is presently being investigated.

Presentation:

1. French, G. R. Korean hemorrhagic fever, Presented, NIH Symposium, 28 Jun 78.

Publications:

None

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6416	78 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	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
78 04 21	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	62776A	3M162776A841	00	055
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 78-7.2.1, 3, 6			

11. TITLE (Include with Security Classification Code)

(U) Immunologic studies with typhus fever rickettsiae

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	CONT	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 78	0.3	140.0
FISCAL YEAR CURRENT 79	0.5	19.4

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
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21. GENERAL USE

Foreign intelligence considered

20. PERFORMING ORGANIZATION

NAME: Rickettsiology Division USAMRIID
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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Gonder, J. C.
NAME: Anderson, Jr., G.W. POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Vaccines; (U) Laboratory animals; (U) Immunopathogenesis; (U) Tyhus fever

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

23 (U) Develop an effective, inactivated whole organism, cell-culture grown, nonreactogenic vaccine for immunophylaxis of epidemic, louse-borne typhus fever. This disease is a major epidemic problem for military personnel.

24 (U) Develop purified master and working seeds of Rickettsia prowazekii in leukosis virus-free eggs. Simultaneously, growth characteristics will be examined. Testing will be conducted in appropriate laboratory animals.

25 (U) 77 10 - 78 09 - Prototype epidemic typhus vaccines were prepared from chick tissue culture cells and from MRC-5 cells (human diploid). In guinea pigs these vaccines were as efficacious as, but not superior to, the present commercial vaccine. Cynomolgus monkeys were found to be a suitable subhuman primate model for epidemic typhus. Future research effort with epidemic typhus vaccine prophylaxis has been halted pending approval of proposed recommendations.

* Available to contractors upon requestor's approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498-1 NOV 65 AND 1498-2 MAY 68 ARE USABLE FOR ARMY USE AND OBSOLETE

* U.S. GPO 1974-540-843:8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 055: Immunologic Studies with Typhus Fever Rickettsiae

Background:

Epidemic typhus fever (caused by Rickettsia prowazekii) has affected mankind since ancient times; it is usually associated with wars and famine. The disease was believed to occur naturally only in man and human body and head lice, but recent evidence suggests that flying squirrels may harbor the organism (1). The disease is debilitating and prolonged with a mortality rate in untreated cases of ∿20% (2). Tetracycline therapy is extremely effective, but diagnosis of the disease is often delayed. Recrudescence may occur many years after the original infection (Brill-Zinsser disease); such an occurrence could conceivably initiate an epidemic (3). Relative infectivity of R. prowazekii in some small laboratory animals has been examined (2), but data are sparse on infectivity for subhuman prinates.

There are 2 vaccines available for protection against epidemic typhus, the first, a commercial product, is a chicken yolk sac-grown formalin-inactivated product. This vaccine reduces mortality to 0%, but contains large amounts of egg proteins, and may not reduce the incidence of disease (4). The second type (Madrid E strain) is a live, attenuated vaccine, but safety remains an issue; it is still considered an investigational drug.

Our purpose is to develop and test an efficacious cell-culture-grown inactivated epidemic typhus vaccine. In concert with vaccine development, a suitable small laboratory animal model will be chosen, and the feasibility of a primate model will be examined.

Progress:

Vaccine studies. Using the Breinl strain of R. prowazekii, several tissue culture cells suitable for vaccine production were screened for optimal propagation of rickettsiae; (a) FRhL-103 (fetal rhesus lung cells), (b) MRC-5 (human diploid cells), (c) chick embryo cells (CEC) and (d) duck embryo cells (DEC). All are suitable for human vaccine production. Results are shown in Table I.

TABLE I. PROPAGATION OF R. PROWAZEKII (BREINL STRAIN) IN VARIOUS CELL CULTURES.

Count	COUNTS IN CELL CULTURES			
	CEC	DEC	MRC-5	FRhL
Total (direct, acridine orange dye)	2.0×10^8	2.3×10^8	3.0×10^8	9.0×10^7

Since direct rickettsial counts from each of the 4 candidate cell cultures were not significantly different, duck and FRhL cell cultures were eliminated. Pathogen-free duck eggs are difficult to obtain and FRhL cells grow poorly in roller cultures. Two prototype formalin-inactivated (0.1%) vaccines were prepared from rickettsiae grown in CEC and MRC-5 cell cultures and compared in guinea pigs with commercial yolk sac-grown epidemic typhus vaccine. Guinea pigs were vaccinated with a single IP inoculation and challenged 3 weeks later with 10^7 PFU yolk sac-grown R. prowazekii (Breinl strain). Mean areas under the fever curves are shown in Table II.

TABLE II. COMPARISON OF 3 EPIDEMIC TYPHUS VACCINES IN 4 GUINEA PIGS

VACCINE	MEAN AREAS UNDER FEVER CURVE ± SE
Commercial	
Undiluted	0.6 ± 0.3
1:10	0.9 ± 0.4
1:100	3.4 ± 0.9
1:1000	3.6 ± 0.4
CEC	
Undiluted	1.1 ± 0.2
1:10	1.9 ± 0.4
1:100	3.0 ± 0.9
1:1000	3.8 ± 0.2
MRC	
Undiluted	0.7 ± 0.3
1:10	1.7 ± 0.9
1:100	1.6 ± 0.5
1:1000	2.4 ± 0.7
None	3.0 ± 0.5

There appears to be little difference between the 3 vaccines. Neither of the cell culture vaccines appears superior to the present commercial vaccine. Since titers approaching 5 x 10^9/ml can be attained in chick yolk sacs, superior efficacy from cell culture-grown vaccines may be difficult to achieve. This differs from spotted fever rickettsiae where titers from cell cultures considerably exceed those from yolk sac cultures. A recommendation for action has been prepared by Dr. Osterman concerning future vaccine research efforts with epidemic typhus at USAMRIID. Research with epidemic typhus vaccine prophylaxis has been halted pending a decision.

Animal studies. Small laboratory animals (rabbits, guinea pigs, rats, mice, gerbils and hamsters) inoculated with the Breinl strain R. prowazekii showed varying degrees of clinical illness (Table III). Hartley strain guinea pigs showed overt signs of illness with increased rectal temperature, scrotal swelling, anorexia, depression and death in the high-dose group. Rats showed mild susceptibility to infection, with inconsistent fevers and deaths. Cotton rats appear to be a lethal model for epidemic typhus, but only when high doses (10^7 PFU) of rickettsiae are used. Of the 20 strains of inbred and 4 strains of outbred mice inoculated with 10^6 PFU rickettsiae, none has shown susceptibility. Further screening of inbred mice is under way.

Four species of primates were examined as models for epidemic typhus infection (Table IV). African green monkeys (Cercopithicus aethiops) and capuchin monkeys (Cebus apella) showed only slight increases in rectal temperature on days 1 and 2 following IV inoculation with 10^7 PFU R. prowazekii. No clinical illness or changes in hematological or serum chemical values were noted. Of 4 rhesus monkeys (Macaca mulatta) inoculated IV with 10^7 PFU and 4 with 10^5 PFU, 3 monkeys in each group became ill with anorexia, depression, and fever. Transient lymphopenia and neutrophilia were noted 2 days after infection in both groups. Total leukocyte counts remained within normal limits. Cynomolgus monkeys (Macaca fascicularis) became severely ill following IV inoculation with 10^4 PFU. One monkey was moribund on day 11 and was euthanized. Microscopic examination revealed typical "typhus nodules" in the brain of this monkey. All 4 cynomolgus monkeys had a marked leukocytosis and increases in blood urea nitrogen, serum glutamic pyruvate transaminase, and serum alkaline phosphatase values. These data indicate that the cynomolgus monkey may be most suitable for further studies with R. prowazekii. Additional studies to define better this model are planned.

TABLE III. RESPONSE OF SMALL LABORATORY ANIMALS TO BREINL STRAIN *R. PROWAZEKII*

ANIMAL	NO.	DOSE (PFU)	FEVER (Days)	NO. DEAD	MEAN DAYS TO DEATH (RANGE)
Guinea pigs	6	10^3	5	0	
Hartley	7	10^5	6	0	
	7	10^7	7	1	8
Rabbits	3	10^3	2	0	
	3	10^5	2	0	
	4	10^7	2	0	
Rats					
F344	6	10^3	1	0	
	7	10^5	2	0	
	7	10^7	3	2	2.6 (1-4)
WF/fMai	5	10^7	4	0	
LEW/fMai	6	10^7	3	0	
ACI/fMai	5	10^7	2	0	
Wistar	11	10^7	3	0	
Cotton rats	6	10^3	ND[a]	0	
	6	10^5	ND	2	7.5 (6-9)
	6	10^7	ND	5	7.0 (5-10)
Gerbils	7	10^3	ND	0	
	7	10^5	ND	0	
	7	10^7	ND	6	1.0

[a] Not done.

An attempt was made to induce recrudescence of typhus fever in primates. Two cynomolgus and 2 rhesus monkeys, previously infected with epidemic typhus, were given 6.25 mg methylprednisolone daily for 2 weeks, continuing for 2 additional weeks on decreasing doses. Rectal temperatures remained normal throughout the study and no changes were noted in hematologic and serum chemistry values. Failure to cause recrudescence may have been due to continuing high circulating antibody levels. Steroid treatment levels may also not have been sufficiently high, although the dose administered was well above recommended therapeutic levels. At a later date the use of stronger immunosuppressants such as antilymphocytic serum or cytoxan may be evaluated.

TABLE IV. RESPONSE OF SUBHUMAN PRIMATES TO BREINL STRAIN R. PROWAZEKII

MONKEY	NO.	DOSE (PFU)	ANOREXIA DEPRESSION (%)	FEVER %	FEVER Mean days to onset (range)	FEVER Mean days duration (range)
Rhesus	4	10^5	100	75	4.3 (4-5)	5 (4-5)
	4	10^7	100	100	3.0 (2-4)	5 (3-7)
Cynomolgus	4	10^7	100	100	2.0 (1-3)	4.5[a] (4-6)
African green	2	10^5	0	50	9.0	2.0
	3	10^7	100	100	2.0	3.0
Capuchin	3	10^5	67	67	6.0 (5-7)	5.0 (4-6)
	4	10^7	50	50	2.5	3

[a] euthanized, day 11

Publications:

None.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6417	78 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
78 04 21	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	62776A	3M162776A841	00	056
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(U) Effects of respiratory infections on selected nonrespiratory functions of the lung

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	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		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.0	115.0
	CURRENT 79	1.0	122.2

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Kishimoto, R. A.
NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Immunology; (U) Respiratory diseases; (U) Lung physiology; (U) Tularemia; (U) Klebsiella

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish 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.

24 (U) Study activities of alveolar macrophages obtained by lung lavage from a variety of laboratory animals during experimental respiratory infection.

25 77 10 - 78 09 - Conditions needed in vitro to determine optimal phagocytosis by cultured alveolar macrophages obtained from living rhesus monkeys were investigated. In vivo activity of the alveolar macrophage was evaluated in cynomolgus monkeys infected with Klebsiella pneumoniae. The effect of nonspecific stimulation of macrophages on infection was studied in rats treated with glucan prior to infection with SCHU S4 Francisella tularensis. Mortality was significantly reduced when glucan was administered IV and rats were infected by IP inoculation or small-particle aerosol exposure.

Publication: Am. Rev. Resp. Dis. 117:245, 1978.
Am. J. Vet. Res., in press, 1978.

*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.

* U.S. GPO 1974-540-843/8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 056: Effects of Respiratory Infections of Selected Nonrespiratory Functions of the Lung

Background:

Infections in U.S. military and civilian populations result from operational use of biological weapons by enemy forces; they are likely to be induced by inhalation of infectious agent aerosols. Infection of the respiratory tract, therefore, may be the initial manifestation of illness caused by organisms which are either natural respiratory pathogens or which do not normally enter the host by way of the respiratory tract.

The lung performs numerous nonrespiratory functions in addition to its primary function of gas exchange. Important among these is the protection of the lung from infection. Recent investigations have indicated that the lung is an organ of immunological significance and that respiratory defenses include both humoral and cellular components. The present investigations were designed to examine the effects of respiratory infections on selected nonrespiratory functions of the lung, with particular emphasis on the immunologic defense mechanisms, and to develop means for enhancing the protective mechanisms of the lung.

Progress:

Techniques for the measurement of the phagocytic properties of cultured alveolar macrophages were developed. Alveolar macrophages were obtained from live rhesus monkeys by lung lavage (1). Leukocytes contained in lung wash fluids were concentrated by centrifugation, counted and resuspended in Earle's 199 medium containing penicillin, streptomycin, and 5% homologous serum. Suspensions of leukocytes containing 10^6 macrophages were inoculated onto 35-mm tissue culture dishes. Pure cultures of alveolar macrophages were obtained by attachment of the cells to the dishes during overnight incubation at 37°C in 5% CO_2. After washing, dishes routinely contained more than 7×10^5 adherent macrophages.

Studies were completed to determine the conditions *in vitro* for optimal phagocytosis by cultured alveolar macrophages. Three ratios of indicator yeast cells and macrophages were investigated (Table I). These data indicate the 10:1 ratio of yeast to macrophages permitted more phagocytosis than the 1:1 or 1:10 ratios.

TABLE I. EFFECT OF YEAST:MACROPHAGE RATIO ON PHAGOCYTOSIS OF KILLED YEAST BY CULTURED ALVEOLAR MACROPHAGES INCUBATED 30 MIN

	MEAN ± SD		
YEAST CELLS: MACROPHAGES	% Macrophages containing yeast	Yeast Cells/100 macrophages	Yeast cells/ phagocytic macrophage
10:1	35.0 ± 9.7	74.4 ± 28.6	2.1 ± 0.5
1:1	20.3 ± 5.5	31.5 ± 11.4	1.5 ± 0.3
1:10	16.1 ± 9.5	25.3 ± 16.7	1.5 ± 0.3

TABLE II. PHAGOCYTOSIS OF KILLED YEAST BY CULTURED ALVEOLAR MACROPHAGES

		MEAN ± SD		
AGE OF CULTURE (hour)	INCUBATION TIME (min)	% Macrophages containing yeast	Yeast cells/100 macrophages	Yeast cells/ phagocytic macrophage
24	30	39.8 ± 6.6	63.8 ± 18.1	1.58 ± 0.23
48	30	41.4 ± 15.3	55.1 ± 10.9	1.45 ± 0.13
72	30	37.3 ± 5.6	62.3 ± 12.4	1.67 ± 0.25
24	60	40.4 ± 6.0	67.8 ± 18.5	1.66 ± 0.26
48	60	36.2 ± 6.8	68.6 ± 16.5	1.91 ± 0.23
72	60	38.2 ± 7.7	64.3 ± 18.9	1.66 ± 0.24
24	90	39.3 ± 2.6	67.6 ± 12.9	1.7 ± 0.26
48	90	40.0 ± 8.2	61.1 ± 29.1	1.5 ± 0.40
72	90	37.5 ± 7.5	57.3 ± 9.4	1.5 ± 0.12

[a] n = 6; yeast:macrophage ratio 10:1

The ability of the alveolar macrophage to phagocytize Klebsiella pneumoniae organisms in vivo was studied in collaboration with Dr. Richard Berendt, Aerobiology Division; 10 cynomolgus monkeys were inoculated intratracheally (IT) with 5×10^7 live K. pneumoniae. Clinical measurements and observations, as well as throat swabs and lung lavages, were performed on 2 monkeys at 0.5, 1, 6, 24, and 48 hr after inoculation. All throat swabs, except one collected from one monkey at 0.5 hr, were positive for K. pneumoniae. Respiratory rate was significantly increased by 6 hr and remained increased through 48 hr. Leukocytosis was present in the period 6-48 hr, and bacteremia was present in the period 24-48 hr. Fever was observed only in the monkeys examined at 48 hr.

K. pneumoniae organisms were recovered from lung wash fluids at each sample time; the highest recovery was at 6 hr. The number of leukocytes recovered from lung wash fluids increased during the experimental period, as did polymorphonuclear leukocytes (PMN) (Table III).

Smears used for differential leukocyte counts were also examined for phagocytic cells. Slides from all samples contained numerous extracellular K. pneumoniae. Numerous macrophages containing 2-12 K. pneumoniae were observed; however, a high proportion of these cells appeared to be disintegrating. Occasional PMN seen contained a few K. pneumoniae. Results of this study indicate that the alveolar macrophage is capable of ingesting K. pneumoniae organisms in vivo in cynomolgus monkeys and, therefore, may be important in normal defense against this infection. Attempts to demonstrate phagocytosis of K. pneumoniae in vitro have failed thus far. Additional studies are required to elucidate the role of the alveolar macrophage in defense of the lung against infection with K. pneumoniae.

Collaborative studies have been conducted with CPT Reynolds, Animal Assessment Division, and Dr. Joseph Jemski, Aerobiology Division, on the effects of nonspecific stimulation of macrophages with glucan on the course of infection. Glucan, a component of yeast-cell walls, is a potent reticuloendothelial stimulant whose immunobiological activity is mediated in part by an increase in the number and function of macrophages. Glucan was obtained from Dr. N. R. Di Luzio, Department of Physiology, Tulane University School of Medicine. Rats were treated IV or IN 5 days and 1 day prior to IT (10^5) or SPA (10^3) challenge with SCHU S4 Francisella tularensis. Survival of each group is shown in Table IV. Rats treated IV with glucan prior to infection showed significantly greater survival following IP and SPA-induced infection. Rats treated IN with glucan prior to infection showed no protection against IP infection, but showed greater survival after SPA infection than the untreated controls; the difference was not significant. These results are promising, and studies are under way to expand the area of nonspecific enhancement of host defenses.

TABLE III. RESULTS OF LUNG LAVAGE[a] OF CYNOMOLGUS MONKEYS AFTER INOCULATION WITH K.PNEUMONIAE

TIME (hr)	% FLUID RECOVERY	K. PNEUMONIAE/ ml (x 10^6)	TOTAL LEUKOCYTE COUNT (x 10^6)	DIFFERENTIAL LEUKOCYTE COUNT %				% VIABILITY
				Macro.	Lymph.	PMN	Other	
0.5	84.2	0.27	3.45	73	26	1	0	97
0.5	80.8	0.28	3.80	56	36	6	2	98
1.0	78.3	0.21	6.88	53	34	11	2	98
1.0	86.7	0.16	2.82	56	39	4	1	97
6.0	79.2	16.2	44.27	52	34	12	2	84
6.0	73.3	26.7	7.90	54	41	4	1	74
24	79.2	3.20	5.27	50	37	8	5	92
24	80.0	0.13	5.42	47	43	15	7	96
48	79.2	3.30	6.24	49	38	11	2	95
48	71.7	1.73	11.94	52	27	16	5	87

[a]Total volume of saline used in each lavage = 120 ml

TABLE IV. PERCENT SURVIVAL OF GLUCAN PRETREATED RATS AFTER INFECTION WITH SCHU S4 F. TULARENSIS

ROUTE OF INFECTION	% SURVIVAL		
	No glucan	IV glucan	IN glucan
None	-	100	100
IP	0	69	0
SPA	37.5	100	62.5

Presentations:

1. Kastello, M. D. A comparative approach to renal physiology. Presented, Uniformed Services School of Medicine, Bethesda, MD, 19 Apr 78.

2. Kastello, M. D., A. D. Emmert, R. F. Denson, and R. A. Kishimoto. A technique for recovery of alveolar macrophages from monkeys by lung lavage. Presented, American Thoracic Society, Boston, MA, 14-17 May 1978. Am. Rev. Resp. Dis. 117:245,1978.

Publications:

None

LITERATURE CITED

1. Kastello, M. D., A. D. Emmert, R. F. Denson, and R. A. Kishimoto. 1978. A technique for recovery of alveolar macrophages from monkeys by lung lavage. Am. Rev. Resp. Dis. 117:245.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6418	78 10 01	DD-DRAE-AR(6)6

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
78 04 21	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	62776A	3M162776A841	CO	057
b. CONTRIBUTING				
c. ~~XXXXXX~~	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code) (U) Metabolic alterations in fatty acid metabolism during infection 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	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	75.0
FISCAL YEAR CURRENT 79	1.0	119.5

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

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NAME: Neufeld, H. A.
POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Inflammatory stress; (U) Bacterial infection; (U) Viral infection; (U) Lipid metabolism; (U) Therapy

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

23 (U) Identify mechanisms responsible for the limited fatty acid oxidation and ketone body production associated with the inflammatory stress of a bacterial or viral infection. Study the adipose tissue interrelationships of muscle and liver during starvation and militarily important infections. Determine if lipids are potentially useful in supportive therapy 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 model, examine mechanistic changes in lipid metabolism by in vivo and in vitro methods. Extend work to study tularemia and viral encephalitis.

25 (U) 77 10 - 78 09 - The overall capacity for long-chain fatty acid oxidation in homogenates and mitochondria isolated from S. pneumoniae-infected rat liver appears to be unaltered by fasting or infection. Diminished "starvation-induced" ketosis is due to decreased ketogenic capacity of the liver and reduced substrate availability. Isolated liver perfusion studies indicate a decrease in total acid-soluble CoA and an increase in acetylcarnitine during infection. When perfused with oleic acid, long-chain acyl-CoA decreased and long-chain acyl-carnitine increased in infected liver. It is suggested that there are different mechanisms for decreased ketone body production in infected rats and for ketone body regulation in fed rats. The regulating factor appears to be carnitine in fed and acyl-CoA in infected rats.

Publications: Fed. Proc. 37:1505, 1978;
Clin. Chem. 24:32-35, 1978;
Digest, Nuclear Med., in press, 1978.

*Available [illegible] upon request of [illegible] approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1 MAY 74 FOR ARMY USE ARE OBSOLETE

* U.S. GPO 1974-540-833 8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 057: Metabolic Alterations in Fatty Acid Metabolism During Infections of Military Importance

Background:

Some degree of starvation is generally present during a bacterial or viral infection; however, the hormonal responses and substrate availability during sepsis differ from those of starvation alone. Survival during starvation is dependent upon the conservation of body protein as well as on the supply of energy-yielding substrates in the form of ketone bodies. Therefore, the decreased ketogenesis associated with bacterial and viral infections (1) may lead to increased oxidation of amino acids because of an increase in gluconeogenesis and may contribute to the protein-wasting state that accompanies infection (2). Studies on the possible regulatory mechanisms of fatty acid oxidation and ketogenesis during infection have shown: (a) the mitochondrial processes of β-oxidation, the tricarboxylic acid cycle and acetoacetate production are intact in the rats infected with Streptococcus pneumoniae (3); (b) isolated perfused livers from rats infected with S. pneumoniae or Francisella tularensis are less efficient at oxidizing long-chain fatty acids to ketone bodies (3); and (c) hepatic free and total carnitine increase to the same extent in rats infected with S. pneumoniae or F. tularensis as in starved controls, while hepatic short-chain acylcarnitines increase and long-chain acylcarnitines decrease during infection (3). Because of these differences it appears that regulation may exist at the site of any of the key enzymes in the pathway of fatty acid oxidation. Determining changes in concentration of the intermediates, such as CoA and carnitine, is essential to the understanding of the control of these processes in the intact tissue during an infectious illness(4).

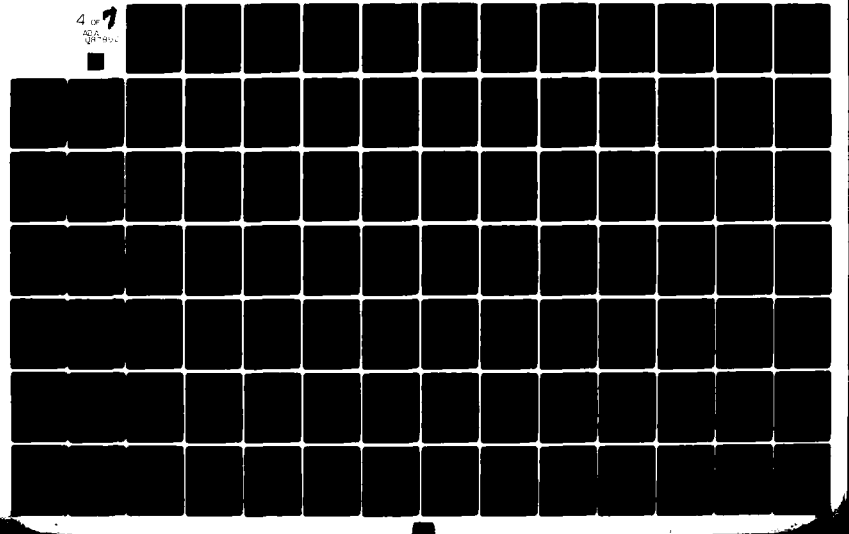
Progress:

In an effort to define more clearly the decreased "ketogenic capacity" observed during a bacterial infection, studies were conducted to measure the ability of the liver to produce ketone bodies when exposed to a given concentration of long-chain fatty acid. Livers from rats infected with 10^4 S. pneumoniae were collected after a 1-hr perfusion with either oleic or octanoic acid or perfusate without added fatty acid. There was a 2-fold decrease in the rate of ketone body production from oleic acid in the infected rat liver. Under all perfusion conditions the total acid-soluble CoA decreased in infected rats. Carnitine, especially acetyl-carnitine, increased above fed values in both fasted and infected rats, and significantly increased in infected compared to fasted rats. When perfused with oleic acid, long-chain acyl-CoA decreased (50%) in infected rats compared to fasted rats. Long-chain acylcarnitine increased (38%) in fasted when compared to fed controls, with a 32% further increase in infected compared to fasted rats. These results suggest that there

AD-A087 852 ARMY MEDICAL RESEARCH INST OF INFECTIOUS DISEASES FR--ETC F/G 6/5
ANNUAL PROGRESS REPORT - FISCAL YEAR 1978.(U)
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4 OF 7
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are different mechanisms for the decrease in ketone body production during infection compared to the decrease in ketone bodies in fed rats. The regulating factor appears to be carnitine in the fed rat and acyl-CoA in the infected rat. These results seem to indicate that since the rate of ketogenesis from long-chain fatty acid is low in the infected rat liver, the predominant fate of acetyl-CoA is oxidation through the Krebs cycle or transfer of the acetyl unit to carnitine.

The calculated value of acetoacetyl-CoA, a precursor in the formation of ketone bodies and cholesterol, decreases significantly under all perfusion conditions during an infection. The acetyl-CoA/CoA, acetylcarnitine/carnitine, acyl-CoA/CoA and acylcarnitine/carnitine ratios were not significantly different in the livers perfused with a fatty acid.

Whole tissue levels of the intermediates in the pathway of fatty acid oxidation have been determined. Carnitine concentrations changed little during a less severe infection with S. pneumoniae. However, acetoacetyl-CoA and acid-soluble CoA were significantly decreased 24 and 48 hr after inoculation.

In other studies, a comparison of the rates of oxidation of oleate and octanoate by rat liver mitochondria and homogenates suggests that both substrates support equal rates of ketogenesis in liver from an infected rat. This apparent discrepancy between whole liver studies and those involving mitochondria or homogenates is under investigation.

Publications:

1. Pace, J. A., R. W. Wannemacher, Jr., and H. A. Neufeld. 1978. Improved radiochemical assay for carnitine and its derivatives in plasma and tissue extracts. Clin. Chem. 24:32-35.

2. Pace, J. A., R. W. Wannemacher, Jr., and H. A. Neufeld. 1978. Improved radiochemical assay for carnitine and its derivatives in plasma and tissue extracts. Digest, Nuclear Med. (condensed version). In press.

LITERATURE CITED

1. 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.

2. Wannemacher, R. W., Jr. 1975. Protein metabolism, pp. 85-153. In Total Parenteral Nutrition: Premises and Promises. (H. Ghadimi, ed.) John Wiley and Sons, New York.

3. Pace, J. A., F. A. Beall, H. A. Neufeld, and R. W. Wannemacher, Jr. 1977. Alterations in carnitine (C) acylation states in S. pneumoniae infected (INF) rats. Fed. Proc. 36:788.

4. Idell-Wenger, J. A., L. W. Grotyohann, and J. R. Neeley. 1978. Coenzyme A and carnitine distribution in normal and ischemic hearts. J. Biol. Chem. 253:4310-4318.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6419	78 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
78 04 21	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	058
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(U) Molecular structure and antigenic determinants of Rift Valley fever virus

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 07	78 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 78	1.0	70.0
FISCAL YEAR CURRENT 79	0	0

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NAME: Rosato, R. R.

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Laboratory animals; (U) Bunyaviridae; (U) Rift Valley fever; (U) Preventive medicine

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

23 (U) Determine the number, molecular weight and immunogenicity of structural polypeptides of Rift Valley fever virus (RVFV). When successful, a new and better vaccine against the disease may be developed which will protect military personnel from either natural or BW transmitted diseases. An existing vaccine does not meet modern safety standards for use in man.

24 (U) Use a variety of physicochemical techniques to separate the virus into its subunits. The same will be done with Bunyamwera, Japanese B encephalitis and Uukuniemi viruses. Immunize rabbits for production of antibodies against the whole virus and its subunits. Determine if protective immunity is induced.

25 (U) 77 10 - 78 09 - Growth, purification, concentration and radiolabeling techniques have been perfected for RVFV. The South African 1951 (SA-51) isolate gave consistently higher tissue yield than did Entebbe, Zagazig 501 or South African 1975 (SA-75). Purified SA-51 has a density of 1-175 in Renographin 60 and when subjected to polyacrylamide gel electrophoresis on 12% tube gels gave 3 major peaks of approximately 60,000, 54,000 and 30,000 daltons; on 8% gels the peaks were 70,000, 65,000 and 23,000 daltons. Minor proteins of 181,000, 125,000, and 87,000 daltons were present. The 125,000-65,000 peaks on 8% gels were glycoproteins.

SA-51 RNA was labeled with 3-H-uridine or 32-P-orthophosphate. The RNA was extracted using a saturated phenol technique and subjected to electrophoresis on 2.4% polyacrylamide gels; 3 peaks of RNA were obtained, with 2.6, 1.7 and 0.6 million daltons.

The studies are terminated since the investigator is leaving the Institute.

*Available to contractors upon originator's approval

DD FORM 1498 MAR 68

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

* U.S. GPO 1974-540-843/8691

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 058: Molecular Structure and Antigenic Determinants of Rift Valley Fever

Background:

Rift Valley fever (RVF) is an acute arthropod-borne viral disease of many species, primarily of sheep, cattle and man, which is characterized by a short incubation period, a short febrile period and focal to diffuse liver necrosis; it is an problem in some parts of Africa and in Egypt.

RFV virus is a proposed member of the family Bunyaviradae. The family consists of viruses characterized by a single stranded RNA in 3 segments, spherical virions 90-100 nm in diameter, and an envelope which contains at least one virus-specified glycopeptide. They develop in the cytoplasma, and bud into smooth-surfaced vesicles in or near the Golgi. The bunyaviruses are taxonomically divided into the Bunyavirus genus, consisting of 11 serologically related groups with Bunyamwera virus being the type species of the genus and family, and a group of 54 additional morphologically similar viruses, which are serologically unrelated to the members of the Bunyavirus genus. RVF virus is tentatively classifed with this group and serologically distinct from all other described arboviruses.

Progress:

The Entebbe strain of Rift Valley Fever virus (RVFV-Ent) was grown in BHK, 1-929, Vero, FRhL and Raji cells; 7 logs PFU were obtained within 48 hr from these cells. It was decided to use BHK cells for the growth of RVFV because this cell line has been used by other investigators. A total of 4 strains of RVFV/Entebbe, (Ent); Zagazig 501, (ZZ501); South Africa 1975, (SA75); South Africa 1951 (SA51) have been grown in BHK cells; SA51 has consistently yielded 8 logs/ml of tissue culture fluid in low passage BHK cells (Pass. 56-64) while the other 3 strains yielded 7 logs. In all cases E-199 with 2% heat-inactivated fetal calf serum (FCS) and 1% pen-strep was used for the growth medium; 1 MOI of 0.001 was used for all strains.

First attempts at purification in sucrose gradients under isopycnic conditions yielded purified nucleoprotein as shown by electron microscopy (EM) and polyacrylamide gel electrophoresis (PAGE). However, when substituting Renographin-60^{R} for the sucrose and using the buffer described by Gentsch and Bishop (1) (0.01 m-tris, 0.15M NaCl, and 0.002 MEDTA, pH 7.4) intact virus was obtained from isopycnic bands.

The best method found for purification and concentration, which yields clean virus with the least amount of centrifugation, is as follows: (a) clarification of whole cells at 2000 rpm for 10 min in a Beckman desk top centrifuge. (b) Clarification of cell debris at 5000 rpm in SS-34 rotor in the Sorval RC-2B centrifuge for 20 min. (c) Fifty ml of clarified harvest

were placed into appropriate tubes and 10 ml of 15% Renographin added with a cannula and syringe. This was spun for 2 hr at 21,000 rpm in a Beckman fixed-angle 21 rotor. (d) Virus pellets were resuspended in 0.5 ml of TNE and put onto a continuous 15-40% Renographin gradient. For small harvest (1-2 roller bottles) the SW 50.1 was used at 40,000 rpm for 4 hr while the SW41S was used at 40,000 for 6 hr for larger volumes. (e) The visible band of virus was harvest in ∿ 0.5-1 ml and diluted with TNE and pelleted with the SW 50.1 rotor at 45,000 rpm for 30 min and step (d) and (e) were repeated again. (f) The 2X concentrated pellets was resuspended in 100 vol TNE and 5 vol removed to determine the radioactivity. Samples were diluted to obtain approximately 10,000 CPM 5 µl and stored at 4 C until used.

The radioactive compounds (New England Nuclear) utilized in these experiments were [^{3}H]L-amino acids (NET-250), [^{3}H] glucosamine (NET-SS7), [^{14}C]L-amino acids (NE C-445), and [^{35}S] methionine (NEG-009T). All labeling experiments were done similarly, after 1 hr absorption the roller bottles were rinsed 3 times with HBSS and E199 and 1/40 concentration of amino acids (Grand Island Biological Co.) was added with 1% pen-strep. After 6 hr the isotope [(^{3}H] - 10 µCi/ml, [^{14}C]-, 2 µCi/ml) and 2% heat-inactivated dialyzed FCS were added. When [^{35}S] methionine was used the E199 1/40 amino acid media was discarded and replaced with BME Diploid media (Grand Island Biological Company) deficient in methionine. Media was harvested when CPE was maximal and ∿ $\leq$ 50% of the monolayer remained attached to the growing surface and handled as described above.

Sample preparation was similar to that previously described (2) except dry heat was used at 100°C for 30 min. Acrylamide gels and buffers were prepared according to the method described by Laemmli (3). Resolving gels of 6, 8, and 12% were used; the 12% gels gave the best resolution of the 3 major peaks. Electrophoresis was started at 1 mA per gel until bromphenol blue dye was starting into the 3% stacking gel, then 2 mA for 15 min. and then 3 mA until 30 min after the bromphenol blue went off the bottom of the gel. Gels were removed by smashing electrophoresis tubes and fixed overnight in 7.5% acetic acid; those gels to be sliced were frozen at -70°C. The frozen gels were sliced in 1-mm sections; each section was placed into a miniscintillation vial and 7 ml of a scintillation cocktail (3 L toluene, 100 ml protocol solutions and 150 ml Liquiflor) were added. The vials were incubated for at least 24 hr at 37°C and DPM determined.

Six proteins were used as molecular weight (MW) markers (phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin) which were purchased from Pharmacia as a Low Molecular Weight Calibration Kit. The 3 peaks of RVFV-SA51 were shown to be approximately 60,000; 54,000; and 30,000 daltons. A dual label experiment with [^{14}C] amino acids and [^{3}H] glucosamine showed that the 60,000 and 54,000 peaks were glycoproteins.

According to the review article by Obijeski and Murphy (4) RVFV-SA51 is similar to other Bunyaviruses in that it has 3 major peaks, 2 of which

are glycoproteins. The MW of these 3 peaks differ from the 120,000; 38,000 and 25,000 daltons of the supergroup viruses. The profile of RVFV-SA51 is most similar to Uukuniemi virus which is the only non-supergroup virus which has had its structural proteins characterized.

Publication:

None.

LITERATURE CITED

1. Gentsch, J., D. H. L. Bishop and J. F. Obijeski. 1977. The virus particle nucleic acids and proteins of four bunyaviruses. J. Gen. Virol. 34:257-268.

2. Rosato, R. R., J. M. Dalrymple, W. E. Brandt, R. D. Cardiff, and P. K. Russell. 1974. Biophysical separation of major arbovirus serogroups. Acta Virol. 18:25-30.

3. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

4. Obijeski, J. F., and F. A. Murphy. 1977. Bunyaviridae: recent biochemical developments. J. Gen. Virol. 37:1-14.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6425	78 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
78 04 21	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	62776A	3M162776A841	00	059
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

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	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PREVIOUS 78	1.0	60.0
FISCAL YEAR CURRENT 79	1.0	118.9

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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 (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To elucidate the mechanism 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-drived 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 produced 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) 77 10 - 78 09 - Some progress has been made regarding the growth of B. anthracis. Cultures have been propagated in synthetic medium using a variety of conditions which include static carboys and controlled fermentations. Supernatants from the various cultures have been tested for toxin activity using animal models. Serologic tests have been run to determine the presence of antigenic components. Thus far the cultures have yielded only small amounts of toxin or antigen. Parameters of growth will be changed in order to improve the yield. In order to demonstrate toxin activity, culture supernatants have been concentrated using ultrafiltration and ammonium sulfate precipitation. Those materials which are reactive have been shown to be very heterogeneous by electrophoretic techniques. Ion exchange chromatography may provide a solution to the partial purification of the anthrax toxin.

Publication: Abstracts, ASM Meeting, 1978, p. 30.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 059: Pathogenesis of anthrax

Background:

Bacillus anthracis, cause of anthrax, has long been considered one of the most potentially threatening organisms available for biological warfare. The high mortality rate from the pulmonary form of the infection, along with the extreme stability of the spores makes it extremely dangerous (1). Therefore, the importance of rapid diagnosis and improved defensive technology against this organism is of prime importance for medical defense in the military.

Earlier work on anthrax involved challenge studies using spores of virulent strains of B. anthracis (2). Although there was some biochemical work done on the toxin (3), most interest was centered on the organisms and establishment of infection. One of the present goals is to study the mechanism of action of the isolated toxin. The pathophysiology of the intoxication studied in the absence of infection may lead to a better understanding of the lethality of anthrax. It has been shown that antibiotic therapy is sufficient to eradicate the organisms, yet death may ensue as a direct consequence of toxin elaboration. Studies using purified toxin will be designed to study the molecular and/or cellular level of action of the toxin alone, or in conjunction with the bacillary infection. Other goals include development of a more efficacious vaccine against anthrax and further biochemical characterization of the anthrax toxin. Production of standard antigen to be used as a vaccine is also a prime goal of this work unit.

Progress:

Various conditions have been tested for production of anthrax toxin. Stock cultures were received from several different sources, including a veterinary pharmaceutical house and Microbiological Research Establishment, (MRE), Porton, England. Thus far work has been limited to avirulent strains of B. anthracis.

Cultures have been grown in several types of media under different cultural conditions. All toxin production in the past utilized synthetic medium, consisting of amino acids, inorganic salts, vitamins and glucose, as a carbohydrate source. We have only recently been able to duplicate precise formulation of this complex medium. Using a standard inoculum of spores, we have grown static and shake cultures. In addition, we have made several runs using 10-L fermentor, and one run using a 50-L fermentor. In these fermentors we can carefully control the agitation rate as well as the sparge and overlay gases.

During these fermentations, samples were removed periodically to study bacterial growth and toxin elaboration. Bacterial growth was estimated by an increase in optical density of the culture, depletion of glucose from the medium, and an increase in total protein concentration in the medium. Toxin production was assayed from these samples using bioassay and serologic procedures. Test specimens were injected IV in rats and mice. These animals were then observed for characteristic symptoms of intoxication, ultimately resulting in death. Additionally, the test preparations were reacted in Ouchterlony gel diffusion against standard anthrax antiserum and antigen supplied by MRE.

Culture supernatants have been consistently low in yield of both total protein and toxin. Generally we have not been able to demonstrate toxin in unconcentrated culture fluids. Concentration methods currently being studied include $(NH_4)_2SO_4$ precipitation, ultrafiltration and non-specific methods, such as carbowax. When concentrated materials are prepared in sufficient quantity, these toxin preparations will be partially purified by ion-exchange chromatography, molecular sieving or other methods.

To date, progress has been achieved in establishing some conditions for optimal production of anthrax toxin. Test procedures have been standardized to compare various methodologies of toxin production. Thus far no real progress has been achieved in purification of the toxin, due mainly to the extremely low yields produced.

Presentation:

Johnson, A. D., L. Spero, and J. F. Metzger. Purification and characterization of two immunologically distinct staphylococcal exfoliative toxins. Presented, annual meeting, American Society for Microbiology, Las Vegas, NV, 14-19 May 1978 (Abstracts-1978, p. 30).

Publications:

None.

LITERATURE CITED

1. Lincoln, R. E., and D. C. Fish. 1970. Anthrax toxin, pp. 361-414. In Microbial Toxins, Vol. III (T. C. Montie, S. Kadis, and S. J. Ajl, eds). Academic Press, New York.

2. Wright, G. G., M. Puziss, and W. B. Neely. 1962. Studies on immunity in anthrax. IX. Effect of variations in cultural conditions on elaboration of protective antigen by strains of Bacillus anthracis. J. Bacteriol. 83:515-522.

3. Fish, D. C., B. G. Mahlandt, J. P. Dobbs, and R. E. Lincoln. 1968. Purification and properties of in vitro-produced anthrax toxin components. J. Bacteriol. 95:907-918.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6426	78 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
78 04 10	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	62776A	3M162776A841	00	060
b. CONTRIBUTING				
c. ~~APPLIED~~ / STOG 78-7.2.1, 3, 6				

11. TITLE (Precede 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 AGENCY	16. PERFORMANCE METHOD
77 11	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	0.5	100.0
FISCAL YEAR CURRENT 79	1.0	120.0

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NAME:

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Bacterial diseases; (U) Chemiluminescence; (U) Rapid detection

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

23 (U) Develop reproducible and reliable techniques for the rapid detection of small numbers of bacteria or viral particles in blood 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) After establishing a good technique, test the system for a variety of potential BW agents, beginning with Francisella tularensis.
25 (U) 77 11 - 78 09 - Studies in other laboratories have shown that detection of small numbers of biological agents are possible by use of the luminescence created by the hemin-catalyzed oxidation of luminol. Moreover studies in laboratories in Israel have shown that the capability exists for the development of a technique of coupling sensitive luminescent procedures with established immunochemical procedures to permit the development of a luminescent-immunoreactive system which will permit rapid identification in small amounts of biological fluids. Preliminary work has established the efficacy of luminescent procedures and work is now in progress on the development of useful peroxidase-antibody complexes which might prove to be useful in rapid identification.

*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

* U.S. GPO: 1974-540-843/8691

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 060: Identification of Potential BW Agents Using Chemiluminescent Immunoreactive Procedure

Background:

In 1965, Neufeld et al. (1,2) reported on the use of the chemiluminescence of luminol as a system which would be suitable for the detection of small amounts of hematin iron and small numbers of microorganisms. In 1971 Neufeld et al. (3) published an in-house report which indicated the feasability of the luminol system as a serious candidate for biodetection. Halmann et al. (4) in 1977 reported on a chemiluminescent system coupled to an immunological technique to identify and to quantitate small numbers of microorganisms.

In November of 1977 this work unit was established with the purpose of extending the observations made by Halmann and his colleagues and to begin the development of a system for the rapid identification of microorganisms. In the spring of 1978, instrumentation was recalibrated and antibodies against Francisella tularensis were prepared. Preliminary investigations were started on the best procedures to couple peroxidase and the antibody.

Progress:

It has been determined that when perioxidase is coupled to antibody, the sensitivity of the chemiluminescent procedure is not impaired.

Problems have arisen in the attempt to apply the antibody to a solid medium: when specific antibodies were applied to Aclar plastic, polycarbonate membranes, glass or plastic test tubes, a large increase in background was noted. At present the high background has been attributed to nonspecific adsorptions of hemin-containing proteins. Various washing methods, preabsorption reagents, and inhibition of nonspecific adsorptions are being examined.

Preliminary work has also started on other luminescent systems such as the substitution of perborate for peroxide. In addition, a new and novel type of instrument which is specifically designed for this type of assay manufactured by the Vitatech Corporation is being evaluated.

Publications:

None.

LITERATURE CITED

1. Neufeld, H. A., C. J. Conklin, and R. D. Towner. 1965. The luminescence of luminol as a tool for biodectection. Technical Report 67, United States Army Biological Laboratories, Fort Detrick, Frederick, MD, (AD467 160).

2. Neufeld, H. A., C. J. Conklin, and R. D. Towner. 1965. Chemiluminescence of luminol in the presence of hematin compounds. Anal. Biochem. 12:303-309.

3. Neufeld, H. A., G. E. Hatfield, and A. D. Brumbaugh. 1971. Investigation of Chemiluminescence Detection Approaches, Project 1W663720D165, Edgewood Arsenal, MD.

4. 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.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6410	78 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
78 06 27	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	62776A	3M162776A841	00	063
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(U) Rapid diagnosis of viral diseases of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 0100100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 06	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	62.8
FISCAL YEAR CURRENT 79	1.0	152.7

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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) Virus diagnosis; (U) Arboviruses; (U) Arenaviruses

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

23 (U) Develop and standardize rapid immunofluorescent techniques for detection and identification of viruses of military significance and of high hazard in tissues and body fluids. There is an urgent need for identification methods for viruses to decrease the time required for diagnosis.

24 (U) Produce specific virus antigens and antisera for use as reagents for virus identification and diagnosis; evaluate clinical specimens for diagnosis of virus infections; develop specific SOPs, systemitize procedures and develop protocols for virus diagnosis.

25 (U) 76 06 - 78 09 - Spot slides of tissue culture cells containing specific virus antigens have been prepared for 15 viruses and test lots of similar slides are available for 6 additional viruses. FA conjugates for direct and indirect tests are available for 16 agents. It is anticipated that FA reagents will be available for the bulk of the 31 viruses of interest within the next year, as will our capability for the serological diagnosis of these same agents. Studies will begin on RIA, SPIRA and/or ELISA techniques as alternate methods for virus diagnosis. We will continue to actively support outside sources (CDC, Universities, local health agencies, etc.) in their efforts to diagnose virus infections in humans and support USAMRIID's high hazard isolation suite whenever personnel are confined.

*Available to contractors upon originator's approval

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* U.S. GPO 1974-540-843 R/01

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BODY OF REPORT

Project No. 3M176776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 063: Rapid Diagnosis of Viral Diseases of Military Importance

Background:

Specific diagnosis of arbo- and arenavirus infections in man is usually made by the isolation and identification of the virus, or by the detection of a significant increase of specific antibodies between acute and convalescent serum samples. The former requires days, the latter, weeks. The most rapid virus diagnosis is made by the detection of specific viral antigen in clinical specimens, such as blood, urine, throat washings, feces and conjunctival scrapings. Biopsy materials are only occasionally available for this purpose. Successful isolation of most arboviruses from patients is the exception, reasons being that the specimen is not obtained soon enough or is not properly handled or transmitted quickly to the laboratory for virus isolation. Viremia for many arbovirus infections in man, if detectable at any stage, generally ceases by the time of, or soon after, onset of symptoms (at which time antibodies are often demonstrated). Mosquito-borne viruses, such as Japanese B encephalitis (JE), and St. Louis encephalitis (SLE) have very rarely been isolated from the circulating blood of patients, while West Nile (WN), yellow fever (YF), dengue (DEN), VEE and the tick-borne viruses have routinely been isolated from sera collected up to a week following onset of symptoms (1). Because of the transient nature or absence of viremia after disease onset, diagnosis is usually dependent upon a rise in antibodies; it is therefore considered to be a retrospective procedure with regard to treatment.

Recent developments have generated increased interest in immunofluorescent (IF) techniques for both the detection of viral antibodies and the direct visualization of viral antigen in clinical specimens. The availability of recently developed equipment, the current state of the art, and our desire to develop procedures for rapid diagnosis of viral diseases of military importance, prompted initiation of this work.

Progress:

Antigen slides. Spot slides prepared with cells containing specific viral antigens are primary reagents for virus diagnosis by IF techniques. Basically, cells are infected, harvested when viral antigen (as detected by IF) is maximal, purified from nonspecific cell debris, adjusted to the desired concentrations, and added to the wells of Teflon-coated slides. After fixation and testing they are stored at -70°C until needed. We have produced working lots (1-200 slides) of Tacaribe (TCR), Pichinde (PIC), Machupo (MAC), Congo (CON), Hazara (HAZ), Korean hemorrhagic fever (KHF) and Lassa (LAS) viruses, and test lots (10-50 slides) of Rift Valley fever (RVF), Dengue-2, -3 and -4 (DEN), Chikungunya (CHIK) and O'nyong-nyong (ONN) viruses.

Working lots of eastern equine encephalitis (EEE), JE, DEN-1, VEE, Langat (LAN), western equine encephalitis (WEE), sandfly fever-Naples (SF-N) and lymphocytic choriomeningitis (LCM) viruses have been received from the Yale Arboviruses Research Unit under contract DADA17-MD-77-C-7035. Each lot has been evaluated, repackaged and added to the regular 3-mon testing schedule for storage stability at -70°C. PIC, LAS, DEN-1 and MAC slides have been used extensively with a variety of human and animal sera for antibody detection and have been completely satisfactory. Working lots of spot slides containing Mayaro (MAY), YF, WN, SLE and LaCrosse (LAC) viruses, as well as those listed previously as test lots, will be produced during the next year, thereby greatly increasing our diagnostic capabilities.

Safety testing for residual live virus has been completed for LCM, CON, HAZ and KHF test slides; all are negative. The SOP for safety testing has been expanded to include animal testing, PFU assay and determination of the presence of viral antigen in blind cell culture passage by fluorescent techniques. Conditions required for virus inactivation are being studied; chlorpromazine with long-wave UV irradiation appears most promising.

Conjugates. Direct conjugates, used to detect the presence of viral antigens in cell cultures, skin biopsies and animal tissues have been prepared against EEE, RVF, YF, DEN-1, LAS, PIC, MAC and VEE viruses. They are also available for Rocky Mountain spotted fever (RMSF), Legionnaire's disease bacterium (LDB), tulemia gamma-globulins of goat and Calomys. Direct conjugates for CHIK, DEN-4, LCM, TCR and Junin (JUN) viruses are being prepared. Indirect conjugates, used for detection of viral antibodies have been prepared against human, rat, guinea pig and goat sera.

Antisera. All available antisera within the division have been located and catalogued. The antiviral activity of sera of immediate interest to the program is being determined by indirect IF and serum neutralization (SN) tests. Testing of lyophilized sera, prepared many years ago and made available to us for use in preparing direct conjugates, indicates that almost all sera are unusable; one exception is a lot of sheep anti-RVF virus.

These sera will be retained for possible use in indirect IF. The unsuitability of these sera for direct conjugation now requires that new lots of sera be produced. Animals have been, or are currently being, immunized with the following viruses: rabbits with CON, HAZ and KHF, monkeys with JUN and YF, guinea pigs with LCM, EEE, VEE, WEE, TCR, CHIK and ONN, and mice with CHIK and ONN. Antisera to MAY and SF-Sicilian (SF-S) will be prepared as virus seed stocks become available.

Virus stocks. In order to approximate the passage levels at which field or clinical specimens will be received, we are attempting to obtainthe lowest possible passage levels of most viruses of interest to the program. Viruses are being obtained from the collections of USAMRIID, WRAIR, ATCC, NIH and Yale. Master seeds for EEE, VEE, MAY, ONN, SF-N,

SF-S, DEN-3, DEN-4 and CHIK have been prepared in suckling mouse brain and/or primary cell cultures. Virus seed stocks have been characterized to the extent indicated in Table I. Sufficient quantities are available to conduct any anticipated studies during the next few years. Seed stocks

TABLE I. VIRUS SEED STOCK CHARACTERIZATION

VIRUS	PROPAGATION HOST OR CELLS[a]	PFU/ml (x 10^7)	ASSAY CELLS	LOG_{10} $SMICLD_{50}$/0.03 ml
WEE	SM	83	CEC	9.6
	CEC	98	CEC	9.3
CHIK	SM	3.3	BHK-21	7.3
	BHK-21	4.0	BHK-21	
		3.0	$LLC\text{-}MK_2$	
		7.0	Vero	
ONN	SM	79	BHK-21	6.9
	BHK-21	40	BHK-21	6.9
		7	$LLC\text{-}MK_2$	
VEE	SM	43	CEC	9.5
	CEC	110	CEC	8.8
EEE	SM	160	CEC	
	CEC	190	CEC	8.5

[a]SM = suckling mouse, CEC = chick embryo cells.

are currently being used to immunize animals, to determine the time-course development of viral antigen in selected cell lines by IF techniques and to infect cells for positive controls.

Cell sensitivity. Studies have been started to assess the sensitivity (i.e., the time-relationship of the development of viral antigen to the inoculated dose) of selected cell lines. As indicated in Table II, it is possible to detect 1 or 10 PFU of WEE virus at 28 hr and 1000 PFU within 12 hr of infection of BHK-21 cell monolayers. VEE virus (Table III) could be detected within 12 hr, if the inoculum contained 20 or 200 PFU, and within 4 hr if 20,000 PFU were present. These studies are most encouraging and will be repeated using BHK-21, Vero and $LLC\text{-}MK_2$ cells and VEE, WEE, EEE, CHIK, MAY and ONN viruses, so as to determine which cell line is most sensitive, and at which minimal level for each virus. Such information will be used to select the appropriate cell line for inoculation with clinical specimens suspected of containing a specific alphavirus.

Model systems. The recent exposure of 2 USAMRIID laboratory workers to DEN-1 virus allowed us to develop techniques for the purification of lymphocytes from heparinized blood. Such purified cells were tested for

the presence of viral antigen by IF. Fortunately, clinical infections did not occur, although the lack of seroconversions has not been demonstrated to date. Identical techniques are to be used in an attempt to demonstrate the presence of viral antigens in purified leukocytes

TABLE II. IF AND CPE FOLLOWING INFECTION OF BHK-21 CELLS WITH WEE VIRUS

TIME (hr)	IF AND CPE (%) BY PFU VIRUS/FLASK							
	0		1		10		1000	
	IF	CPE	IF	CPE	IF	CPE	IF	CPE
6	-	0	-	0	-	0	-	0
12	-	0	-	0	-	0	++++	0
22	-	0	-	0	-	0	+++	0
28	-	0	+++	0	+++	0	++	10
46	-	0	++++	0	++++	50	+	100

TABLE III. IF AND CPE FOLLOWING INFECTION OF BHK-21 CELLS WITH VEE VIRUS

TIME (hr)	IF AND CPE (%) BY PFU VIRUS/FLASK							
	0		20		200		20,000	
	IF	CPE	IF	CPE	IF	CPE	IF	CPE
4	-	0	-	0	-	0	+	0
6	-	0	-	0	-	0	+	0
12	-	0	+++	0	+++	0	+++	0
24	-	0	++++	10	++	10-25	++	50
48	-	0	+	90	+	100	+	100

obtained from monkeys infected with DEC-2, strain PR-159. The study is a cooperative effort between personnel of AR and Virology Divisions. Our data are to be correlated with determinations of viremia and seroconversion, as determined by the presence of serum SN antibodies. Studies using urine, blood, body fluids and biopsy materials will be initiated as specimens become available from cooperative studies within the Institute.

Other studies. The effect of collection of blood in tubes containing oxalate, citrate, heparin, EDTA, fluoride and various amounts of silicone on IF for PIC virus with strongly positive, weakly positive and negative sera was determined. A slight increase in background with negative serum was noted with silicone. We also established that heat-inactivation of serum decreased IF titers substantially against MAC and PIC antigens and should not be used if detection of minimal amounts of antibody is desired. Consequently, an IF laboratory has been established in the MAC Class III cabinet system.

Several detailed SOPs have been written: Procedure for Indirect Fluorescent Antibody Test, Procedure for Direct Fluorescent Antibody Test,

Preparation of Mounting Media for Fluorescent Antibody Tests, Protocol for the Safety Testing of Virus Antigen Spot Slides, and Procedure for Preparation of PBS. Other SOPs currently being written are: Preparation of Cell Culture Antigen Spot Slides for Fluorescent Antibody Tests using Virus Infected Cell Monolayers, Methanol Precipitation of Serum Globulins, Conjugation and Separation of Fluorescein Isothiocyanate Labeled Antibodies, and Procedures for Infecting Leighton Tubes.

Computer literature searches have been completed for the following viruses: SF-S, SF-N, MAY, KHF, LCM, ONN, WN, SLE, JE, RVF and Oropouche. Searches are now being conducted for LAC, CON, JUN and LAN viruses. The purpose of these is to determine the clinical course of the virus disease in man so that decisions can be made as to when and what type of clinical specimens are to be taken for virus isolation and subsequent identification.

Extensive IF support has been given to Dr. T. E. Woodward (RMSF, sera, biopsy materials), Dr. Kenyon (WRAIR specimens of RMSF, sera, biopsy materials; Carlisle, PA, mouse specimens, Q fever), Dr. Gangemi (PIC, indirect and direct IF), Dr. Jahrling (PIC, IF, biopsy materials), Drs Beisel and Hall (indirect and direct IF, YF monkeys), Drs. Hall and Woodward (RMSF), Drs. Hedlund and McGann (LDB) and Dr. Cole and Mr. Hasty (DEN-1, -2, -3, -4, monkey sera).

The program schedule was disrupted by the arrival of the 2 patients who had been exposed to LAS at the Communicable Disease Center. We supplied protocols, replacement personnel, equipment, supplies and specific IF reagents. Our input was extensive and has delayed a number of studies that were either planned or in progress at the time.

In the coming year, antigens, antisera and conjugates will be produced and viability tests conducted for other agents in accordance with Institute priorities. We will begin more comprehensive work on the development of models for early virus detection both in vivo and in vitro and generally refine IF techniques for use with increasingly hazardous agents, as containment facilities, equipment and trained technical help become available. We are currently setting up a high-hazard containment laboratory, including all necessary reagents, euqipment, etc., for the isolation and identification of human infections caused by high-hazard viruses.

Publications:

1. Rosato, R. R., M. R. Elwell, and G. A. Eddy. 1978. Virulence alterations of Tacaribe virus infection in adult mice: lethal model for encephalitis. Arch. Virol. 57: in press.

2. Kuehne, R. W., W. L. Pannier, R. R. Rosato, G. A. Eddy, and E. L. Stephen. 1978. Treatment of Tacaribe virus infection of mice using various antiviral compounds. Abstr. Am. Soc. Microbiol. p. 228.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6416	78 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
78 09 08	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	62776A	3M162776A841	00	065
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2.1, 3, 6			

11. TITLE (Precede 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	CONT	DA	C. In-house

17. CONTRACT GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	0.8	7.0
	CURRENT 79	0.5	135.8

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Stephen, E. L.

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH 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 (Furnish 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) 78 09 - 78 09 - Experimental protocols for this work unit were developed. Required equipment and reagents were ordered. Procedures for the labeling, extraction, purification, hydrolysis and chromatography of m-RNA from tissue culture cells underwent initial evaluation and selection.

*Available to contractors upon originator's approval

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

* U.S. GPO: 1974-540-843/8691

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 065: Mechanism of Action of Antimicrobial Agents

Background:

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been synthesized by others and shown to be a potent in vivo and in vitro inhibitor of RNA and DNA viruses (1). In general, ribavirin is active against 80% of viruses studied in vitro. Its known biological properties are listed in Table I.

TABLE I. BIOLOGICAL PROPERTIES OF RIBAVIRIN (from ref. 5)

1.	Active vs. DNA and RNA viruses.
2.	In vitro anti-DNA virus activity, dependent on cell type.
3.	Active prophylactically and therapeutically.
4.	Greatest efficacy vs. topical, liver and lung infections; little effect on CNS infections.
5.	Activity and toxicity reversed by removal from cell.
6.	Not an interferon-inducer.
7.	Not virus-inactivating.
8.	Not inhibitory to viral attachment or penetration.
9.	Does not prevent viral uncoating.
10.	Inhibits coating of newly-synthesized vaccinia virus DNA.
11.	Inhibits influenza, but not vaccinia, virus protein synthesis.
12.	Inhibits influenza virion RNA and complementary RNA, but not the synthesis of Semliki Forest virus RNA.
13.	Antiviral activity reversed by guanosine (+), xanthosine (+), inosine (+).
14.	Relatively structure-specific.

With regard to the mechanism of action of ribavirin a number of hypotheses have been proposed. Ribavirin has been shown to inhibit inosine monophosphate dehydrogenase activity in intact Ehrlich ascites tumor cells in vitro where the drug is presumably converted to ribavirin-5'-phosphate within the cell. This observation, together with the fact that the antiviral activity against measles virus in cell culture could be substantially reversed by zanthosine and guanosine, led to an early postulation that the antiviral activity of ribavirin was due to inhibition of guanosine monophosphate biosynthesis in the infected cell (2).

The postulation that a phosphorylated form of ribavirin may inhibit influenza RNA-dependent RNA polymerase led to the study of ribavirin-5'-triphosphate and its effect on various polymerase enzymes. These workers showed that ribavirin-5'-triphosphate does not inhibit eukaryotic DNA polymerase α and β, eukaryotic RNA polymerase I and II and eukaryotic poly A polymerase (3). Others, however, have shown that ribavirin-5'-triphosphate selectively inhibits influenza virus RNA polymerase and specifically inhibits guanosine-triphosphate-stimulated influenza virus RNA polymerase. These authors strongly suggest that the prevention of influenza virus multiplication depends on a selective inhibition of the RNA polymerase by ribavirin-5'-triphosphate (4). This concept, however, cannot explain the in vivo and in vitro effects by ribavirin on viruses that do not have a specific polymerase.

Ribavirin is generally ineffective against enteroviruses and viral diseases of the CNS to include those of the alphaviruses. This phenomena must be accounted for by any proposed mechanism of action. The lack of a therapeutic effect of ribavirin on CNS viral infections may represent a distribution or metabolism of the drug by brain tissue incompatible with effective antiviral activity in the CNS. For example, ribavirin is reported to be effective against herpes simplex or vaccinia infection when mouse-tail lesion models are employed but is ineffective against these same viruses when the mouse encephalitic model is used (Table II). Reports that the drug is ineffective against some viruses in vitro may be related to peculiar sensitivity of the cell culture system employed. For example, ribavirin appears to be less effective against most classes of viruses when tested in in vitro systems using Vero cells (Table III).

Recently, ribavirin-5'-triphosphate has been shown to be a potent inhibitor of cap structure formation on vaccinia virus mRNA (R. Smith, personal communication). Cap structures have been found in eukaryotic and viral mRNA and consist of a 5'-terminal oligonucleotide in which a methylated guanylic acid residue is joined to a single or adjacent pair of 2'-O-methyl-nucleotidases. Cap structures appear to be required for binding of mRNA to ribosomes and initiation of translation.

TABLE II. IN VIVO ANTI-VIRUS ACTIVITY OF RIBAVIRIN

VIRUS	ANIMAL	TYPE OF INFECTION	MAXIMUM EFFICACY
Type 1 Herpes	Mouse	Tail lesion	++
	Mouse	Skin lesion	++
	Mouse	Lethal pneumonia	+
	Mouse	Encephalitis	-
Vaccinia	Mouse	Tail lesion	++
	Rabbit	Skin lesion	+
	Rabbit	Karotitis	+
	Mouse	Encephalitis	-
Vesicular stomatitis	Mouse	Tail lesion	+
	Mouse	Encephalitis	-
Semliki Forest		Encephalitis	-
Western Equine		Encephalitis	-

TABLE III. SUMMARY OF VIRAL CPE-INHIBITORY ACTIVITY OF RIBAVIRIN

VIRUS	CELLS	MEAN VIRUS RATING	MIC [a] (μg/ml)
Herpes simplex			
HSV/1	KB	1.1	1
	RK-13	1.3	1
	CE	1.4	0.32
	V	0.4	100
HSV/2	KB	1.1	1
	RK-13	0.8	3.2
	CE	0.4	100
	V	0	∞
Vaccinia (Lederle)	KB	1.0	3.2
	CE	0.6	3.2
	Hela	0.4	32
	V	0.8	320
VSV (Indiana)	KB	0.8	32
	RK-13	1.0	3.2
	V	0.4	320

[a] Minimum inhibitory concentration.

It is proposed that the mechanism of action of ribavirin is related to its inhibition of the guanylation reaction of viral mRNA, resulting in an altered cap structure which would limit or prevent mRNA binding to ribosomes.

Therefore, this work unit will evaluate the in vitro effects of ribavirin on mRNA cap structure formation of RNA viruses. This information is required in order to make rational decisions for the design of new compounds with lower toxicity and improved efficacy toward viral encaphalitic diseases pertinent to the military medical mission.

Progress:

Experimental protocols for this work unit were developed. Required equipment and reagents were ordered. Procedures for the labeling, extraction, purification, hydrolysis and chromatography of mRNA from tissue culture cells underwent initial evaluation and selection.

Publications:

None.

LITERATURE CITED

1. Witkowski, J. T., R. K. Robins, R. W. Sidwell, and L. N. Simon. 1972. Design, synthesis and broad spectrum antiviral activity of 1-β-D-ribofuranosyl-1,2,4 - triazole-3-carboxamide and related nucleosides. J. Med. Chem. 15:1150-1154.

2. Smith, C. M., L. J. Fontenelle, H. Muzik, A. Paterson, H. Unger, L. W. Brox, and J. F. Henderson. 1974. Inhibitors of inosinate dehydrogenase activity in Ehrlich ascites tumor cells in vitro. Biochem. Pharmacol. 23:2727-2735.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6418	78 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
78 04 21	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	001
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 78-7,2,	1, 3, 6		

11. TITLE (Precede with Security Classification Code): (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	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	85.0
FISCAL YEAR CURRENT 79	1.0	66.2

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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) Immunization; (U) Immune response regulation during infection

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish 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 during infection. Propose ways of altering vaccines in order to take advantage of in vivo immunoregulatory phenomena to maximize immunizing effects of vaccines.

25 (U) 77 10 - 78 09 - Suppressor and helper activities have been identified in an infectious disease model. Three phases of these have been identified in AKR/J male mice inoculated with LVS Francisella tularensis. Phases 1 and 3 are characterized by augmentation, while phase 2 is characterized by suppression of induction of an immune response to an unrelated antigen, sheep red blood cells. Phase 3 augmentation continues after signs of illness (including spleen size) subside suggesting long-term effects of LVS inoculation of, as yet, an unknown nature. Induction of an unrelated immune response and the intensity of the suppressive phase 2 produced appear to be closely correlated to infection intensity in this model.

Publication: Fed. Proc. 37:1849, 1978.

*Available to contractors upon originator's approval

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

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BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 001: Effects of Suppressor and Helper T Cell Activities on the Efficacy of Immunization

Background:

The current decade has been one which could, from an immunological standpoint, easily be termed "the decade of T effector cells." Evidence revealing the regulatory functions of T cells both in the humoral and cellular aspects of the immune response continues to be amassed (1). Basic theories of the mechanism of T helper and T suppressor cell actions are being examined, while pathologic states involving these effector cells are being identified and the first halting steps toward incorporation of this knowledge into clinical medicine are being evaluated (2).

Results of research into the actions and capabilities of these regulatory cells during infection has only recently appeared in the literature (3) and manipulation of these effects during infection or vaccination awaits elucidation of these mechanisms. The control of these regulatory phenomena after vaccination could usher in a new era of vaccine design. Vaccine design and dosage has traditionally been chosen empiricly. This process may well be supplemented in the future by more theoretical considerations about T regulatory cells. Some evidence challenging current vaccination methods may already have appeared. Specifically, the use of high antigenic doses appears to be particularly effective in the induction of suppressor activity. This, of course, conflicts conceptually with the use of maximal antigenic doses for vaccination. Thus a paradox has already been shown that warrants investigation.

Progress:

By the beginning of FY 1978, critical assays and techniques needed for this project had been identified and were in use, in basic form, in this laboratory. These techniques included Mishell-Dutton (MD) primary in vitro immunization (4) and Jerne localized Hemolysis in Gel (JHG) assay technique for identification of specific antibody-producing cells (5). Refinement and testing of these techniques were carried out. Then, an infectious disease model was chosen. Live, attenuated vaccine strain of Francisella tularensis (LVS) was inoculated SC into 7-9 week old AKR/J adult, male mice. (Mice at this age are well under the 16 week age considered normal for AKR/J mice as regards onset of thymic lymphoma.) The basic question asked was: what are the effects of LVS vaccination on the ability of AKR/J male mice to mount an immune response to an antigenic stimulus unrelated to LVS? One of the implicit questions contained is: how are regulatory cells of the immune system affected by this vaccination? Highly reliable data have been produced using inoculation of infected and noninfected mice which show that the

capability of the humoral immune system to mount an immune response to an unrelated antigen ("high responder" sheep red blood cells) may be divided into 3 phases based on time after LVS inoculation. The first phase is an augmentation phase characterized by an increased ability to respond which commonly ends by day 6-9 postinoculation. The second phase which follows immediately, is a suppressive phase characterized by a decrease in the ability of the mice to respond, that ends on day 16 or 17. Phase 3 follows and is an augmentative phase similar to phase 1, except that it continues to persist long after mice have returned to healthy status and spleen sizes are back to normal. The effects noted in phases 2 and 3 are especially pronounced when the spleen size, i.e., harvestable numbers of spleen cells, of vaccinees are compared to controls. Spleens from infected mice are increased in size 2- to 3-fold from days 9-26. The large magnitude of the suppression in phase 2 is unexpectedly impressive when the spleen size variations are taken into account.

Clear data show that all phases can be reproduced in culture. This is taken as further evidence that subpopulations of cells in the spleens of infected mice change during and after infection. This change could be in the numbers or types of regulatory cells, noncommitted antibody producing cells, or both.

The augmentation seen in phase 3 cannot be attributed simply to increased spleen size, although this may be a factor. The fact that augmentation persists after spleen size returns to normal suggests a basic change in subpopulations of cells within the spleen. It also presents a further phenomenon of LVS vaccination, and raises attendant questions, such as, how long does this augmentation persist? What effect does it have on infections contracted during this time? What effect does it have on a secondary immune response to LVS? What alterations are there in immune surveillance and does this imply a treatment such as holding or slow release of LVS antigen?

A further problem encountered in research has been the identification of a good assay of infection intensity in mice. Subtle alterations in dose, e.g., $0.9 - 1.5 \times 10^3$ organisms/mouse, are not detectable biologically. Subjective evidence of illness and death remain the modes of choice to predict infection intensity rather than dose or antibody titer to LVS. Using the biological assay of induction of an unrelated immune response during LVS infections appears to correlate well with observations of severity of illness in a group of mice. Time of first appearance of antibody titer to LVS may also roughly correlate with biological bases of infection severity.

Presentation:

Howell, H. M., and D. W. Seburn. Humoral immune response in tularemia-infected AKR/J mice. Presented, Annual Meeting, American Association of Immunologists, Atlanta, GA, 4-8 Jun 1978 (Fed. Proc. 37:1849, 1978).

Publications:

None.

LITERATURE CITED

1. Benacerraf, B. April 1978. Suppressor T cells and suppressor factor. Hosp. Practice 13:4:65-75.

2. Waldmann, T. A., R. M. Blaese, S. Broder, and R. S. Krakauer. 1978. Disorders of suppressor immunoregulatory cells in the pathogenesis of immunodeficiency and autoimmunity. Ann. Intern. Med. 88:226-238.

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4. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. J. Exp. Med. 126: 423-442.

5. Dresser, D. W., and M. F. Greaves. 1973. Assays for antibody producing cells, pp. 27.1-27.9. In Handbook of Experimental Immunology, 2nd ed. (D. W. Weir, ed.). Blackwell Scientific Publishers, Oxford.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OG6419	78 06 27	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
77 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	002
b. CONTRIBUTING				
c. ////////	STOG 78-7,2,	1, 3, 6		

11. TITLE (Precede with Security Classification Code) (U) Transfer mechanisms for cell-mediated immunity using a murine tularemia study

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	78 06	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 78	0.8	34.4
FISCAL YEAR CURRENT 79	0	0

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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) Lymphokines; (U) Tularemia; (U) Cellular immunity

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

23 (U) Develop in vitro tests of cellular function of B and T lymphocytes and macrophages which may correlate with an immunized animal's ability to survive lethal tularemia infection. This will lead to information regarding protection of Armed Forces personnel against potential BW agents.

24 (U) AKR-J mice vaccinated against tularemia at varying intervals will be examined for changes in cell populations and cell functions which correlate with the quality of protective responses.

25 (U) 77 10 - 78 06 - Bacterial capability of murine peritoneal exudate cells against Francisella tularensis was measured as well as temporal changes in splenic T and B cell populations after vaccination with LVS. In the latter study the method of labeling the spleen cells has caused some difficulty, since more than 100% of the spleen cells are labeled with fluorescein. Some preliminary experiments were conducted on delayed type hypersensitivity in AKR/J mice previously vaccinated with LVS using Foshay vaccine injected into the footpad. The greatest amount of swelling occurred 42 days postvaccination. Two papers are being prepared for publication.

The investigator has left the Institute; no further work will be conducted.

*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

* U.S. GPO: 1974—546-843/860

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 002: Transfer Mechanisms for Cell-Mediated Immunity using a Murine Tularemia Study

Background:

Cell-mediated immunity has primary responsibility for protection against intracellular microbial pathogens. Francisella tularensis, a representative of this group of pathogens, routinely produces lethal infection in unprotected AKR mice, whereas mice previously immunized with live vaccine strain show varying degrees of resistance to challenge. In this murine model, humoral immunity has never been shown to provide protection against infection with fully virulent strains of F. tularensis, SCHU S4 and SCHU S5R.

With increasing time after vaccination, AKR mice become less resistant to challenge with SCHU S4, as measured by a decrease in survival ratio. This measurable decay in immunity makes it possible to examine cell populations, especially lymphocytes and macrophages, and cell functions and to determine in vitro correlates for the quality of the protective response.

Since this work unit was initiated, efforts have concentrated on functional correlates of the cell-mediated immunity response, in particular antigen-dependent lymphocyte blast transformation and bactericidal abilities of macrophages. Although these tests have been extensively used for qualitative evaluation of foreign antigen recognition, the objective is to quantitate in vitro responsiveness and to relate degree of response to resistance of the cell donor.

Progress:

Bacterial capability of murine peritoneal exudate cells against F. tularensis was measured as well as temporal changes in splenic T and B cell populations after vaccination with LVS. In the latter study the method of labeling the spleen cells has caused some difficulty, since more than 100% of the spleen cells are labeled with fluorescein. Some preliminary experiments were conducted on delayed type hypersensitivity in AKR/J mice previously vaccinated with LVS using Foshay vaccine injected into the footpad. The greatest amount of swelling occurred 42 days postvaccination. Two papers are being prepared for publication.

The investigator has left the Institute; no further work will be conducted.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6417	78 09 14	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
78 09 14	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	004
b. CONTRIBUTING				
c. [illegible]	STOG 78-7,2,	1, 3, 6		

11. TITLE (Precede with Security Classification Code): (U) Characterization of arenaviruses and their structural components for vaccine development

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
75 03	78 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 78	1.0	123.8
FISCAL YEAR CURRENT 79	0	0

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TELEPHONE: 301 663-7241
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Rice, R. M.
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Biophysics; (U) Electron microscopy; (U) RNA viruses; (U) Vaccine development; (U) Arenaviruses

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

23 (U) Isolate and characterize by physical and biochemical means virion subpopulations of arenaviruses so that their infection and structural characteristics may be better understood; identify polypeptides of the virions which have potential as subunit vaccines for diseases of military importance in BW defense.

24 (U) Prepare milligram quantities of viruses; concentrate and study by a variety of biochemical and biophysical means. Examine the immunizing potential of subunit polypeptides.

25 (U) 77 10 - 78 08 - Glycoprotein (GP) and nucleoprotein (NP) subunits of Pichinde (PIC) virus and Machupo (MAC) virus were carefully fractionated and purified. PIC virus subunits (GP, MW 65,000 and 38,000; NP, MW 68,000) and MAC virus subunits (GP, MW 50,000 and 41,000; NP, MW 68,000) were examined by SDS-polyacrylamide gel electrophoresis, immunoelectrophoresis, and RIA procedures. Only subunit preparations found free of contaminating polypeptide species were used to immunize animals. Guinea pigs inoculated with purified NP or GP subunit antigens of either PIC or MAC virus survived homologous, but not heterologous, virus challenge. Animals inoculated with NP developed CF and immunofluorescent (IF) antibody (Ab) titers but no plaque reduction (PRN) or virion surface Ab as detected by RIA. No viremias were detected; most animals survived lethal virus challenge with homologous virus. Animals inoculated with GP developed CF, IF, PRN, and RIA Ab. All animals survived homologous challenge with no viremia. It is unknown which GP species (G1 MW 65,000; G2 MW 38,000) of PIC virus or MAC virus (G1 MW 50,000; G2 MW 41,000) was immunogenic. Animals may be protected against lethal arenavirus infections with either NP or GP subunit antigens.

The investigator has left the Institute. Work is terminated.

Publications: J. Biol. Standard. 6:117-120, 1978; Abstracts, Ann. Mtg. ASM - 1978, p. 250; J. Gen. Virol., in press, 1978.

*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

* U.S. GPO: 1974—546-843/8691

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 004: Characterization of Arenaviruses and Their Structural Components for Vaccine Development

Background:

The arenaviruses are a unique group of ether-sensitive RNA viruses which are morphologically characterized by ribosomes enclosed in their unstructured interior (1). Both human pathogenic and nonpathogenic members of this group appear identical when examined by several biophysical techniques (e.g., electron microscopy, sedimentation rate, buoyant density), but are serologically distinguishable by CF and immunofluorescence techniques (2). The genetics of arenaviruses are poorly understood; nonetheless, the observation of at least 2 and possibly 3 or more genome segments in the viruses so far examined, reveals the potential genetic flexibility (i.e., recombination complementation) which they possess (3, 4). This flexibility appears to be a major factor in determining their unique biological characteristics.

Varying degrees of cross-reactivity occur between arenavirus members in CF tests; however, little if any cross-reactions occur with neutralizing antibody (5). These observations suggest the presence of at least one common and one distinct structural protein. In agreement with these serological observations, biochemical analysis of both human pathogenic and nonpathogenic varieties has revealed some similarities as well as differences in structural polypeptide compositions (in press). The purpose of this research was to identify and characterize virus subunits which may be useful immunogens in the development of arenavirus vaccines.

Progress:

Glycoprotein and nucleoprotein subunits of Pichinde (PIC) and Machupo (MAC) viruses have been carefully fractionated and purified. The biochemical purity of these preparations was examined by SDS-polyacrylamide gel electrophoresis, immunoelectrophoresis, and RIA procedures. Only subunit antigens found free of contaminating protein species were used as immunogens in the studies conducted this year. Purified PIC virus glycoprotein (G1, MW 65,000 and G2, MW 38,000) or nucleoprotein (NP, MW 68,000) was mixed with adjuvant and inoculated into guinea pigs, as were purified MAC virus glycoprotein (G1, MW 50,000 and G2, MW 41,000) and nucleoprotein (MW 68,000). Table I lists the antibody responses observed and the protection afforded these animals. As illustrated, animals inoculated with nucleo- or glycoprotein subunits survived lethal virus challenge with homologous virus, but did not survive heterologous challenge (data not shown). Guinea pigs inoculated with nucleoprotein subunits developed sub-

TABLE I. IMMUNIZATION OF GUINEA PIGS WITH PIC (AN3739) OR MAC (CARVALLO) VIRUS SUBUNIT ANTIGENS[a]

OBSERVATION	UNIMMUNIZED	NUCLEOPROTEIN	GLYCOPROTEIN
Pichinde (n)	(13)	(16)	(13)
Mean antibody response[b]			
CF	< 10	1,800	800
IFA	< 10	5,000	4,000
PRN_{50}	< 10	< 10	200
RIA-1	< 50	< 50	3,200
RIA-2	< 50	< 50	1,000
Protection against lethal challenge[c]			
No. ill/Total (onset day)	13/13 (10-12)	8/16 (9)	0/16
No. viremic/Tested (log_{10} PFU/ml)	7/7 (3.7-5.8)	0/11	0/11
No. dead/Total (day)	13/13 (11-15)	0/16	0/13
% Survival	0	100	100
Machupo (n)	(13)	(6)	(6)
Mean antibody response[b]			
CF	ND	ND	ND
IFA	< 10	1,900	320
PRN_{50}	< 10	< 10	100
RIA-1	< 50	< 50	800
RIA-2	ND	ND	ND
Protection against lethal challenge[c]			
No. ill/Total (onset day)	13/13 (10-13)	1/6 (7)	0/6
No. viremic/Tested (log_{10} PFU/ml)	6/13 (3-4)	0/3	0/3
No. dead/Total (day)	13/13 (15-17)	1/6	0/6
% Survival	0	83	100

[a] 600-700-gm guinea pigs were immunized with 100 μg nucleoprotein or 25 μg of glycoprotein antigen in Freund's adjuvant; 3 IM inoculations at 10-day intervals. 1st: antigen 1:1 with Freund's complete adjuvant; 2nd and 3rd: antigen 1:1 with Freund's incomplete adjuvant.

[b] RIA-1 = ^{14}C-labeled purified virions; RIA-2 = ^{14}C-labeled glycoprotein subunits. Virus-antibody complexes were detected by the addition of *Staphylococcus aureus* protein A; titers >50 (limit of sensitivity due to prophase phenomenon) reflect interactions of antibody and virion surface antigens.

[c] 8th passage PIC (4763) or 5th passage MAC (Carvallo) in guinea pig spleens, 10 PFU SC.

stantial CF and immunofluorescent (IF) antibody titers but little if any plaque reduction (PRN) or virion surface (RIA) antibodies. No viremias were detected and most of the animals survived virus challenge. In contrast, animals inoculated with glycoprotein developed CF, IF, PRN, and RIA antibody titers. No viremias were detected and all of the animals survived. These results were repeated twice in guinea pigs and once in MHA hamsters. The glyco- and nucleoprotein subunit antigens used in all of the studies were prepared by polyethylene glycol/dextran liquid-phase separation of Triton X-100-disrupted virus proteins (3). The possible presence of residual live virus in fractions was excluded by cell culture techniques. No viable virus was ever recovered from subunit fractions continuously passaged in the most sensitive indicator, Vero cells.

The specificity of the antibody produced in response to inoculation with either nucleo- or glycoprotein subunits was examined by fluorescent microscopy (Table II). Guinea pig anti-PIC virus nucleoprotein cross-reacted vigorously with Tacaribe (TCR), Parana (PAR) and MAC virus-infected cells; however, anti-PIC glycoprotein serum reacted only with PIC virus-infected cells, illustrating the group-specific nature of the nucleoprotein component and the species specificity of the glycoproteins.

TABLE II. CROSS-REACTIVITY IN GUINEA PIGS IMMUNIZED WITH NUCLEO- OR GLYCOPROTEIN SUBUNITS OF PIC VIRUS

SUBUNIT	RECIPROCAL GEOMETRIC MEAN FA TITER				
	PIC	PAR	TCR	MAC	LCM
Nucleoprotein	10,240	640	640	640	40
Glycoprotein	5,120	80	80	10	10

These results indicate that animals may be protected against lethal arenavirus infections with either nucleo- or glycoprotein subunit fractions. It is still not clear how nucleoprotein protects, since it fails to induce SN or RIA antibodies. In contrast, glycoprotein appears to protect by inducing substantial levels of SN antibody; however, no attempt was made to determine the glycoprotein species (G1 or G2) responsible for this effect. Future experiments should attempt to answer the following: (a) identify the glycoprotein species responsible for the induction of neutralizing antibody; (b) characterize the cellular response to subunit antigens and its contribution to immune protection; and (c) examine the immune phenomenon observed following inoculation with nucleoprotein components of the virus.

In addition to these studies on the immunizing potential of arenavirus subunit polypeptides, we initiated a collaborative study with Dr. Marcos Rodriguez of the National Institute for Medical Research, Buenos Aires, Argentina, on the use of ELISA (enzyme-linked immunoadsorbent assay) for the rapid detection of Junin virus infections. Preliminary

results with human sera containing antibody to Junin or PIC virus indicated that this assay is as sensitive as immunofluorescence and less time-consuming. The specificity and sensitivity of this procedure are now being refined by the use of subunit test antigens (e.g., glycoproteins or nucleoprotein). Field diagnostic tests with the antigen preparations provided by our laboratory will be conducted by Dr. Rodriguez by the end of this year and should provide some interesting data as to the usefulness of ELISA in the diagnosis of arenavirus infections.

The investigator left the Institute. No further work will be done under this work unit.

Presentations:

1. Gangemi, J. D., P. B. Jahrling, E. V. Connell, and G. A. Eddy. Protection of guinea pigs against lethal Pichinde virus infection by immunization with Pichinde virus subunits. Presented, Annual Meeting, American Society for Microbiology, Las Vegas, NV, 14-19 May 1978. (Abstr., Annu. Meeting, 1978, p. 250).

2. Gangemi, J. D. Biophysical and biochemical studies on arenaviruses. Presented, University of South Carolina School of Medicine, Columbia, SC, 1 June 1978.

Publications:

1. Gangemi, J. D., and F. E. Cole, Jr. 1978. Venezuelan equine encephalomyelitis virus aggregation and immunogenicity following freeze-drying. J. Biol. Standard. 6:117-120.

2. Gangemi, J. D., R. R. Rosato, E. V. Connell, E. M. Johnson, and G. A. Eddy. 1978. Structural polypeptides of Machupo virus. J. Gen. Virol. 41: in press.

LITERATURE CITED

1. Murphy, F. A., P. A. Webb, K. M. Johnson, S. G. Whitfield, and W. A. Chappell. 1970. Arenaviruses in Vero cells: ultrastructural studies. J. Virol. 6:507-518.

2. Buckley, S. M., and J. Casals. 1970. Lassa fever, a new virus disease of man from West Africa. III. Isolation and characterization of the virus. Am. J. Trop. Med. Hyg. 9:680-691.

3. Vezza, A. C., G. P. Gard, R. W. Compans, and D. H. L. Bishop. 1977. Structural components of the arenavirus Pichinde. J. Virol. 23: 776-786.

4. Carter, M. F., N. Biswall, and W. E. Rawls. 1973. Characterization of nucleic acid of Pichinde virus. J. Virol. 11:61-68.

5. Webb, P. A., K. M. Johnson, and R. B. MacKenzie. 1968. The

measurement of specific antibodies in Bolivian hemorrhagic fever by neutralization of virus plaques. Proc. Soc. Exp. Biol. Med. 130:1013-1019.

6. Gangemi, J. D., R. R. Rosato, E. V. Connell, E. M. Johnson, and G. A. Eddy. 1978. Structural polypeptides of Machupo virus. J. Gen. Virol. 41: in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB6429	78 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
78 04 21	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	006
b. CONTRIBUTING				
c. [illegible]	STOG 78-7,2,	1, 3, 6		

11. TITLE (Precede 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	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	115.0
FISCAL YEAR CURRENT 79	1.0	107.2

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 (Furnish individual paragraphs identified 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) 77 10 - 78 09 - Antigen-binding capacity measurements of the polypeptides obtained by limited trypsin digestion of SEB and SEC-1 disclosed that 2 antigenic determinants on each enterotoxin were capable of reacting with heterologous antibody, one on the first 57 amino acids and one of the last 150 residues of the polypeptide backbone. The determinant on the amino terminal polypeptides is thought be be primarily responsible for the strong reciprocal binding of the intact enterotoxins. The site for mitogenic activity of SEC-1 was located on the amino terminal polypeptide and the site for emetic activity on the larger, carboxyl terminal fragment. A study of the circular dichroic spectra of SEA, SEB, and SEC-1 revealed a remarkable similarity of both secondary and tertiary structure. A scheme for the secondary structure of SEB has been devised.

Publications: J. Immunol. 120:86-89, 1978;
J. Biol. Chem. 253: in press, 1978.

*Available to contractors upon originator's approval

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

* U.S. GPO: 1978-[illegible]

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 006: Enzymatic and Chemical Alteration of Microbial Proteins for Toxoid Production

Background:

The staphylococcal enterotoxins are simple proteins elaborated by Staphylococcus aureus which cause emesis and diarrhea in a limited number of mammalian species. All appear to consist of a single polypeptide chain with one disulfide bridge and to have a MW of ∿ 28,000. Although SEB and SEC_1 do not cross-react in classical measurement of immunodiffusion, common antigenic determinants were demonstrated by antigen-binding capacity and by inhibition of the antigen-antibody reaction in radioimmunoassay. We have been investigating partial enzymatic breakdown as a means of separating and identifying antigenic and toxic sites. SEA is resistant to the action of trypsin but SEB and SEC_1 (1 of 2 isoelectrically different variants of SEC) undergo limited digestion with this enzyme. From SEB, 2 carboxamidomethylated (Cam) peptides were obtained and antigenic determinants were demonstrated on both. Antigenic determinants were also demonstrated on 2 polypeptides, again comprising the entire molecule, obtained from SEC_1.

Progress:

The study of the reactions of the tryptic peptides from the SEB and SEC_1 with both homologous and heterologous antisera has continued. The 6 polypeptides utilized are: 6,500 MW - an amino terminal peptide from SEC_1 comprising ∿ 57 amino acid residues with the indicated molecular weight; 22,000 MW - the remainder of the molecule; Cam 4000 - an amino terminal fragment from 22,000 MW with the cysteine residue alkylated; Cam 19,000 - the remainder of 22,000 MW also with an alkylated cysteine residue; Cam 1-97 - the first 97 residues of SEB of MW 11,500, with the cysteine residue alkylated; and Cam 98-239 - the remainder of the SEB molecule with MW 17000 and also having a carboxamidomethylated cysteine residue.

The peptides were all radiolabeled with ^{125}I in 6 M guanidine hydrochloride and diluted into 10% bovine serum albumin (BSA) to maintain solubility in the test system. Antigen-binding capacity was determined with antisera prepared against SEB, SEC_1 and the 22,000-MW polypeptide. It was quite evident that the binding capacity of the larger fragments, the 22,000 MW polypeptide, Cam 19,000 and Cam 98-239, for each homologous antiserum is very strong. Nearly complete precipitation of label is readily achieved at high dilutions of the antisera. Homologous reactions of the smaller polypeptides are considerably weaker. However, the most striking observation was that there are significant heterologous interactions and that the reciprocal reaction between the whole enterotoxins

with each other's antiserum is reflected in their constituent polypeptides. Consider first the large polypeptides from the carboxyl terminal end of the amino acid backbone and comprising more than half the enterotoxin molecules. Cam 98-239 from SEB binds to anti-SEC_1 and the 22,000 MW polypeptide and Cam 18,000 from SEC_1 bind to anti-SEB. The binding capacities of the latter 2 polypeptides are in the same ratio to each other for all 3 antisera suggesting that the cross-reacting determinant of the 22,000 MW polypeptide is contained totally within Cam 18,000. This is supported by the lack of binding of Cam 4,000 to either anti-SEB or to anti-SEC_1.

Each of these 3 carboxyl terminal polypeptides binds about 2 orders of magnitude more efficiently to its homologous antiserum than to the heterologous antiserum. Moreover, a comparison of the binding of the structurally analogous polypeptides from the 2 enterotoxins, Cam 18,000 and Cam 98-239, with anti-SEB or with anti-SEC_1 yields similar estimates of binding efficacy. The quantitative estimates show quite clearly that an antigenic determinant exists on Cam 18,000 and Cam 98-239 which, although capable of reacting with the heterologous antiserum, is not functionally identical. It is probably also reasonable to infer that they differ structurally in the native enterotoxins.

Unexpectedly, very excellent binding of Cam 98-239 to the antiserum to the 22,000 MW polypeptide was obtained. This must reflect reaction with antibody arising from non-native determinants on the 22,000 MW polypeptide. It also suggests that Cam 98-239 and the 22,000 MW polypeptide possess regions of common structure in addition to that responsible for the common determinant described above.

The binding capacity of the amino terminal polypeptides, the 6,500 MW polypeptide and Cam 1-97, is very much weaker than the larger carboxyl terminal polypeptides even to the homologous antiserum. However, the relative binding efficacy with heterologous antiserum is much greater than that observed with the larger polypeptides. This suggests a high degree of similarity in the structure of this determinant in SEB and SEC_1, which is presumably contained in the first 57 amino acid residues of the amino acid sequence.

There was one apparent anomaly in these observations, the failure to find binding of Cam 1-97 to anti-SEC_1. To test the possibility that this was due to inadequate or improper folding of labeled Cam 1-97 upon dilution into 10% BSA, the technique used for all the peptides in this study, several other methods of renaturation were attempted. In all but one there was no significant change. However, when the labeled antigen in 6 M guanidine hydrochloride was dialyzed directly against phosphate buffered saline, the affinity to anti-SEB was increased to 80-fold, and where no reaction had previously been found with the anti-22,000 MW or with anti-SEC_1 excellent binding was now seen. We attribute this enormous change largely to aggregation of the antigen. This effect was reported earlier and is attested to here by an increase in nonspecific precipitation to about 40% of the total radioactivity. The essential point is that this

experiment does demonstrate the heterologous binding of Cam 1-97 to antiserum to SEC_1.

Thus, we have demonstrated the presence of 2 antigenic determinants on SEB and SEC_1 capable of reacting with heterologous antibody. It is, however, difficult to reconcile the weak heterologous binding of the larger polypeptides and the weak homologous binding of the smaller amino terminal peptides with the extremely high binding capacity of the intact enterotoxins for heterologous antibody. Only 4 times the amount of antibody is required to give equivalent binding with the heterologous antigen compared to the homologous antigen for both enterotoxins.

One may invoke the concept of a native format determinant proposed by Sachs et al. (1). All the components of the determinant may be present in a limited length of the polypeptide chain but may exist in solution in equilibrium among a variety of random conformations. One factor in the overall binding constant is a conformational equilibrium constant; the larger polypeptides would appear to have a high constant. They bound very well to the homologous antibody. In addition, circular dichroic (CD) spectra in the far ultraviolet of the 22,000 MW polypeptide and Cam 19,000 are similar to that of the native enterotoxin indicating a significance refolding to a native-like confirmation. Conversely the CD spectrum of the 6,500 MW polypeptide indicates it to be in a random-chain conformation and this fragment would therefore have a comparatively low conformational constant, accounting for its weak antigen-binding capacity.

The CD spectra of the SEB tryptic polypeptides are not, however, consistent with this simple explanation. Both Cam 1-97 and Cam 98-239 have a sufficiently stable folding to provide spectra indicative of significant secondary structure. Furthermore, these spectra differ from those of the parent enterotoxin. The weak binding of Cam 1-97 and the good binding of Cam 98-239 may be interpreted not in terms of conformational constants but as a reflection of the correctness of folding, the latter peptide being in a relatively native conformation and the former having its determinant in a distorted conformation.

It is suggested that the weak heterologous binding of the larger polypeptides is due to an actual compositional difference in the SEB and SEC_1 determinants. The weak homologous binding of the amino terminal polypeptides is considered to be due to a random conformation for the 6,500 MW polypeptide and to an incorrect folding for Cam 1-97. Finally, since the ratio of binding capacity of the 6,500 MW polypeptide with anti-SEB compared to anti-SEC_1 and the ratio for Cam 1-97 with anti-SEC_1 compared to anti-SEB are both very small, it is proposed that the determinant on these fragments is primarily responsible for the excellent heterologous binding in the intact enterotoxins.

It was earlier reported that the 6500 MW polypeptide possessed mitogenic activity for mouse splenic lymphocytes, but the 22,000 MW polypeptide was inactive. We have now demonstrated that the 22,000 MW fragment retains some of the intrinsic biological activity of the native

enterotoxin, i.e., it produces low but significant diarrhea in rhesus monkeys. We have suggested that a residual mitogenic activity of SEB, detoxified by treatment with formaldehyde, implied that the mitogenic and emetic sites of this enterotoxin were not identical (2). The present studies point to a similar conclusion and provide a general localization of the sites responsible for these 2 activities in the closely related SEC_1.

We are attempting to define further the antigenic determinants of SEB; the approach selected is to block the lysine residues making them resistant to trypsin digestion so that only the arginine residues would be susceptible to hydrolysis by this enzyme. The 5 arginine residues in SEB are fortuitiously separated so that the polypeptide fragments would be of suitable size and location in the amino acid sequence.

The first reagent studied was citraconic anhydride. Essentially complete reaction with the ε-amino group of the lysine residues of SEB was obtained. Unfortunately the derivatized toxin was more unstable than had been described for other proteins; during the reaction with trypsin several of the blocking groups were released and about twice as many peptide bonds as desired were cleaved.

A secondary methodology has been investigated more intensively. This is the reaction of an imidoester with amino groups to form amidines. A relatively small group is introduced with preservation of the positive charge. Reaction of SEB with methyl acetimidate hydrochloride yielded a product with no detectable free amino groups. It was, however, almost completely insoluble and in order to hydrolyze it to its constituent polypeptides it was necessary to suspend it in 2 M urea. Tryptic digestion products have not yet been completely analyzed but it has been established that no material of the original molecular size remained. The resultant peptides were still insoluble; either urea or a detergent was required to solubilize them. Although efforts to fractionate the amidinated polypeptides will continue, it is planned to introduce another acylating agent in these studies. Maleic anhydride gives a more stable derivative and the negative charge on the lysine groups usually makes the protein and peptides prepared from it much more soluble at neutral and slightly alkaline pH values.

We have been in correspondence with Dr. Patrick Argos, Purdue University, concerning the use of predictive methods for the secondary structure of SEB. Dr. Argos has computerized 5 different methods for this purpose (3). It is felt that a consensus has greater reliability than any one individual scheme. The results are obtained as a histogram which we have converted to a structural scheme. In many respects it is very similar to the one proposed earlier on the basis of a single methodology. The most striking feature is the extensive anti-parallel β-pleated sheet surrounding the disulfide bridge.

A detailed comparison of the CD spectra of SEA, SEB, and SEC_1 has been carried out. In the far-UV similar curves are apparent containing

one major negative extremum at ∿ 215 nm and one positive band at ∿ 200 nm. A small positive band 235 nm seen in SEB and SEC_1 is missing in SEA. The major negative band is close to the CD band of β-pleated sheet at 217 nm. There is no evidence of the strong negative bands centered at 207 and 222 nm characteristic of α-helix. The ellipticity of the negative band for all 3 enterotoxins is weak, with a maximum amplitude of ∿ -3×10^3 deg-cm^2/dmol. The problem of interpretation of these spectra in terms of the content of α-helix or β-pleated sheet is that it would take about 50% β-pleated sheet to overcome the intense negative band generated by random chain at about 200 nm. The intensity of this observed band at 215-217 nm is far too low for the required amount of β-sheet. Nevertheless 2 attempts were made to fit the data to the responses of various combinations of the 3 contributing structures, α-helix, β-pleated sheet and random chain. The first was a least-squares method (4) and the second an integral method (5). Mr. Higbee, Computer Science Office, attempted the computer solution by the least-squares method and Dr. Irvin Isenberg, of Oregon State University, was kind enough to run out data through his computer program. In neither case was a meaningful fit obtained. The best estimate of secondary structure is obtained from the predictive methods. For SEB this is 12% α-helix and 29% β-pleated sheet.

CD spectra in the far-UV reflect principally the secondary structure of a protein. Above 240-250 nm CD bands also reflect features of secondary structure but are characteristic primarily of tertiary structure. In this region there is a striking similarity in the spectra of the 3 enterotoxins. There are 2 positive overlapping bands between 250 and 260 nm, a small negative band at 263 nm, a small shoulder at 269 nm and two strong negative bonds at 280 nm and 285 nm which overlap. There is finally an extremely weak positive band at 292 in SEA at 297 nm in SEC_1 and ∿ 305 nm in SEB. These results demonstrate a common secondary structure for the enterotoxins and also that they have the same basic tertiary structure. The differences between SEB and SEC_1 are minimal. Some difference is apparent between them and SEA and this probably reflects differences in the number and/or environment of aromatic residues.

A study has been initiated to identify the CD bands in the near-UV. SEB has 6 tyrosyl residues which titrate reversibly between pH 9 and 11.2. When pH in this range is increased a moderate augmentation of ellipticity is seen in the positive bond at 237 nm and a very large increase is seen at 248 nm. The small positive band at 300-305 nm is changed in sign and becomes a weakly dichroic negative band. Similar results were obtained with SEC_1 which has been reported to have 5 exposed, reversibly titrating tyrosyl residues. The band at 237 nm in SEC_1 is weaker than the comparable band in SEB (235 nm) and does not appear to be significantly affected by the alteration of pH. A strong new positive CD band was again seen at 248 nm. Its ellipticity was, however, somewhat less than in SEB (450 compared with 800). The changes in both of these bands are readily attributable to tyrosyl ionization. A change in the 300-nm region was also observed which was about twice as strong as in SEB. When difference spectra between the neutral and alkaline enterotoxin were calculated the extrenum for the band was located at 295-298 nm corresponding exactly with the wave-length

of maximum absorption of ionized tyrosyl residues.

Another methodology used to identify dichroic amino acid residues is the determination of the effect of chemical modification upon CD spectra. We have used the reaction of protein with tetranitromethane. This reagent has a high degree of specificity for tyrosyl residues and has been reported to modify the exposed tyrosines in SEB and SEC_1. In our hands a somewhat greater number reacted, 7 instead of 6 for SEB[1] and 6 instead of 5 for SEC_1. Nitrotyrosine has a greatly reduced pK and the effect of titrating the nitrated derivatives from pH 5 to 8 was determined. The results are remarkably similar for both enterotoxins. The band at 235 nm is clearly involved; however, virtually no change is seen at 248 nm where the greatest alteration occurred in the native enterotoxins. Unanticipated changes were also seen in the 260-290 nm range. The bands in this region had been considered to arise from buried tyrosyl residues (and probably tryptophan). Similar studies with SEA are in progress.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 04 21	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	007
b. CONTRIBUTING				
c. [illegible]	STOG 78-7,2,	1, 3, 6		

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

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.5	222.0
	CURRENT 79	1.0	209.2

19. RESPONSIBLE DOD ORGANIZATION
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POC:DA

21. GENERAL USE
Foreign intelligence considered

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 (Furnish individual paragraphs identified by number. 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 carbohydrate metabolism. The data obtained will be utilized to develop nutrient therapy to prevent the body wasting which is associated with infectious disease. This, in turn, should shorten the time 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.

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) 77 10 - 78 09 - Because of reduced hepatic ketogenic capacity and fatty acid supply the infected host utilizes amino acids as source of energy and for the synthesis of glucose via gluconeogenic pathways. Glucose is utilized at an increased rate in the infected host, especially in pentose phosphate shunt pathways. The use of body proteins to meet the increased energy requirements of the infected host can be prevented by an adequate endogenous supply of amino acids and calories. An endogenous lipid supply can be utilized as a source of energy by an infected host but is less efficient than carbohydrate calories.

Publications: J. Parent. Enteral Nutr. 1:14a, 147-151, 1977; Clin. Res. 26:283A, 587A, 1978; Am. J. Clin. Nutr. 31:700, 1978; Fed. Proc. 37:894, 1315, 1978. J. Parent. Enteral Nutr. 2: in press, 1978.

*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. BS03 00 007: Therapeutic Reversal of Abnormal Host Amino Acid, Protein and RNA Metabolism During Infectious Disease of Unique Military Importance

Background:

The increase in urinary nitrogen which is characteristic of infectious disease is the result of increased breakdown of body protein (especially skeletal muscle) to supply amino acids for anabolic processes associated with host defense and maintenance of hemostasis during periods of reduced food intake (1). To develop rational nutritional therapy to prevent the wasting of body protein, models have been developed in rats and monkeys to study the interaction between substrate supply and defense against infectious disease (2). Even a mild, infectious, 2-3-day illness can result in severe loss of body protein; it will take 2-3 weeks to return to original work capacity (3). Thus, 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 to enter an epidemic area.

Progress:

Glucose and Alanine Metabolism During Bacterial Infections

Studies in rats and rhesus monkeys over the past years on the effects of bacterial infection on glucose and alanine (ALA) metabolism (2) can be summarized as follows:

(a) Glucose synthesis and utilization are increased 2-3-fold during clinical illness as a result of pneumococcal infection. Glucose can be recycled via the Cori cycle, which involves the breakdown of glucose to lactate and pyruvate in peripheral tissues and the resynthesis of glucose from lactate and pyruvate in the liver and to some extent in the kidney. While recycling is increased, it still accounts for only a small percentage of the total glucose synthesis in the infected host. Therefore, the majority of the newly synthesized glucose must come from either glycogenolysis or gluconeogenesis of amino acids (mainly ALA).

(b) By use of a pulse-dose technique in the rat or continuous infusion procedures in the monkey, it was established that the rate of gluconeogenesis of labeled ALA was significantly increased during the acute and agonal stages of a pneumococcal infection. This increase of the gluconeogenic rate may be related to the elevated amounts of circulating glucagon and other glucogenic hormones.

(c) When given an ALA load and/or stimulated with mannoheptulose to measure the maximum gluconeogenic capacity, those rats in the agonal stages of the infection synthesize glucose at a reduced rate compared to fasted controls or those in the acute stage of infection. Similarly, livers from the rats during the agonal stages produce significantly less glucose from 10 mM ALA, pyruvate, or lactate than do fasted controls or those in the acute phase of infection. Thus, in the terminal stages of pneumococcal infection maximum gluconeogenic capacity of the liver is significantly reduced compared to fasted controls or those from rats in the acute stages of illness. This effect in the agonal stages of the infection does not appear to be related to alteration of the intracellular redox state as suggested by Curnow et al. (4). Further, this reduced gluconeogenic capacity did not prevent the livers from rats in the agonal stages of infection from responding to the glucogenic stimuli associated with infectious disease.

(d) During clinical illness from pneumococcal infection in rhesus monkeys, ALA production and utilization were increased almost 2-fold above that seen in control monkeys or during early convalescence. Almost 90% of the ALA produced was utilized for glucose synthesis. It can be concluded that increased glucose synthesis in the infected host is a result of elevated substrate availability (lactate, pyruvate, and amino acids) and rates of hepatic gluconeogenesis.

(e) The increase in glucose utilization appeared to be related to elevated rates of oxidation, especially via the pentose phosphate shunt pathway. The rate of oxidation increased with the severity of both the pneumococcal and tularemia infections in the rat. Since the oxidation of glucose via this pathway is a major source of energy for a number of cells of the body as well as playing a key role in RNA synthesis, the increased glucose production associated with infectious disease may not be merely functional wastage to remove excess nitrogen, but a necessary process in the host defense mechanism.

Studies on the Mechanism of the Inhibition of "Starvation-ketosis" in the Septic Host

For the past 2 years, collaborative studies have been conducted (Work Units BS03 00 008 and A841 00 057) to elucidate the mechanisms responsible for the inhibition of "starvation-ketosis" in the septic host. In man, rhesus monkey, and rat, sepsis associated with bacterial or viral infections prevents the normal increase in plasma ketone bodies associated with fasting. Since ketone body production from the 2 carbon fragments of β-oxidation of fatty acids (FA) only takes place in the liver, the infection-related inhibition of "starvation-ketosis" can be the result of one or a combination of the following: (a) a reduced rate of release of FA from adipose tissue; (b) a decrease in the transport, via serum albumin, of the FA from the adipose tissue to the liver; (c) a reduced rate of hepatic production of ketones from FA; and/or (d) an increased peripheral utilization of ketones. These various possibilities have been studied by a number of experiments which can be summarized as follows:

(a) In vitro rates of lipolysis and esterification in epididymal fat pads were measured in fed or fasted rats exposed to either live or heat-killed Streptococcus pneumoniae. The weight of the fat pads decreased with fasting at the same rate in both control and infected rats. Fasting increased glycerol production (an indicator of elevated rate of lipolysis) and reduced the percentage of free FA esterification. However, no differences were noted between fasted-control and infected rats. When the body fat was prelabeled with [^{14}C], the in vivo rate of oxidation, as measured by production of [$^{14}CO_2$], was significantly increased in fasting; again, no differences were noted between control and infected rats. Further, no differences were noted in the in vivo rates of oxidation of labeled palmitic or oleic acid in fasted-control or infected rats. These observations suggest that the inhibition of "starvation-ketosis" in an S. pneumoniae infection in the rat is not related to reduced rates of lipolysis, increased rates of esterification, or decreased rates of release of FA from adipose tissue; nor is there an increase in the rate of tissue utilization of FA.

(b) During S. pneumoniae infection in the rat, plasma albumin and free fatty acid concentrations were both significantly reduced when compared to fasted controls. The molar FA/albumin ratio, however, was not altered by infection. Thus, in this infection, the decrease in plasma free FA concentration appeared to be related to the reduction in plasma albumin, the major transport component for FA in the blood. This decrease in free FA concentration did not appear to influence the rate of whole body oxidation of prelabeled fat stores in infected rats. Studies involving the rate of infection on hepatic extraction of free FA will be required to determine what effect this reduced supply has on the rate of liver uptake and ketone production.

(c) When infected rats were injected IV with a lipid load, serum triglycerides disappeared at an increased rate; no difference was noted in the rate of production and utilization of free FA, but the rate of increase of β-hydroxybutyrate and cholesterol was significantly less in infected rats compared to fasted-controls. These data suggest that infection has altered the liver's ability to utilize free FA for ketone body production. To test this conclusion, livers from pneumococcal and tularemia-infected rats were perfused with either a long-chain (oleic) or a short-chain (octanoic) FA. Fasting increased the rate of ketosis from a long-chain FA; this rate was similarly reduced by 48 hr in the S. pneumoniae infection and by 24 or 48 hr in the Francisella tularensis (LVS) infection. No significant alterations were noted in the rates of ketosis when fasted or infected rats were perfused with octanoic acid. These data demonstrate that the liver from the infected host had a reduced ability to synthesize ketones when supplied with an excess of long-chain FA. No differences were noted in the rate of uptake of the oleic acid by the infected or control livers. By use of labeled FA it was shown that ketone body production was reduced by 50% in livers from infected rats, while there was almost a 2- to 3-fold increase in the label associated with the hepatic lipids compared to fasted controls. Further, livers from infected rats had a higher lipid content and an increased amount of radioactivity associated with the lipid fraction when the

livers were perfused with [^{14}C] octanoic or oleic acid. Since octanoic acid must first be broken down to 2 carbon fragments which are then synthesized to FA and triglycerides, it can be concluded that the S. pneumoniae infection stimulated an increase in both synthesis of the acids and formation of triglycerides, and a decrease in the rate of ketosis.

(d) By measuring the rate of [$^{14}CO_2$] production after IV injection of labeled hydroxybutyrate or acetylacetate it was determined that no difference was detectable in the rate of oxidation of these 2 ketones during an acute pneumococcal infection and fasted controls but was reduced in the agonal stages of the infection. Because of the reduced extracellular pool of these ketones, tissue utilization of these substrates was markedly reduced. Thus, the reduced plasma ketone concentration in infected rats was not the result of increased utilization.

From these studies it can be concluded that the infection-related inhibition of "starvation-ketosis" is the result of decreased hepatic production and perhaps a reduced substrate supply because of the decrease in plasma albumin concentration.

Rat Parenteral Nutrition Model

Utilizing the rat parenteral nutrition models described previously (2), studies have been completed on alterations in protein metabolism during S. pneumoniae infection in rats maintained on a number of different solutions. As reported previously (2), when rats were maintained on 25% dextrose plus 4.25% amino acid 4 days postsurgery, they were all in positive nitrogen balance at the start of the experiments (day 1). When switched to a solution supplying electrolytes and vitamins only, both infected and control rats went into a severe negative balance with nitrogen losses being significantly greater in the infected group. The addition of 5% dextrose also resulted in negative balances with little difference between infected and control rats. When rats were infused with a 4.25% amino acid solution, they were in positive balance the first day after exposure; on the second day they went into negative nitrogen balance significantly different from that observed in heat-killed controls. The addition of 5% dextrose to the amino acid mixture maintained both the infected and noninfected rats in positive nitrogen balance for both days of the experiment with the infected group being in a slightly less positive balance on day 2. Similar results were observed when the amino acid solution was supplemented with 25% dextrose.

In man (5), the addition of hydrocortisone to a 6.5% dextrose, 4.25% amino acid (P-900) solution reduced the irritation produced when this solution was infused via a peripheral vein. In the rat, hydrocortisone reduced nitrogen retention compared to the 5 or 25% dextrose plus amino acid solutions and added to the nitrogen wasting in the infected group. When this P-900 solution was infused for an additional 4 days both control and penicillin-treated groups went into marked negative balances. In contrast, both infected and heat-killed controls infused with 25% dextrose plus amino acids were in positive balance throughout the 6-day experimental period.

The addition of 10.4% of 1,3-butanediol to the P-900 solution improved the nitrogen retention slightly, but was again inferior to the amino acid plus 25% dextrose solution. Butanediol can be rapidly converted to ketones and has the caloric density of approximately 6 cal/gm. Thus the addition of 10.4% to the P-900 made it isocaloric, with a standard hyperalimentation solution. When fed the former solution rats excreted approximately 0.5 mmol of β-hydroxybutyrate/day in urine and had plasma concentrations of ∿ 2.5 mmol/ml. Thus, both infected and noninfected rats were able to convert butanediol to ketones with equal efficiency, but tissues were not able to utilize these ketones effectively which resulted in a spilling in the urine. There is some indication that the rats adapted and were able to utilize the ketones more effectively as an energy source in the later stages of the experiment. Butanediol has the advantage of being water soluble with a higher caloric density than dextrose. The compound is rapidly converted to ketones but it may take several days for the cells in the body to adapt to utilization of these high quantities of ketones as an energy source. These studies may have been complicated by the fact that it was put into the P-900 solution which contained hydrocortisone, which was infused at the rate of 1.25 mg/kg/day. The data indicate that this amount of hydrocortisone apparently stimulated catabolism of peripheral tissues and may have prevented the effective use of ketones as an energy source.

Of interest was the fact that the plasma albumin concentrations were higher in the P-900 plus butanediol solution than in the standard hyperalimentation solution. Apparently the hydrocortisone stimulates a flux of amino acids from peripheral tissues which can then be utilized for synthesis of some of the visceral proteins. In contrast, this solution stimulates insulin release, and sequestration of amino acids in peripheral tissues and favors synthesis of proteins in tissues such as skeletal muscle. Studies are currently in progress to evaluate further the use of butanediol as an energy substrate in hyperalimentation solutions which do not contain hydrocortisone.

Monkey Parenteral Nutrition Model

Studies have now been completed on alterations in protein metabolism during S. pneumoniae infection in rhesus monkeys maintained (2) on a solution that supplies 0.5 gm of amino acid nitrogen/kg/day plus 85 cal/kg/day from dextrose. These results are compared with the previously reported data (2) when the monkeys were maintained on a solution that supplied the same amount of amino acid nitrogen/kg/day with or without 85 cal/kg/day from Intralipid.

When noninfected monkeys were infused with the amino acid mixture alone, they lost 2.3 ± 0.7% of their body protein over the 6-day experimental period. In contrast, the infusion of dextrose or Intralipid with the amino acid mixture resulted in retention of nitrogen equivalent to 2.7 ± 0.6% and 1.1 ± 0.4%, respectively, of their initial body protein over the 6 days. Thus, in the noninfected monkey lipid calories were utilized as a source of energy but may not have been as effective as an isocaloric infusion of dextrose.

Infected monkeys infused with amino acids alone lost 2.6 ± 0.5 gm nitrogen/kg or 12.5 ± 2.5% of their body protein over the 6-day experimental period. In contrast, the addition of dextrose plus amino acids resulted in a significant retention of nitrogen equivalent to approximately 1.1 ± 0.7% of the initial body protein, while the amino acid plus Intralipid infusion resulted in a loss of 1.8 ± 0.7% of the body nitrogen in the septic group. These data support the earlier conclusion that lipid calories with amino acids significantly reduce the nitrogen wasting associated with the infectious process but they are less efficient than the dextrose plus amino acid solution. Monkeys receiving this latter solution developed a slight negative balance during the illness phase of the infection which quickly reversed during the recovery phase so that they had a net positive balance not significantly different from that observed in noninfected monkeys infused with the amino acid-dextrose solution.

As reported previously (2), infected monkeys infused with the amino acid solution alone had severely reduced concentrations of plasma and urinary β-hydroxybutyrate and plasma free FA values. This resulted in a 4-fold increase in utilization of branched-chain amino acids by skeletal muscle as an energy source with a marked increase in the production of ALA and glutamine (GLU) in these tissues. This reduction in ketone body production and free FA mobilization was not correlated with a consistent alteration in peripheral plasma insulin concentrations. Thus, in the absence of exogenous calories, the infected host breaks down body protein to utilize amino acids as an energy source resulting in a rapid wasting of body protein during an acute infection of rather short duration. When the infected monkey was infused with amino acids and lipids, plasma free FA increased to approximately 4 mmol/L, plasma β-hydroxybutyrate was maintained throughout the infection at approximately 1.5 mmol/L, with a slight decrease in urinary β-hydroxybutyrate concentration. Thus, the infected monkey was able to utilize endogenous free FA for ketone body production; the higher free FA concentrations and lower β-hydroxybutyrate values suggested reduced efficiency for ketone body production as compared to noninfected controls. Also, infected monkeys infused with the amino acids plus lipid developed severe hypertriglyceridemia compared to the noninfected group. Thus, while the infusion of amino acids and lipids markedly reduced the wasting of body proteins associated with infectious disease, the reduced efficiency of conversion of the FA to ketones in these monkeys resulted in a slight breakdown of body protein with the utilization of the amino acids as an energy source.

When infected and control monkeys were infused with amino acids plus dextrose, plasma free FA and β-hydroxybutyrate concentrations were reduced to very low values, while plasma insulin was increased nearly 10-fold. Under these conditions the dextrose became the exclusive substrate utilized by most cells as an energy source. During the febrile phase of the infectious illness the monkeys developed slight negative balance suggesting that their caloric requirements exceeded 85 cal/kg/day.

In both infected and control monkeys maintained on amino acids and dextrose, plasma albumin progressively decreased over the experimental

period. A marked decrease in plasma albumin was also observed in infected monkeys given amino acids alone. In contrast, plasma albumin was maintained or slightly increased in the infected and noninfected monkeys maintained on the amino acid plus lipids. These observations, along with nitrogen balance, suggest that the hyperinsulinemia observed in the monkeys infused with dextrose stimulates a flux of amino acids to peripheral tissues and the increased synthesis of proteins associated with these tissues, while the amino acid and lipid solution tend to favor synthesis of visceral proteins.

Energy Utilization by Skeletal Muscle During Sepsis in Rhesus Monkeys

Skeletal muscle can utilize a number of substrates as a source of energy, including glucose, fatty acids, total ketones (hydroxybutyrate and acetylacetate), and branched-chain amino acids. In the monkey parenteral nutrition model, a catheter was inserted into the femoral vein and passed to the vena cava proximal to the bifurcation of the iliac vein and was used to obtain venous blood samples from the lower hind-quarter. A catheter was inserted into the carotid artery and passed to the level of the aorta for obtaining arterial samples. Thus, blood samples obtained during the course of study from the carotid artery and femoral vein catheters were utilized to determine arterial-venous differences mainly across the skeletal muscle of septic and control monkeys infused IV with a solution that supplied either 0.5 gm of amino acid nitrogen/day or one that supplied amino acid nitrogen plus 85 cal/kg/day from either dextrose or a lipid emulsion.

When infused with amino acid alone both infected and control monkeys took up glucose from skeletal muscle, while FA appeared to be released, perhaps as a result of lipolysis in adipose tissue. Lactate was released from skeletal muscle as a result of metabolism of the glucose; its rate of release was significantly increased during illness. The monkeys developed starvation ketosis as indicated by an uptake of β-hydroxybutyrate on day 0 before starting the amino acid infusion. Muscles from control monkeys continued to take up β-hydroxybutyrate; in the infected group during illness, and even recovery, there was a significant decrease in its utilization by skeletal muscle. Total amino acids were lost from muscles throughout the study with an increase observed during illness in the septic group. ALA and GLU were also lost from skeletal muscle, at increased rates in the septic monkey. In contrast, branched-chain amino acids were taken up by the skeletal muscle, at an increased rate during illness in the septic group.

The addition of dextrose to the infusion media significantly increased the uptake of glucose by skeletal muscle. This uptake was even more marked during illness in septic monkeys. Lactate was again lost from skeletal muscle but no difference was noted between control and infected groups nor was there any effect by adding dextrose. The addition of dextrose markedly reduced lipolysis and ketosis so that there was very little exchange of free FA or β-hydroxybutyrate in monkeys infused with this solution. The dextrose plus amino acid mixture stimulated uptake of amino acids by skeletal muscle. The septic monkeys continued to release ALA and GLU, while in the controls there was essentially no signifi-

cant exchange of amino acids. The infusion of dextrose into septic or control monkeys resulted in an uptake of branched-chain amino acids with no significant difference between groups. It may be concluded that when the monkey is infused with amino acids and adequate carbohydrate calories, skeletal muscle utilizes glucose as a major metabolic fuel. Since lactate production was not increased most of the glucose was probably oxidized completely to CO_2 and water. This parenteral solution also stimulated the uptake of amino acids by skeletal muscle but the reduced excretion of alanine and glutamine indicated that amino acids did not enter pathways of transamination and oxidation but were probably utilized for protein synthesis. During pneumococcal septicemia there was a slight increase in ALA and GLU excretion which was not as marked as that seen when animals were given amino acids alone. Therefore, it appears that the infectious process increases the metabolic needs of the skeletal muscle, which under the conditions of our study resulted in slight utilization of amino acids as energy substrates.

The infusion of amino acids plus lipid resulted in a significant uptake of glucose compared to fasting values. Lactate production was significantly increased during illness in septic monkeys. Despite a 5- to 10-fold increase in arterial free FA concentrations, skeletal muscle of both septic and control monkeys did not take up these acids during the course of the experiment. In contrast, skeletal muscle did take up β-hydroxybutyrate, suggesting that the muscle of rhesus monkeys did not effectively utilize free FA as an energy substrate. This may be related to the fact that monkeys were infused with an amino acid solution relatively high in branched-chain amino acids. When monkeys were infused with amino acids plus lipids there was very little uptake or release of total amino acids in control or septic monkeys. In the control monkeys, ALA release tended to decrease, while in septic monkeys it was elevated during illness. Very little change was noted in GLU metabolism in either control or septic monkeys. In contrast, branched-chain amino acids were taken up at increased rates during illness in the septic group. Thus, during pneumococcal sepsis in the monkey infused with the amino acid plus lipid, skeletal muscle increased its uptake of branched-chain amino acids, which are transaminated to form ALA and utilize the carbon skeleton as an energy source.

Trace Metal Metabolism During Pneumococcal Sepsis in the Monkey

Most parenteral nutrition solutions are deficient in Zn, Fe, and Cu; therefore, a study was initiated to determine what effect pneumococcal sepsis would have on metabolism of these trace elements when used as supplements to parenteral nutrition solutions. Preliminary observations indicate that when no supplement was added to the IV alimentation solutions, marked negative balances were noted for these metals in both control and septic monkeys. During oral feeding the rhesus monkey requires approximately 1 mg/kg/day of Zn. When monkeys were infused with 0.38 mg/kg/day there was marked positive balance, resulting in accumulation of more than 1 mg of Zn during a 6-day experimental period in both control and septic monkeys. Similar marked uptake of Fe and Cu were observed when they were added to the infusion media. Supplementation with them

resulted in only slight increases in plasma concentration while appreciable accumulation of Cu was observed in the septic monkeys. Results suggest that these trace elements are taken up and rapidly sequestered within the various tissues of both infected and control monkeys and that plasma concentrations do not necessarily reflect this uptake. Since fecal losses are variable and sporadic in a monkey supported by a parenteral nutrition solutions, the major loss in trace elements during sepsis is via the urine. If one bases the requirements on urinary excretions of both control and septic monkeys during IV infusion, then they require 150 mg of Zn, 100 mg of Fe, and 30 mg Cu/kg/day. These values are about 1/10 the requirements for oral feeding. Future studies will involve supplying these concentrations of trace elements to determine if they will maintain body stores. Since the intestinal tract plays an important role in regulating their intake, care should be exercised when giving these trace elements by IV alimentation, to prevent overloading the body, perhaps resulting in toxicity.

Presentations:

1. Wannemacher, Jr., R. W. The unique role of amino acid metabolism during infectious illness. At a symposium on "Metabolic Response to Infection" at the InterScience Conference on Antimicrobial Agents and Chemotherapy Meeting, New York, NY, 12-14 Oct 1977.

2. Wannemacher, Jr., R. W., M. V. Kaminski, H. A. Neufeld, R. E. Dinterman, and C. L. Hadick. Protein sparing therapy during pneumococcal sepsis in the rhesus monkey. Presented, American Society for Parenteral and Enteral Nutrition, Houston, TX, 2-4 Feb 1978 (J. Parent. Enteral Nutr. 1:14A, 1977).

3. Wannemacher, Jr., R. W., R. E. Dinterman, and C. L. Hadick. Metabolic fuel utilization by skeletal muscle of rhesus monkeys during pneumococcal sepsis. Presented, Annual Meeting, FASEB, Atlantic City, NJ, 9-14 Apr 1978 (Fed. Proc. 37:894, 1978).

4. Wannemacher, Jr., R. W., E. C. Hauer, and C. L. Hadick. Zinc (Zn), iron (Fe), and copper (Cu) metabolism during intravenous hyperalimentation (HA) in septic and non-septic rhesus monkeys (RM). Annual Meeting, American Society for Clinical Nutrition, San Francisco, CA, 28 Apr 1978 (Clin. Res. 26:587A, 1978, Am. J. Clin. Nutr. 31:700, 1978).

5. Wannemacher, Jr., R. W., R. E. Dinterman, K. D. Burman, and L. Wartofsky. The possible role of thyroid hormone in regulating the rates of protein degradation in skeletal muscle. Annual Meeting, American Society of Biochemists and American Association of Immunologists, Atlanta, GA, 4-8 Jun 1978 (Fed. Proc. 37:1315, 1978).

Publications:

1. Wannemacher, Jr., R. W., and W. R. Beisel. 1977. Metabolic response of the host to infectious disease, pp. 135-159. In Nutriitonal Aspects of the Care of the Critically Ill (J. R. Richards and J. M. Kinney, eds). Churchill-Livingstone, Edinburgh.

2. Kaminski, Jr., M. V., N. P. Dunn, R. W. Wannemacher, Jr., R. E. Dinterman, R. DeShazo, W. W. Wilson, and D. E. Carlson. 1977. Specific muscle protein-sparing postoperative dextrose-free amino acid infusions. J. Parent. Enteral Nutr. 1:147-151.

3. Bistrian, B. R., D. T. George, G. L. Blackburn, and R. Wannemacher. 1978. Metabolic response to yellow fever vaccine during a modified fast. Clin. Res. 26:283A.

LITERATURE CITED

1. Wannemacher, Jr., R. W. 1977. Key role of various individual amino acids in host response to infection. Am. J. Clin. Nutr. 30:1269-1280.

2. U.S. Army Medical Research Institute of Infectious Diseases. 1 October 1977. Annual Progress Report, FY 1977, pp. 442-449. USAMRIID, Fort Detrick, Frederick, MD.

3. Beisel, W. R. 1977. Magnitude of the host nutritional responses to infection. Am. J. Clin. Nutr. 30:1236-1247.

4. Curnow, R. T., E. J. Rayfield, and F. A. Beall. 1974. Impaired hepatic gluconeogenesis (GNG) caused by altered intracellular redox state (IRS) during pneumococcal (P) infection in rat. Diabetes 23(Suppl 1):369.

5. Isaacs, J. W., W. J. Millikan, J. Stackhouse, T. Hersh, and D. Rudman. 1977. Parenteral nutrition of adults with a 900 milliosmolar solution via peripheral veins. Am. J. Clin. Nutr. 30:552-559.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0F6412	78 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
78 04 21	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	008
b. CONTRIBUTING				
c. 7/7/8/7/7	STOG 78-7,2,	1, 3, 6		

11. TITLE (Precede with Security Classification Code)

(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	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.5	290.0
	CURRENT 79	1.0	147.9

19. RESPONSIBLE DOD ORGANIZATION

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TELEPHONE: 301 663-2833

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

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TELEPHONE: 301 663-7181

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Beall, F. A.

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Inflammation; (U) Infection; (U) Metabolism; (U) Ketone bodies; (U) Lipids; (U) Trauma; (U) Stress

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

23 (U) To maintain by appropriate therapy the body stores which are 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 utilizes its own fat and protein to supply energy to various cells. Decreased ability of the host to utilize its fat stores during infectious disease could explain the marked protein-wasting associated with illness. An understanding of these metabolic changes can lead to effective nutrient therapy which would reduce the protein-wasting and promote rapid recovery.

24 (U) Microanalytical methods are applied to the study of various metabolites and their alterations caused by infection.

25 (U) 77 10 - 78 09 - Studies have continued on the inhibition of ketone body production which occurs during all inflammatory stresses examined. The stresses which have been examined are: bacterial infection (Streptococcus pneumoniae, Francisella tularensis, and Salmonella typhimurium); viral infections (2 strains of VEE); induction of a sterile turpentine abscess; and administration of endotoxin. The fact that inhibition of starvation ketosis accompanying inflammation does not occur in rats made diabetic implicated insulin as playing a major role. In addition, it has now been found that inhibition of ketosis accompanying inflammation also does not occur in hypophysectomized rats. These data suggest that inflammation causes a direct effect on the pituitary gland which, in turn, stimulates the pancreas to release insulin during the anorectic state, causing the decrease in the level of circulating ketone bodies.

Publications: J. Lab. Clin. Med. 91:255-263, 1978.
Fed. Proc. 31:1505, 1978.

*Available to contractors upon originator's approval

DD FORM 1498 (MAR 84) PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 84 (FOR ARMY USE) ARE OBSOLETE

* U.S. GPO: 1974—540-843/8691

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BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 008: Therapeutic Correction of Energy Metabolism Alterations During Infection of Unique Importance in Military Medicine

Background:

Previous reports from this laboratory and others in recent years have shown that one of the prime metabolic alterations accompanying infection is extreme protein wastage. Accompanying this negative nitrogen balance are 2 extremely important and related metabolic alterations. With every inflammatory stress there is an increase in the rate of gluconeogenesis which persists until the animal reaches the moribund state, at which time the rate decreases; and there is inhibition of the ketosis which normally occurs during the fasting state. The major emphasis of this study has been to assess the importance of the inhibition of ketone production, to attempt to learn as much as possible about the mechanism of this response, and to assess the role of this inhibition in the treatment of the inflammatory states.

Progress:

In normal mammals fasting or starvation causes a rapid and dramatic increase in the plasma concentration of acetoacetate and β-hydroxybutyric acid, the ketone bodies. This high concentration of ketone bodies serves to spare nitrogen and also provides a fuel for such vital tissues as heart, skeletal muscle, and brain. The presence of any inflammatory stress so far tested in rats causes a marked decrease in the concentration of ketone bodies during the anorexia which accompanies it. Among the inflammatory stresses so far examined are the following: (a) bacterial infections as typified by Streptococcus pneumoniae, Francisella tularensis, and Salmonella typhimurium; (b) viral infections, as typified by infection with 2 strains of VEE; (c) induction of a sterile turpentine abscess; and (d) induction of endotoxemia.

It is of great interest and significance to note that accompanying the decrease in the concentration of ketone bodies is a decrease in the concentration of free fatty acids (FA). These 2 aberrations have, in our experience, been noted singly only when rats were subjected to noninflammatory stresses, such as screen restraint or femoral fracture, where an inhibition in the level of free FA was observed but not an inhibition in ketone body concentration.

The decrease in plasma free FA has been correlated with the decrease in plasma albumin. In the plasma of animals subjected to inflammatory stress the decrease in free FA is accompanied by a decrease in plasma

albumin. The ratio, however, of free FA to albumin remains relatively constant. Data such as these seem to indicate that the decrease in free FA during inflammatory stress may be due to the fact that during this stress the concentration of plasma albumin declines by over 50%.

When, however, livers of infected animals are perfused with the same concentration of oleic acid as the livers of healthy controls, the rate of ketone body formation is markedly depressed. These data suggest, then, a correlation between plasma albumin concentration and plasma free FA concentration but a lack of correlation between free FA concentration and ketone body concentration.

In Annual Report, FY 1977 (1), it was reported that there was a relationship between insulin and the inhibition of starvation-induced ketosis during periods of inflammatory stress. In the past year much more substantial information has been collected which tends to confirm the earlier report. In rats made diabetic by the administration of streptozotocin and then subjected to an inflammatory stress, no inhibition of fasting ketosis could be detected. Moreover, if small amounts of insulin were administered to diabetic rats already ill, the level of ketosis was reduced by the same amount on both infected and control rats.

In another experiment, insulin was administered IV to fasted normal rats whose plasma ketone level was already elevated. In this case there was a very rapid decline in the level of plasma ketones.

These data tend to support the hypothesis that there is a strong link between the level of insulin in plasma and the degree of ketosis. More credence to the relationship between insulin and the inhibition of fasting ketosis observed in the inflammatory state is given by the fact that rats subjected to a variety of inflammatory stresses all show a dramatic rise in the level of plasma insulin despite the fact that no food is provided the animals. As yet, no data is available which gives a clue as to the mechanism of action of insulin in this instance.

More recently, data have been obtained which show that when hypophysectomized rats are subjected to an inflammatory stress there is no inhibition of fasting ketosis. Moreover, the level of insulin in stressed hypophysectomized animals is very low.

All of these data have allowed us to formulate the following hypothesis: induction of an inflammatory stress causes the release of some factor which travels via the blood to the hypophysis. Some type of pituitary stimulation, possibly hormonal, then reaches the pancreas causing a release of insulin which, by a mechanism not yet understood, causes an inhibition of fasting ketosis.

Presentations:

1. Kaminski, M. V. and H. A. Neufeld. Specific metabolic similarities and differences between septic and non-septic stress. Presented, Western Hemisphere Nutrition Congress V, Quebec City, Canada, Aug 1977.

2. Neufeld, H. A. The effect of inflammatory stress on the ketosis induced by fasting. Presented, Department of Biochemistry, University of Maryland Dental School, Apr 1978, Baltimore, MD.

3. Neufeld, H. A., J. A. Pace, M. V. Kaminski, D. T. George, R. W. Wannemacher, and W. R. Beisel. The role of insulin in the inhibition of starvation ketosis by inflammatory stress. Presented, Annual Meeting, American Society of Biological Chemists, Atlanta, GA, Jun 1978 (Fed. Proc. 37:1505, 1978).

4. Neufeld, H. A. The mission of USAMRIID. Presented, Israel Institute of Biological Sciences, Ness-Ziona, Israel, Jun 1978.

5. Neufeld, H. A. The detection of air-borne biologicals by luminescent procedures. Presented, Israel Institute of Biological Sciences, Ness-Ziona, Israel, Jun 1978.

6. Neufeld, H. A. The effect of inflammatory stress on ketone body formation in the rat. Presented, Tel-Hashomer Hospital, Tel-Aviv, Israel, Jun 1978.

Publication:

Neufeld, H. A., M. C. Powanda, A. DePaoli, J. A. Pace, and P. B. Jahrling. 1978. Host metabolic alterations during Venezuelan equine encephalitis in the rat. J. Lab. Clin. Med. 91:255-263.

LITERATURE CITED

1. U.S. Army Medical Research Institute of Infectious Diseases. 1 Oct 1977. Annual Progress Report, FY 1977. pp. 451-455. Fort Detrick, Frederick, MD.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6422	78 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. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 04 21	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	013
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 78-7,2,	1, 3, 6		

11. TITLE (Precede 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	CONT	DA	C. In-house

17. CONTRACT GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.0	110.0
	CURRENT 79	1.0	310.5

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
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SOCIAL SECURITY ACCOUNT NUMBER:

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NAME: McCarthy, J. P.
NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Leukocytes; (U) Polymorphonuclear leukocytes; (U) Laboratory animals; (U) Bacteria; (U) Viruses

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

23 (U) Determine extent to which agents of BW interest can cause changes in circulating leukocytes; determine response of leukocytes to opsonized pathogens for antigen-antibody complexes to see if specificity or sensitivity is enough to use for rapid diagnosis.

24 (U) Using a rat model with S. pneumoniae, F. tularensis or Venezuelan equine encephalitis measure leukocyte functions as measured by chemiluminescence, phagocytosis and chemotaxis.

25 (U) 77 10 - 78 09 - Preliminary results obtained in studies using rats infected with LVS F. tularensis suggest that this infection induces enhancement of polymorphonuclear leukocyte chemiluminescence prior to onset of fever and other overt clinical signs of illness. These results differ from those reported by others using LVS and S. pneumoniae infections in rats. The reason for this apparent discrepancy is at present unknown. Work is planned to resolve these diverse findings.

*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

U.S. GPO 1971—840-847/8601

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 013: Changes in Leukocyte Function During the Course of Viral and Bacterial Infections

Background:

The polymorphonuclear leukocyte (PMN) is one of a number of circulating cells which play an essential role in defense against infection. It has as its primary function the movement to, ingestion and destruction of, microorganisms as well as other foreign bodies.

The physiology of PMN is controlled primarily by cell surface receptors. These receptors control random mobility, directed chemotaxis, phagocytosis of foreign material and the exopinocytosis of lysosomal enzymes and destroyed or inactivated microbes. Surface receptors also control azurophilic and specific granules which contain and release into the phagosomes, enzymes and other proteins involved in killing and digestion of cells. Stimulation of glucose metabolism and the release of activated oxygen superoxide anion, singlet oxygen, hydroxyl radical, and hydrogen peroxide also takes place at the plasma membrane (1). The return to stable state of the excited oxygen species results in the emission of photons. This phenomenon, termed chemiluminescence, was first observed by Allen et al. (2).

The surface receptors of leukocytes can be activated by immunoglobulins (IgG, IgA), complement components (C_3b, C_5a, and C567), tripeptides, such as Form-MET-LEU-PHE, and certain other compounds which include bacterial and host lipids. In addition to activation of certain PMN functions by various agents, inhibition is also known to occur. Schlisinger et al. (3) and others (4) noted that influenza virus particles at a ratio of 10 viral particles/cell inhibited PMN chemotaxis and phagocytosis but no effect on hexose monophosphate shunt was observed.

Although several functions of PMN will be investigated during the course of various infections, initial emphasis has been placed on determining the usefulness of PMN chemiluminescence for the early detection of infectious diseases of importance to military medicine. The possibility exists that differential effects on various aspects of PMN physiology may provide information useful as an aid in diagnosis and agent identification.

Progress:

Considerable effort during this period has been devoted to the identification and control of physical parameters which influence the measurement of PMN chemiluminescence. For example, it has been observed that temperature of the reaction mixture must be strictly controlled to achieve reproducible results with respect to the extent of photon emission. Other

factors which may influence results are currently under investigation and include cell separation methods, reaction medium composition, time-course characteristics of photon emission, and detector geometry.

Studies have been initiated using LVS Francisella tularensis infection in rats to provide PMN for the performance of initial work cited above. Although highly preliminary results suggest that this infection induces a significant enhancement of chemiluminescence in circulating PMN prior to onset of fever and other overt clinical signs of illness. These results differ considerably from those reported by the previous investigator (Dr. F. Abeles, in 2nd Quarterly Report FY 78) using LVS and Streptococcus pneumoniae infections in rats in which a decrease in chemiluminescence was observed. The reason for this apparent discrepancy is at present unknown. Work is planned to resolve these diverse findings.

Publications:

None

LITERATURE CITED

1. Henson, P. M. 1976. Membrane receptors on neutrophils. Immunol. Commun. 5:757-774.

2. 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.

3. Schlesinger, J. J., C. Ernst, and L. Weinstein. 1976. Inhibition of human neutrophil chemotaxis by influenza virus. Lancet 1:650-651.

4. Larson, H. E., and R. Blades. 1976. Impairment of human polymorphonuclear leucocyte function by influenza virus. Lancet 1:283.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6411	78 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
78 04 21	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	015
b. CONTRIBUTING				
c. //////////	STOG 78-7,2,	1, 3, 6		

11. TITLE (Precede 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	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.0	125.0
	CURRENT 79	1.0	146.0

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) Lipogenesis; (U) Plasma membranes; (U) Enzyme regulation; (U) Macrophages; (U) Cytochemistry

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish 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 membrane.

25 (U) 77 10 - 78 09 - Several basic functions of cell membranes were investigated during Streptococcus pneumoniae infection in rats. These include the synthesis of Golgi and plasma membranes; synthesis, intracellular transport, and secretion of plasma proteins; and the binding of radiolabeled hormones. It was shown that during infection the liver maintains its capability to perform the basic functions of protein synthesis, intracellular transport and secretion, and that newly synthesized plasma proteins follow the same intracellular pathway in both control and infected rats.

In addition, initial experiments suggest that there is increased synthesis of liver cell plasma membranes during infection. Since this is a secretory organelle, it may be reasonable to assume that more of this organelle is required during infection since the liver is processing more secretory proteins.

Publication: J. Cell Biol. 75:364a, 1977.
Infect. Immun. 22: in press, 1978.

*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. * U.S. GPO 1974—540-843/8501

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BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 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:

Streptococcus pneumoniae infection in rats causes a significant number of metabolic changes in the liver (1); the liver, in addition to its many other functions, is responsible for the synthesis of many plasma proteins. It is known that liver cell membranes are involved at every stage in the synthesis, transport, packaging, and secretion of plasma proteins and that S. pneumoniae infection significantly alters the concentration of many of these proteins in plasma. A series of experiments was designed which would not only answer questions concerning the effects of infection on the function of liver cell membranes but also the effects of infection upon the basic liver function involving protein synthesis, transport, packaging, and secretion.

A pulse of radiolabeled protein was followed through the intracellular secretory pathway of the liver. To perform these experiments, it was first necessary to isolate the secretory components from control and infected rat liver homogenates. Although these components have been isolated from control animals and partially characterized by others, this work represents the first isolation and partial characterization of rough microsomes, smooth microsomes, and Golgi from infected rats. Since it has been shown that S. pneumoniae infection not only alters the enzyme complement (2), but also the number (3) and density (4) of hepatic organelles, this effort was especially important.

Groups of 6 rats were inoculated with 3×10^5 heat-killed (control) or live (infected) S. pneumoniae and 40 hr later injected with 100 µCi of [^{3}H]leucine. At 90 min the animals were sacrificed. The specific activity of the label in the intracellular secretory components [rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), and Golgi] of the liver and plasma was determined.

It was first necessary to isolate and characterize RER, SER, and Golgi from infected animals. It was shown by the use of enzyme markings and electron microscopy that the isolated components were pure.

Following infection the specific activity of label in liver homogenates and cell fractions isolated from the liver was significantly increased. In both control and infected animals the kinetics of labeling suggested that the secretory pathway of the newly synthesized protein was consistent with the conventional pathway, i.e., RER, to SER, to Golgi, and finally to the circulation. It is concluded that following S. pneumoniae infection the liver still maintains its capability to perform the basic functions of protein synthesis, intracellular transport and secretion and that newly synthesized secretory protein follows the same intracellular pathway in both control and infected rats.

In order of study the synthesis of groups of 6 rats were inoculated with 3×10^5 heat-killed (control) or virulent (infected) S. pneumoniae; 40 hr later they were injected via the dorsal penile vein with 100 μCi of [^{3}H]leucine. At various time intervals thereafter, plasma membranes were isolated from both groups of rats (5); radioactivity incorporated into plasma membrane protein was determined. When the activity was expressed as CPM/mg plasma membrane protein there was a slight but significant increase in incorporation of label into plasma membrane protein during infection. However, when it was expressed as CPM/total liver plasma membrane protein, a very significant increase in the incorporation of label was observed during infection. The explanation for this larger increase can be explained by the fact that the size of the liver increased significantly during infection. These results suggest increased synthesis of liver plasma membranes during infection. Since the plasma membrane is involved in secretion of plasma proteins, it may be reasonable to assume that more of this secretory organelle is required during infection since the liver is processing more secretory proteins.

To study the synthesis of Golgi membranes after pneumococcal sepsis, rats were inoculated with S. pneumoniae and injected with radioisotope as described. At various time intervals after the injection of isotope, Golgi were isolated from both groups. Radioactivity was determined. There was a peak in Golgi of both groups at about 20 min after the injection of the isotope and then a subsequent decrease. This decrease was due to secretion of plasma proteins from the Golgi. It must be noted, however, that the measured radioactivity is derived from the Golgi membrane proteins and secretory proteins located within the cisternae. Therefore, to study synthesis of the membranes, it is necessary to show that radioactivity in only Golgi membrane proteins is being measured. This could be accomplished by either separating the Golgi membranes from the labeled secretory proteins or by measuring radioactivity at a time point when secretory proteins have been excreted from the Golgi and are in the circulation. Radioactivity in the Golgi would then be due only to labeled Golgi membrane proteins.

Since it is not possible to separate quantitatively Golgi membranes from labeled secretory proteins, it was decided to study the Golgi membranes at times when label due to secretory protein was secreted from the Golgi. Initial experiments showed that at 20 min, when Golgi are maximally labeled, ~50-70% of the counts can be precipitated with antibody to control plasma. These results show that secretory protein in the Golgi is antigenic and that a significant amount of the radioactivity measured at early time points is due to labeled secretory proteins. It is now necessary to show that at later time points, little or no radioactivity is precipitated with antibody. This result would be expected if all of the labeled secretory protein had been secreted. If this is found to be valid, then measured radioactivity would be due to labeled Golgi membrane proteins only and the synthesis of Golgi membranes isolated from control and infected animals could be studied.

Studies designed to determine the effect of S. pneumoniae infection on the number of insulin receptors and affinity of insulin receptors for insulin have been initiated. To date only a few experiments have been completed. Plasma membranes were isolated from control rat liver homogenates and incubated in an appropriate medium with [^{125}I]insulin. These preparations bound labeled hormone which could be displaced with an excess of cold insulin. The observations suggest that these preparations contain a specific receptor for insulin.

Studies designed in collaboration with MAJ Harold P. Hawley (Work Unit BS03 00 002) to determine the pathology of liver in rats following S. pneumoniae infection have continued. These studies were described in detail in the Research and Technology Work Unit Summary DD 1498, FY 77. Satisfactory liver sections were prepared and examined by light microscopy.

After a 40-hr S. pneumoniae infection, livers of infected rats are approximately 3 g heavier than the livers of control rats. In order to more correctly express biochemical results, experiments were devised to determine what this increase in liver weight was due to.

Groups of 10 rats were inoculated with 3×10^5 heat-killed (control) or live (infected) S. pneumoniae. At 40 hr postinoculation the animals were sacrificed; RNA, DNA, protein, lipid, dry weight, water weight, and total liver weight were determined. There was a significant increase in liver weight and in total liver RNA, protein, and lipid. Total liver DNA was not changed. There was also significant increases in the cold dry weight and total water. It is important to note that similar changes were not observed when the results were expressed per gram liver. In this case water content increased, protein and lipid did not change, and RNA and DNA decreased. Results emphasize that significant differences may be observed when biochemical results are expressed per gram liver or per total liver. The increase in the amount of water per gram liver actually acts as a dilution phenomenon.

After infection the percent of the dry weight that was protein, DNA or lipid was not changed. These results are consistent with the hypothesis that there is hypertrophy of the liver cell after infection. This hypothesis is also in agreement with pathology studies. A 20% increase in liver cell

volume and a significant increase in sinosoidal space was observed after infection. No increase in mitotic activity was seen. These results are consistent with the increases in dry weight, chemical constituents, and water weight, and with the DNA results.

Presentation:

Little, J. S. Synthesis, intracellular transport, and secretion of plasma proteins by the livers of control and Streptococcus pneumoniae-infected rats. Presented, Am. Soc. Cell Biol., San Diego, CA, 15-18 Nov 77 (J. Cell Biol. 75:364a, 1977).

Publications:

None.

LITERATURE CITED

1. Powanda, M. C. 1977. Changes in body balances of nitrogen and other key nutrients: description and underlying mechanisms. Am. J. Clin. Nutr. 30:1254-1268.

2. Canonico, P. G., E. Ayala, W. L. Rill, and J. S. Little. 1977. Effects of pneumococcal infection on rat liver microsomal enzymes and lipogenesis by isolated hepatocytes. Am. J. Clin. Nutr. 30:1359-1363.

3. Canonico, P. G., J. D. White, and M. C. Powanda. 1975. Peroxisome depletion in rat liver during pneumococcal sepsis. Lab. Invest. 33:147-150.

4. Canonico, P. G. 1971. Lysosomal responses during infection, pp. 75-90. In Annual Report to the Armed Forces Epidemiological Board. Commission on Epidemiological Survey, Washington, D. C.

5. Little, J. S. 1975. Isolation and partial characterization of plasma membranes from the livers of control and Streptococcus pneumoniae-infected rats. Infect. Immun. 16:628-636.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6429	78 06 27	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DIST'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 04 21	K. COMPLETION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. LEVELS	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	018
b. CONTRIBUTING				
c. ////////	STOG 78-7,2,	1, 3, 6		

11. TITLE (Procede with Security Classification Code)

(U) Cellular membrane alterations in staphylococcal enterotoxin 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
74 07	78 06	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE — EXPIRATION

b. NUMBER: NA

c. TYPE — d. AMOUNT:

e. END OF AWARD — f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	0.8	69.1
	CURRENT 79	0	0

19. RESPONSIBLE DOD ORGANIZATION

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RESPONSIBLE INDIVIDUAL

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NAME: Altenbern, R. A.

TELEPHONE: 301 663-7211

SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign Intelligence considered

POC:DA

22. KEYWORDS (Procede each with Security Classification Code)

(U) Fatty acids; (U) Military medicine; (U) BW defense; (U) Cerulenin; (U) Physiology; (U) Enterotoxin; (U) Staphylococcus; (U) Bacterial genetics

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

23 (U) Investigate the influence of specific biochemical alterations in composition of the bacterial cell membrane on control of synthesis and/or secretion of staphylococcal toxins, expecially enterotoxin of all serotypes, since they are serious problems to the military. Study the basic mechanisms of production of the many toxic extracellular proteins produced by Staphylococcus will lead to prevention of their secretion in food products or, perhaps, in human infections and also may lead to stimulation of their production in laboratory cultures thus simplifying isolation of toxins and evaluation of their effects on experimental animals, and the development of safe, effective toxoids.

24 (U) Study amounts of enterotoxin produced under a variety of experimental conditions by certain specific mutants induced in the parent strains.

25 (U) 77 10 - 78 06 - Studies continued on growth of S. aureus and SEB production at various temperatures with and without added saturated and unsaturated fatty acid supplementation. Incubation temperatures of 30-57 C are best. No matter what manipulation of media ingredients was carried out, no SEB production was detected at temperatures of 20-22 C. It is concluded that foods implicated in food poisoning were undoubtedly held at temperatures greater than about 20-22 C, permitting SEB formation by neglect or mishandling.

The investigator has retired from Federal Service.

Publication: FEMS Microbiol. Letters 3:199-202, 1978.

*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

* U.S. GPO: 1974—540-843/8601

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BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 018: Cellular Membrane Alterations in Staphylococcal Enterotoxin Production

Background:

The recently developed antibiotic, cerulenin, inhibits bacteria by preventing the synthesis of fatty acids (FA), which are incorporated into phospholipids, the major structural component of the cell membrane. Cerulenin-inhibited bacterial cells will resume growth when supplemented with preformed saturated (SFA) and unsaturated (UFA) FA. Very recent reports have implied that cell membrane fluidity plays a crucial role in synthesis and release of exoproteins from the cell. Fluidity of the cell membrane can be modulated by growing cells in the presence of an inhibitory concentration of cerulenin plus the proper combination of SFA and UFA. Application of these principles to Staphylococcus aureus has shown that production of enterotoxin B (SEB) is markedly sensitive to the kind and amounts of SFA UFA provided, although growth of the organism can be attained by almost any combination of FA.

Progress:

Studies continued on growth of S. aureus and SEB production at various temperatures with and without added SFA and UFA supplementation. Incubation temperatures of 30-57 C are best. No matter what manipulation of media ingredients was carried out, no SEB production was detected at temperatures of 20-22 C. It is concluded that foods implicated in food poisoning were undoubtedly held at temperatures greater than about 20-22 C, permitting SEB formation by neglect or mishandling.

The investigator has retired from the Federal Service.

Publication:

Altenbergn, R. A. 1978. Protease inhibitors suppress enterotoxin B formation by Staphylococcus aureus. FEBS Microbiol. Lett. 3:199-202.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6424	78 10 01	DD-DRAP-1654

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
78 04 21	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	019
b. CONTRIBUTING				
c. [illegible]	STOG 78-7,2,	1, 3, 6		

11. TITLE (Precede with Security Classification Code)
(U) Mechanism of action of bacterial exotoxins

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical medicine; 004900 Defense; 016800 Toxicology; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
75 11	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.0	205.0
	CURRENT 79	1.0	158.0

19. RESPONSIBLE DOD ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER:

21. GENERAL USE
Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME:
NAME:

POC:DA

22. KEYWORDS (Precede EACH 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 (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)

23 (U) Elucidate the mechanisms of action of militarily significant bacterial exotoxins in order to develop prophylactic or therapeutic measures. Two toxins are under investigation; Pseudomonas aeruginosa exotoxin A (PE), which has been implicated as a (the?) causative agent in the mortality of recuperating burn casualties who develop Pseudomonas infections; staphylococcal enterotoxin B (SEB) which has a demonstrated BW potential.

24 (U) Find and characterize cell line(s) or tissue(s) susceptible to the toxin of interest; study the toxin-induced biophysical and biochemical events which lead to the 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.

24 (U) 77 10 - 78 09 - Receptor binding systems have been established for both PE and diphtheria toxin (DE). With DE, monkey kidney cells (Vero, BS-C-1 or LLC-MK-2) were used and toxin was radioiodinated, utilizing chloramine T. Radiolabeled DE bound to cells in a time-, temperature- and concentration-dependent manner. The binding exhibited saturation, high affinity and reversibility. The interaction was blocked at both temperatures by unlabeled DE, but fragment B or CRM-197, a nontoxic mutant toxin containing an intact fragment B but an inactive fragment A. Extent of binding correlated with the sensitivity of various cell lines to the cytotoxic action of DE and was altered by pH or exogenous nucleotides in a manner paralleling the biological effects. The PE system used mouse liver cells and lactoperoxidase-catalyzed iodinated toxin. PE-cell binding was also time-, temperature- and concentration dependent. PE binding was blocked by unlabeled PE but not by DE or abrin. Binding was affected by pH in a manner paralleling pH effects on PE-induced bytotoxicitv.

Publications: Abstracts, ASM, 1978, p. 25; J. Biol. Chem. 253: in press, 1978.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE AND 1498 1 MAR 68 FOR ARMY USE ARE OBSOLETE

U.S. GPO 1973 543-843/8601

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 019: Mechanism of Action of Bacterial Exotoxins

Background:

Bacterial toxins mediate most of the harmful effects of many bacterial infections. In some instances, morbidity results from preformed toxins, e.g., 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 in at least one case (diphtheria), 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 most toxins are bipartitie, one portion mediating the binding specificity and the other carrying out an enzymatic function. Moreover, it is of considerable interest that, of the 3 toxins whose molecular mechanisms of action are known, all have similar enzymatic (ADP-ribosylating) activity. Thus, there is a good chance that insights gained with any one toxin might find application with others as well.

During the past year, most of our efforts have focused on a comparative study of Pseudomonas and diphtheria exotoxins (PE and DE). Very significant and important breakthroughs were achieved when receptor binding systems were developed for both toxins. The availability of such systems should facilitate a detailed analysis of receptor composition and a study of how the toxins (toxin-receptor complexes) are transported intracellularly. Chronologically, we discovered the DE receptor first, so more is known about that system. However, much of the technology developed with DE has been directly applicable to the PE system, so we anticipate rapid progress with that system as well.

DE: A year ago we published a paper (1) in which the sensitivities of 24 cell lines to PE and DE were examined. Beginning with the assumption that cell line sensitivity is proportional to the number of toxin

receptors which a cell possesses, we examined the interaction of labeled DE with our most DE-sensitive lines. We observed uptake of label by the cells in a time- and temperature-dependent manner. Uptake at 37° was biphasic; label was rapidly taken up for 1-1 1/2 hr followed by a decrease to a steady-state by about 3-4 hr. The steady-state level was approximately 50% of the maximum measured association. At 4°C, uptake was slower and not biphasic; association of label with cells followed a classical time-dependent increase to a steady state level at 10 hr. The maximum 4°C uptake was 2-3 X the 37°C level. At both 4 and 37°C, uptake of label could be blocked by addition of excess unlabeled toxin. In fact, all our experiments are run with and without a 100-fold excess of unlabeled DE, the difference of which we refer to as "specific binding."

Once we felt comfortable with the assay and its specificity, we ran a series of experiments to correlate cell association of label with the biological effects of DE. We found that binding of the label was proportional to the sensitivity of individual cell lines (Table I).

TABLE I. COMPARISON OF RADIOLABEL UPTAKE TO TOXIN SENSITIVITY[a]

CELL LINE	SENSITIVITY[b]	SPECIFIC RADIOLABEL UPTAKE (cpm/10^6 cells)	
		4°C	37°C
Vero	0.01	18,300	6,600
BSC-1	0.01	23,900	11,600
LLC-MK_2	0.02	5,590	2,360
BHK-21	0.15	0	1,750
WI-38	0.20	100[c]	140[c]
HeLa	1.50	160[c]	150[c]
L929	> 1000.00	0	490[c]

[a] ^{125}I-toxin (0.03 µg/ml) uptake by cells in 24-well tissue culture plates at 4°C (12 hr) or 37° C (1 hr).
[b] Reference (1).
[c] Results are not statistically greater than zero.

We found that the B fragment of DE blocked label uptake while the A fragment did not. Moreover, CRM-197, a mutant form of DE with full binding but no toxic capability, also blocked uptake of label. Finally, some time ago we observed that pH had a marked effect on the cytotoxicity of DE. In higher pH environments (~8.5), cells were protected from DE relative to lower pH (~5.5). A similar pH dependence was noted with our binding assay since the magnitude of label uptake at pH 9.0 is about 1/100 that at pH 5.5. Thus, we now have a reliable binding system which correlates well with known biological events and we feel safe in referring to the cell-bound label as toxin binding to its receptor.

Some time was spent studying the effects of NH_4Cl on DE binding. It was reported some time ago by Kim and Groman (1) that NH_4Cl would also protect certain cell lines from PE. Kim and Groman reported indirect evidence indicating that NH_4Cl works not by preventing DE binding to the cell, but by blocking its subsequent internalization. We now have direct evidence demonstrating that such is the case. At 4°C, we found little or no effect of NH_4Cl on the binding of ^{125}I-DE to Vero cells. At 37°C, we found that the binding of DE was not inhibited, but the biphasic nature of binding was blocked. This is an important observation in that it tells us the biphasic nature of binding at 37°C is a manifestation of the expression of toxicity. Put another way, we find that whenever the biphasic pattern is blocked (by NH_4Cl or other chemicals) the expression of DE cytotoxicity is also blocked. This finding should be of interest to others in the Institute since Kim and Groman cite other studies showing that NH_3 ($NH_4^+ \rightleftharpoons NH_3 + H^+$) affects the reproduction of some mammalian cell viruses.

Efforts were made towards characterization of the physicochemical parameters of the binding. We found that the binding was saturable, and when plotted in the manner of Scatchard, produced straight-line patterns indicating a single class of binding sites. Estimates of binding affinity yielded values of $5\text{-}8 \times 10^8$ 1/mol for the association constant and the number of binding sites were calculated to be in the range of $1\text{-}2 \times 10^5$/cell.

Another important point to demonstrate is binding reversibility. This must be done at a temperature below which internalization of the toxin takes place. We, therefore, performed a classical "chase" type experiment at 4°C. Toxin was bound to cells at 4°C for 12 hr. Then a large excess of unlabeled toxin (chase) was added and the level of radiolabeled toxin-cell association was followed as a function of time. We obtained data which appears to be a straight line (correlation co-efficient = 0.94) and which has a slope (i.e., disassociation rate constant) of 2×10^{-3} min^{-1}. Thus, this experiment not only demonstrated reversibility of the toxin-cell association but also allowed a measure of the off rate.

In another effort to demonstrate the biological relevance of the ^{125}I-DE-cell association, we compared the effect of exogenous nucleotides on binding and cytotoxicity. The results (Table II) demonstrate a direct correlation between effects on cytotoxicity and the level of binding. The concentrations of adenine nucleotides required to block 50% of the binding or protect 50% of the cells were very similar. Furthermore, the same general pattern of potencies was seen in both cases, i.e., tetraphosphate >> triphosphate > diphosphate > monophosphate. These data provide even further proof that the association measure is binding to the biologically relevant diphtheria toxin receptor.

To better define the nature of nucleotide block of DE-receptor binding, we carried out a limited structure-activity analysis of the effect. We found that there was a base specificity for protection. Adenosine, guanosine and thymidine triphosphates were the most potent; uridine and inosine triphosphates were intermediate in potency, and cytosine triphosphate was no more active than tripolyphosphate alone. In each case, the

nucleotide protection from DE-induced cytotoxicity paralleled the nucleotide block of ^{125}I-DE-cell binding. An intact nucleotide was required for full potency, as was demonstrated by the following experiment. Thymidine triphosphate is effective at protecting cells and blocking DE-cell binding at 0.1 mM. When similar levels of thymidine and tripolyphosphate were used, little or no effects were observed indicating a covalent link of the phosphate oligomer and the nucleotide are required.

TABLE II. EXOGENOUS NUCLEOTIDE EFFECTS ON LABEL-CELL ASSOCIATION

NUCLEOTIDE	CONCENTRATION (mM) REQUIRED TO:	
	Block 50% of specific label-cell association[a]	Protect 50% of the cells[b]
Adenosine 5'-tetraphosphate	0.02	0.03
Adenosine 5'-triphosphate	0.10	0.25
Adenosine 5'-diphosphate	0.25	0.50
Adenosine 5'-monophosphate	2.00	> 3.00

[a]Nucleotides were prepared in 0.1 M Hepes buffer (10 X final concentration) and titrated to pH 7.0. Various concentrations of each nucleotide in the range 0.01-5 mM were added to cells. ^{125}I-Toxin (0.03 µg/ml) or ^{125}I-toxin plus unlabeled toxin (3 µg/ml) were added immediately and toxin-cell association measured at 37°C (1 hr).
[b]Nucleotides added to cells as in part a. Cells were then challenged for 3 hr at 37°C with 1 µg/ml diphtheria toxin. Cell monolayers were then washed, complete media added and cytotoxicity measured after 48 hr.

Finally, when a double-reciprocal analysis of nucleotide binding inhibition was carried out, the results were consistent with a competitive inhibition. We concluded that nucleotides bind to the receptor at the toxin binding site or at an allosteric control site. Thus, nucleotides offer an extremely valuable tool for future DE-receptor work in that their effects are specific, demonstrate biological relevance of binding and can be used to bring about a specific disassociation of the toxin-receptor complex. The use of nucleotides as specific "elutors" in an affinity chromatography approach to receptor purification is obvious and currently under investigation. The use of nucleotides to demonstrate biological relevance of binding has been applied by Dr. David Neville at NIH and acknowledged by him as a important part of his work.

PE: A PE-receptor binding system was developed late in the year. One key to the work was provided by the efforts of Dr. Leppla and Ms. Dorland. They spent some time studying the conditions necessary to radiolabel PE with I without affecting its biological activity. The second key to the advance was the establishment of liver cell lines very sensitive to the toxin. This approach was taken since we knew that the liver is apparently the most PE-sensitive organ in animals challenged with the toxin.

By analogy with the DE work, we reasoned that liver cells might be more sensitive because they have more PE receptors. Employing the liver cell lines and ^{125}I-PE we demonstrated (a) PE binds to the cells in a time-, temperature- and concentration-dependent manner; (b) unlabeled PE competes for the binding of ^{125}I-PE while unlabeled DE and abrin do not; (c) the liver cells which are not sensitive to DE do not bind ^{125}I-DE; (d) pH affects both the binding of PE to cells and the sensitivity of cells to the toxin in a similar manner. Obviously, the PE system is not yet as developed as is that for DE binding but now represents our major efforts.

Presentations:

1. Middlebrook, J. L., R. B. Dorland, S. H. Leppla, and R. Altenbern. Receptors for diphtheria toxin on cultured mammalian cells. Presented, Annual Meeting, American Society of Microbiology, Las Vegas, NV 14-19 May 1978 (Abstracts, 1978, p. 25).

2. Middlebrook, J. L. Diphtheria toxin receptors on vero cells. Presented, Gordon Conference on Microbial Toxins, Andover, NH 23-28 Jul 1978.

Publications:

Middlebrook, J. L., R. B. Dorland, and S. H. Leppla. 1978. Association of diphtheria toxin with Vero cells. Demonstration of a receptor. J. Biol. Chem. 253: in press.

LITERATURE CITED

1. Middlebrook, J. L., and R. B. Dorland. 1977. Response of cultured mammalian cells to the exotoxins of Pseudomonas aeruginosa and Corynebacterium diphtheriae: differential cytotoxicity. Can. J. Microbiol. 23:183-189.

2. Kim, K., and N. B. Groman. 1965. Mode of inhibition of diphtheria toxin by ammonium chloride. J. Bacteriol. 90:1557-1562.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6414	78 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
78 04 21	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	021
b. CONTRIBUTING				
c. [illegible]	STOG 78-7,2,	1, 3, 6		

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	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	1.0	50.0
	CURRENT 79	1.0	115.0

19. RESPONSIBLE DOD ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Wannemacher, Jr., R. W.
NAME: POC:DA

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) 77 10 - 78 09 - The appearance of acute-phase proteins in the serum of rats subjected to inflammatory agents has led to the study of hepatic RNA regulation which results in their production, and the subsequent isolation of several of these proteins in order to study their regulation and function. Increased rates of RNA production which in turn result in increased production of serum proteins account for most of this activity. However, it appears that certain of these acute-phase proteins respond independently of increases in transcriptional rates. Also, soluble cytoplasmic factors may be involved in translational regulation independent of the quality of ribosomes present. Isolation and development of antibodies to alpha-1 and alpha-2 macroglobulin, albumin and several other acute-phase proteins are currently underway for use in future studies on their regulation and involvement in the acute-phase process.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 021: Regulation and Involvement of Acute-Phase Proteins in Infections of BW Importance

Background:

Studies on the sequence of host biochemical events which occur in response to infection or inflammatory substances support the theory that these stimuli induce an increase in hepatic production of RNA which is directed predominantly to the bound ribosome fraction (1). This in turn results in a subsequent increased synthesis of acute-phase serum proteins (2), each of which presumably plays a role in the host's defense against the inflammatory substance. However, not all of the acute-phase proteins are regulated by increased rates of RNA transcription (3). Therefore, studies of the regulatory mechanisms for the production of hepatic RNA and protein in response to inflammatory substances are necessary to understand the host's response to infection better.

The involvement of several acute-phase proteins in such processes as antiproteolitic activity, wound healing, protection of hemoglobin transport of metal ions, and regulation of the immune response has been reported by several authors (4). However, little is known about the specific function of each of the acute-phase proteins and their overall interrelationship in providing a defense for the host against inflammatory substances.

Progress:

Studies on the comparison of response in hepatic RNA production among Streptococcus pneumoniae, Salmonella typhimurium and its endotoxin in rats have been completed. An analysis of the results obtained from these 3 studies indicates that there is an early endotoxin response involving elevated intracellular production of proteins and redistribution of cytoplasmic RNA into the free ribosome fraction. This is followed by a typical "infection" response of newly formed RNA directed to the bound ribosome fraction for the production of proteins for export from liver. Studies on the effects of these 2 organisms on the rate of protein synthesis of ribosomes in an in vitro amino acid incorporation system has shown no difference from control levels. However, slight differences in rates were observed when using the supernatants from both S. pneumoniae- and S. typhimurium-infected hepatic cells. This implies that translational regulation may not be strictly dependent on ribosomal numbers but may also involve certain soluble factors. Additional studies on cytoplasmic factors from endotoxin treated hepatic cells are currently being carried out to evaluate these findings better.

Techniques for the isolation of the first 2 acute-phase proteins of interest, α_1- and α_2-macroglobulin of rats, have been carried out. The

3-step process of Gordon (5) was used to isolate and separate them. A major improvement in the last step of isolation was made by changing the elution of the macroglobulins off an ion exchange column from gradient to step-wise elution. This resulted in a better separation of the macroglobulin peaks, improving both yield and purity of product. Identification and characterization were carried out by acrylamide gel electrophoresis, SDS flat-bed electrophoresis, electrofocusing plates, determination of nitrogen content by the Kjeldahl method and amino acid composition on the automated amino acid analyzer. Patterns on the gels, and values determined from the other procedures compare favorably with published information on these 2 macroglobulins. Improvements in electrofocusing techniques established pI values of the 2 bands for each at around 4.4-4.5 for the double bands of α_2-macroglobulin and 4.8 and 5.3 for the 2 separated α_1-macroglobulin bands.

These purified proteins were then used to develop specific antibodies in goats. The serum from the goats was tested against both macroglobulins on Ouchterlony plates for titers and cross-reactivity, isolated, and stored for future use in the study of the involvement of these proteins in the acute-phase response.

A 3-step procedure for the isolation of the antibodies was attempted. These were $(NH_4)_2SO_4$ precipitation, and 2-stage ion exchange and affinity chromatography using the purified antigens bound to a matrix for isolation of the antibodies. Several affinity chromatography support systems were tried but each resulted in nonspecific binding of proteins to the α_1 and α_2 macroglobulins. This is probably due to their large size which makes them unacceptable for use on affinity columns. Therefore, antibody to these 2 proteins was purified only through the first 2 steps of the procedure and stored frozen for future use. Aliquots of each were tested for purity on crossed immunoelectrophoresis plates against inflamed rat serum. Negligible contamination of other antibodies was noted.

Since a decrease in serum levels of albumin is noted in response to infection, it was of interest to isolate and develop specific antibodies to rat albumin for future acute-phase response studies. Fractions from a G-150 column separation of serum from a rat with induced inflammation were compared to albumin standards and the peak containing the albumin was run on a preparative flat-bed electrofocusing plate. The resulting albumin band plus 6 other protein bands were isolated from these plates. Albumin purified in this manner is currently being used to stimulate antibody production in a goat.

The use of electrofocusing and SDS flat-bed acrylamide gels along with specific definitive tests will be used to identify several other acute-phase proteins of interest. They can then be isolated using similar techniques to those used for albumin and studied for their involvement in the response of interest.

Several additional related projects are currently underway. Antibodies are being developed to total serum from rats with inflammation in an additional goat to aid in the studies. Comparison of normal and "inflamed" serum fractions from a G-150 Sephadex separation is being made

on thin-layer electrofocusing plates to help identify some of the acute-phase proteins. A kit recently developed by New England Nuclear containing all the necessary ingredients for endogenous cell-free translational mRNA studies using the reticulocyte lysate system has been purchased. This will make future studies of isolated mRNA translational activity much easier to carry out.

Publications:

1. Masters Thesis for Hood College. 1978. Isolation and characterization of rat α_1 and α_2 macroglobulin and the development and purification of their specific antibodies, Hood.

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3. Thompson, W. L., F. B. Abeles, F. A. Beall, R. E. Dinterman, and R. W. Wannemacher, Jr. 1976. Influence of the adrenal glucocorticoids on the stimulation of synthesis of hepatic ribonucleic acid and plasma acute-phase globulins by leucocytic endogenous mediator. Biochem. J. 156:25-32.

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5. Gordon, A. H. 1976. The α macroglobulins of rat serum. Biochem. J. 159:643-650.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6420	78 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
78 04 21	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	022
b. CONTRIBUTING				
c. [illegible]	STOG 78-7,2,	1, 3, 6		

11. TITLE (Precede with Security Classification Code)

(U) Amino acid sequence analysis of pathological agents

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 08	CONT	DA	C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	90.0
FISCAL YEAR CURRENT 79	1.0	68.8

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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) Pseudomonas; (U) Staphylococcal enterotoxin C; (U) Amino acids

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with 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) 77 10 - 78 09 - During the past year the basic organization of a laboratory specializing in determining the amino acid sequence of proteins has been completed. A number of proteins and a peptide have been analyzed on the Beckman sequencer. The exotoxin from Pseudomonas aeruginosa was subjected to "sequencing" once in its native state and once in a derivatized form. Sequence analysis of the Staphylococcus aureus exfoliative toxins DI and TA has so far yielded the first 26 residues of DI and the first 18 of TA. Beginning work was started on the determination of amino acid sequence of S. aureus enterotoxin C-1. The first 32 residues of the entire protein have been sequenced, while 20 residues from an internal peptide have been identified.

* Available to contractors upon originator's approval

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

* U.S. GPO: 1974-540-847/8691

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 022: Amino Acid Sequence Analysis of Pathological Agents

Background:

The rationale for the investigation of protein amino acid sequences depends upon the fact that proteins are rich in biological information. For example, the reconstruction of genetic events and the description of evolutionary history and mechanisms is possible through the use of amino acid sequences of similar proteins from different species (1). The elucidation of structure-function relationships in proteins is also under investigation by the use of amino acid sequences (2).

Since amino acid sequences are such a multifaceted source of fundamental biological information, the last several years have seen an explosive increase in the number of proteins subjected to such determinations. Considerable work and ingenuity have been put into the improvement of the techniques involved. Even so, the principles employed are still the same as originally developed in late 1940s and early 1950s (3). This process involves the fragmentation of the protein chain into peptides, the purification of these peptides, and then the determination of the amino acid sequence of each peptide. By using semiautomatic Edman equipment, such as the Beckman 890C sequencer, the work involved proceeds much more rapidly than if accomplished by manual methods alone.

Of the purified bacterial toxic proteins which are readily available and of potential BW importance, the enterotoxin C_1 of Staphylococcus aureus (SEC_1), is of greatest interest for future work. The interest in this bacterial toxin is due to the role it plays in several diarrheal diseases (4). Other purified proteins are available, such as the exotoxin of Pseudomonas aeruginosa and the S. aureus exfoliative toxins (5). Investigation of the amino acid sequences of these proteins will continue, depending upon the availability of the proteins and consideration of the time involved.

Progress:

During the past year the basic organization of a laboratory specializing in the determination of the amino acid sequence of proteins has been completed. Installation of the 2 main units of equipment, the Beckman 890C sequencer and the Hewlett-Packard gas chromatograph (GC) was completed. These units are now in use.

A number of procedures which are necessary to complete the sequencing process have also been established. After the protein or peptide has been subjected to analysis on the sequencer, the resulting phenylthiohydantoin

(PTH) amino acid must be identified. Currently in use for identifying the derivatized amino acid are GC and 2 different types of thin layer chromatography (TLC). Acid hydrolysis of the PTH amino acid, resulting in the original amino acid, is used as the last step in identification.

A number of different proteins and a peptide were "sequenced" on the Beckman sequencer. The number of residues "sequenced" varied for each protein or peptide. Each resulting PTH amino acid was identified by the procedures already mentioned.

The P. aeruginosa exotoxin has been subjected to sequence analysis once in its native state and one after being carboxymethylated. Only a few amino acid residues were identified from the native state attempt. More information was derived from carboxymethylated protein. It is assumed the derivatization "opened" the protein up to the sequencing process.

Further work on Pseudomonas aeruginosa exotoxin will be attempted when time allows. At present the following amino acid sequence has been derived: H_2N-Ala Glu Glu Ala Phe Asp Leu (Ala) (Asp) Glu (Gly) Ala (Lys) ? ? ? ? ? ? (Asp) (Gly) ... - COOH. Those code names in parenthesis represent tentative conclusions, while the others are more definitive. The question mark (?) represents a residue for which no intelligent identification can be made at the present time. H_2N- indicates the amino-terminal end of the protein or peptide, while -COOH indicates the carboxy-terminal.

Continuing effort has been put into the sequence analysis of the S. aureus exfoliative toxins DI and TA. Analysis of the data from the first 3 runs of the DI protein on the Beckman sequencer yielded more information than initially suspected. The following amino acid sequence for DI has been deduced: H_2N-Lys Glu Tyr (Ala) (Ala) Glu Glu Ile (Arg) Lys Leu Lys (Gln) Lys Phe Glu Val (Trp) (Pro) Thr (Asp) Lys Glu Leu Tyr Thr... -COOH. Preliminary data from the 4th run of the protein on the sequencer has so far confirmed the proposed amino acid sequence. If time permits, efforts will continue to increase the number of sequenced residues after clarifying the tentative results.

For S. aureus exfoliative toxin TA, the amino acid sequence from the 1st run is as follows: H_2N-Glu Val (Thr) Ala Glu Glu Ile (Lys) ? ? Glu Glu (Lys) ? (Asp) Lys ? (Tyr)... -COOH. Due to an error in the sequencing procedure, an attempt at clearing up the ambiguities has yielded more unclear data. At least a 3rd analysis on the sequencer will be needed to define the amino acid sequence better.

Information gathered from the analysis of the SEC on the Beckman sequencer has been partially analyzed. The following sequence has been determined so far: H_2N-Glu (Val) (Gln) Pro Asp Pro Thr Pro Asp Glu Leu ? (Lys) (Ser) (Ser) (Lys) (Phe) (Thr) ? (Leu) ? ? (Asp) ? ? (Val) (Leu) (Tyr) (Asp) ? (Asp) (Tyr)... -COOH. As can be seen, further work with this protein will be necessary and is planned. Continuing analysis of the native protein is part of the process.

Yet another step in the determination of the amino acid sequence of the SEC[1] has been started. Two runs on the Beckman sequencer of a peptide from the protein have been accomplished. As a result, the following amino acid sequence has been derived: H_2N-Asn Tyr Asp Lys Val (Lys) Thr Glu Leu Leu Asn Glu (Gly) Leu Ala Lys Lys Tyr Lys (Asp) ... -COOH. This peptide will be placed in the sequencer again for further analysis.

Publications:

None.

LITERATURE CITED

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2. Niall, H. D., H. Keutmann, R. Sauer, M. Hogan, B. Dawson, G. R. Aurbach, and J. Potts, Jr. 1970. The amino acid sequence of bovine parathyroid hormone. I. Hoppe Seylers Z. Physiol. Chem. 351:1586-1588.

3. Brown, H., F. Sanger, and R. Kitai. 1955. The structure of pig and sheep insulins. Biochem. J. 60:556-565.

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5. U.S. Army Medical Research Institute of Infectious Diseases. 1 Oct 1977. Annual Progress Report, FY 1977, pp. 183-185, Fort Detrick, Frederick, MD.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OH6427	78 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
78 06 20	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	023
b. CONTRIBUTING				
c. //////////	STOG 78-7,2,	1, 3, 6		

11. TITLE (Precede with Security Classification Code)*

(U) Biochemical events at the cellular level: possible early indicators of infection

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

003500 Clinical medicine; 004900 Defense; 012300 Biochemistry; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 11	CONT	DA	C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	0.5	115.0
	CURRENT 79	0.5	198.4

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME:
NAME:

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Bacterial diseases; (U) Rapid diagnosis; (U) Blood platelets

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

23 (U) Identify metabolic and functional changes in circulating platelets that might be useful in early detection of infections of importance in BW defense. Since platelets are among the first of the host's cells to interact with invading organisms, this study will monitor alterations in platelet parameters to determine if any are of value for detection of infection well in advance of clinical symptoms, such as fever.

24 (U) Isolate platelets from infected and control animals during the early stages of illness and measure biochemical parameters which are probably early indicators of infection.

25 (U) 77 11 - 78 09 - Of the platelet parameters examined to date for use as early indicators of infection, the most promising is fatty acid (FA) analysis. Gas chromatography of platelet FA seems to indicate that changes in linoleic and arachidonic acid levels occur early in infection. Results depend upon whether free or total FA are measured; if free platelet FA are analyzed, arachidonic acid decreases relative to linoleic acid in infected vs. control rats. In the total platelet FA concentration, the reverse is true. The relative amounts of serum FA do no change early in infection. Total amounts of serum free FA and serum albumin both decline only as clinical symptoms of illness manifest themselves. Some changes were observed for beta-glucuronidase, serine esterases, acid phosphatase, and peroxidase, but no statistically significant results were apparent before the onset of zinc depression and fever.

*Available to contractors upon originator's approval

☆ U.S. GPO 1971-543-843/8601

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 023: Biochemical Events at the Cellular Level: Possible Early Indicators of Infection

Background:

Increased research into platelet function has indicated their role in defense of the host against infection. Platelets have been recognized as potential phagocytic cells (1), as well as being possible contributors to inflammation and disseminated intravascular coagulation (DIC) (2). Inflammatory actions have been attributed to release of platelet lysomosomal cationic proteins, vasoactive amines, and prostaglandins, while the adhesion and aggregation mechanisms contribute to intravascular coagulation. By following the levels of inflammatory agents in the platelet in infected animals, and by monitoring changes in the aggregating ability of circulating platelets these studies aim to use the platelet as a means for early detection of illness.

In infected and control rats differences were measured between the platelet concentrations of metabolites likely to be detected early in the course of the platelets' response to infection. These changes could be due to actual changes in them, or to replacement of a fraction of the platelets with fresher ones having a different complement of proteins and other constituents.

Progress:

The live vaccine strain of Francisella tularensis was chosen as the infectious bacterium because of the military significance of more virulent strains of the same organism, and because a sublethal dose of the bacterium could be administered in such a way that fever did not appear until the next day, allowing adequate time for studying the early phases of the infection. Sprague-Dawley rats were chosen as the animal model host.

Platelets from individual rats were used for assaying differences between infected and control rats in the parameters investigated. This allowed better statistical analysis of the results than preliminary studies done by Dr. Mapes on this project using pooled platelet samples. In addition to the Sprague-Dawley rats injected with live LVS F. tularensis, there were 2 types of control used: rats injected with saline and rats injected with heat-killed LVS. Food was removed 24 hr before the start of the experiment.

Fatty Acid Analysis. There are several good reasons for consideration of platelet fatty acids (FA) as possible early indicators of infection. First of all, arachidonic acid is important because of its role as a precursor for prostaglandins, the concentrations of which increase during infection. Arachidonic acid is utilized in the formation of endoperoxides and thromboxanes, which are of importance in platelet aggregation and release reactions. Also, as a constituent of the phospholipids comprising the membranes of the platelet, arachidonic acid values may reflect membrane changes occurring during these aggregation and release reactions. Furthermore, other FA may change their concentrations in platelets and serum as a host animal responds to infection; the interconversion of ketone bodies and FA is affected by illness. Because arachidonic acid in the platelet is predominantly in the esterified form, mainly in phospholipids, and can be rapidly liberated to the free acid to fulfill the platelet's metabolic needs, it is important to monitor both free and total FA values.

Our work currently shows that FA, either free or total, can be measured in platelets taken from individual rats. The analyses agree with reported results, showing, for instance, that arachidonic acid comprises 25-35% of the fatty acids present. The method of analysis is gas-liquid chromatography of the FA, after methylation. For total FA analysis, the lipids isolated are first saponified with KOH/methanol.

Initial results seem to indicate that platelet arachidonic and linoleic acids are useful early indicators of infection. The values of these 2 acids appear to change (in opposite directions relative to each other) as early as 6 hr after infection, whereas fever is detectable beginning at 16-24 hr. In platelet free FA analyses, arachidonic acid concentrations decrease relative to linoleic acid. In platelet total FA analyses, the linoleic acid values appear to decrease relative to arachidonic and oleic acids. In the serum, arachidonic acid values are reduced relative to other fatty acids beginning with the onset of fever. Total serum free FA and albumin concentrations also decline as fever appears.

An attempt will be made to modify the extraction procedures so that lower molecular weight (C_8-C_{12}) FA from platelets and serum can be examined as well as those currently investigated. In one instance, thin-layer chromatograms were run of the platelet and serum lipids extracted for FA analysis before saponification and methylation. Good separation was achieved between simple and complex lipids in the system used (Silica Gel support with chloroform:methanol:water = 65:25:4 as solvent) but there were no obvious differences between lipids from infected and control rats.

Platelet Enzyme Activities. Several enzymes which were thought to be representative of the lysosomal proteins secreted by platelets during the release reaction were investigated as early indicators of infection. These were β-glucuronidase, serine esterase, peroxidase, and acid phosphatase. Platelets were collected from individual rats for these assays,

which were then run in triplicate. It was found that there was no statistically significant difference in the activities of any of these enzymes between infected and control rats until the onset of fever.

Other enzymes which may be more promising can be investigated instead. Lysozyme, for instance, is an obvious choice and will be investigated next.

Aggregating Ability. If platelets respond to the presence of bacteria either directly or via mediators, it is possible that one could detect a change in aggregating ability. Studies were undertaken to do so. The following were found to be suitable aggregating agents which could be used to monitor changes in rat platelet aggregating ability: adenosine diphosphatase (ADP), collagen, and phospholipase-C. Salmonella typhimurium endotoxin was found to cause platelet aggregates of a different type than the other agents just listed; endotoxin-induced aggregates remain in suspension until mechanical stirring is halted. With ADP we have not been able to show a difference in aggregating ability between platelets of infected and control rats. Studies on the others are continuing.

In an effort to correlate arachidonic acid changes with changes in platelet aggregating ability, platelets which had been allowed to aggregate and then disaggregate were analyzed for FA content in comparison with unaggregated platelets handled in the same way. Unfortunately, there was too much sample-to-sample variation to conclude that aggregation caused any change in FA composition.

Emphasis was also placed upon studies which used phospholipases as aggregating agents. It has been found (3) that phospholipase A2, which should liberate arachidonic acid from platelets and cause aggregation in the same manner as externally added arachidonic acid, does in fact do so. We obtained the same negative results with phospholipase A2 derived from Naja naja, and Crotalus venoms, or beef pancreas. There is, however, an interesting result in that these phospholipases hindered aggregation by other agents, such as collagen and arachidonic acid.

Platelet Counts. Manual counts of platelets in dilutions of platelet-rich-plasma seem to indicate a transient, barely statistically significant rise in platelet number at about 8 hr after infection. Because of the errors involved in manual counts it would be preferable to pursue these studies on a suitable machine, preferably one which would give information of size distribution as well. Meanwhile, the protein content of the platelet suspensions was checked and found not to vary between infected and control rat samples.

Publications:

None.

LITERATURE CITED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUMRY	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
78 06 20	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO. CODEN	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
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b. CONTRIBUTING				
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11. TITLE (Precede with Security Classification Code)
(U) Diagnosis and pathology of Legionnaires' disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
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22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; Laboratory animals; (U) Special containment facilities; (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) Develop rapid serologic method for diagnosis of Legionnaires' disease organism (LDO), determine optimal conditions for growth and survival of LDO and define factors involved in pathophysiology of the disease. If successful, study aerosol infectivity in appropriate laboratory animals. This study will allow the assessment of the military importance of this organism in either natural infections or biological warfare and may well proceed to vaccine development.

24 (U) Starting with information, procedures and sera from CDC, develop a serum bank, assess strain differences, and test a variety of growth media. If successful, use subhuman primates for virulence studies.

25 (U) 78 03 - 78 09 - Direct and indirect immunofluorescence assays, microagglutination, and ELISA assays have been established. A variety of artificial media in addition to the modified Mueller-Hinton media have been employed. The goal of maintaining virulence on artificial media remains a universal problem. AKR/J mice, new animal model, die within 24 hr after lethal challenge and can be protected by pretreatment with LDO antibodies or LDO soluble antigen. Aerosolization studies are underway in small animals as well as primates.

* Available for contractors upon originator's approval

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BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 024: Diagnosis and Pathology of Legionnaires' Disease

Background:

In the summer of 1976, 221 American Legion conventioneers in Philadelphia were afflicted by an acute febrile illness that ended in death for 34. Although the disease is thought of in epidemic proportions the fact is that of the 884 presently confirmed cases, 275 have appeared as isolated incidents. Evidence now strongly supports researchers' earlier beliefs that the Legionnaires' disease organism (LDO) is spread by aerosolization and most commonly by means of air conditioning units.

Although the typical victim is middle-aged, younger age groups have been affected. The mean age of the 95 of 100 employees who worked in the Oakland County Health Department, Pontiac, MI, in 1968 was 33.

From the incomplete data gained thus far it appears that LDO is probably a ubiquitous soil organism (30 states and portions of Europe) which can be aerosolized. Our laboratory experience has taught us that the severity of the illness produced in animals depends upon the amount of inoculum, route of administration and the virulence of the particular strain used. We assume all these factors play a role in the development and severity of the human disease.

Progress:

Part I: Cultures of LDO were obtained from J. E. McDade, CDC: frozen suspensions of infected yolk sac (YS) from embryonated eggs and MH agar cultures freshly inoculated with YS suspensions were supplied for the 4 Philadelphia strains (L1, L2, L3, L4) and the Pontiac strain, and YS-inoculated MH cultures for the Washington, Flint, Knoxville and Vermont strains. Ten-day growth harvested from the MH agar cultures into 50% normal rabbit serum-tryptose-saline diluent (NRS-TS) and stored at -70°C served as inocula for subsequent preparation of seed and working stock suspensions. Subcultures prepared from the first subculture, designated as "2nd passage," were used routinely for further studies. First passage subcultures remain viable after 10 mon storage at -70°C; and 8-mon-old "2nd passage" subcultures on MH slants covered with sterile glycerol and stored at -10°C are still viable. Cultures stored at 4°C or in the 35°C, 5% CO_2 incubator did not survive longer than 1 mon. The Pontiac and Legionnaire #2 strains were chosen as prototypes for most further studies. These strains survived for less than 3 days at 25°C in sterile Fort Detrick sand or soil, but are still viable after 4 mon at 25°C in gel-saline (pH 7), tryptose-saline (pH 7), phosphate buffered saline (pH 7) or pond water, and after 3 mon in tryptose

saline at 4°C. Exposure for 5 min at a distance of 3 feet from standard bactericidal UV lights within a Blickman hood sterilized MH agar plates freshly seeded with 10^8 LDO suspended in tryptose saline, but exposure for 1 hr did not sterilize fully grown colonies on MH agar.

Many standard bacteriologic media, tissue culture media, specially formulated media, and media supplements have been tested individually as solid and liquid media, and as supplements to the CDC-recommended MH agar, for their efficiency in initiating colony formation from individual organisms and for stimulating more rapid growth of LDO. To date the following solid media appear to be more efficient than MH agar in promoting relatively rapid growth (2 days for visible colony formation compared with 4 days on MH agar), and in initiating growth from individual LDO cells (Legionnaire #2 isolate): (a) glucose-cysteine blood agar; (b) Difco brain heart infusion agar + 1% hemoglobin + 2% BBL Isovitalex; (c) Difco purple agar + 1% hemoglobin + 2% BBL Isovitalex; (d) Difco mycoplasma agar + 1% hemoglobin + 2% BBL Isovitalex; and (e) Casman agar + 1% hemoglobin + 2% Isovitalex (Table I). Unlike MH agar, most of these media do not contain starch but do have added NaCl; otherwise they are very similar to MH agar.

The total count (Petroff-Hauser method) of LDO from 6-day-old cultures on MH agar suspended in tryptose-saline (pH 7) when compared with the number of CFU following inoculation of the suspension onto MH agar revealed that >95% of the organisms were viable. Addition of Vancomycin, Colicin, and Nystatin to produce a selective medium as recommended by CDC, however, prevented the growth of LDO from inocula containing as many as 10^8 organisms (Table I). Substitution of cysteine and glutamate for Isovitalex in MH agar supported growth almost as effectively as the original medium; however, although a variety of inorganic Fe compounds could substitute for hemoglobin, and cysteine alone could substitute for Isovitalex, none of these experimental media were as effective as the original.

A suitable liquid medium continues to be elusive. The only effective liquid medium to date is part of a biphasic system consisting of MH broth + 2% Isovitalex + 0.01% hemoglobin layered over an equal volume of MH agar in Ehrlenmeyer flasks. Static incubation of this system at 35°C in 5% CO_2 permitted growth from an inoculum of ∿100 LDO (Legionnaire #2 isolate, original subculture stored at -70°C). Visible turbidity was noted in about 12 days, and a density equivalent to ∿1 x 10^9 LDO/ml was achieved in 14 days. An inoculum of about 1 x 10^8 LDO, by direct microscopic count, in 50 ml of liquid medium in the biphasic system produced the following growth pattern and pH changes: (a) a lag phase lasting 3 days, 2.8 x 10^6/ml viable organisms on MH agar with pH 6.85 decreasing gradually to pH 6.75; (b) an exponential growth phase lasting 3 days, with viable organisms increasing from 2.8 x 10^6/ml to 7 x 10^9/ml and pH 6.75 decreasing to pH 6.48; (c) a stationary phase lasting 3 days, pH 6.48 decreasing to pH 6.25; (d) an exponential death phase lasting 4 days, with viable organisms decreasing

TABLE I. COMPARISON OF MEDIA EFFECTIVENESS IN RECOVERY OF VIABLE LDO IN TRYPTOSE-SALINE SUSPENSION

MEDIUM [a]	VIABLE COUNT/ml [b]			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
MHA + Isovitalex + Hemoglobin	3.9×10^9	1.2×10^9	7.8×10^9	3.5×10^8
MHA + Isov + Hemo + VCN	0	-	-	0
MHA + Isov + $FePO_4$	3.0×10^8	2.2×10^6	2.5×10^9	-
MHA + Isov + $FePO_4$ + VCN	? $\times 10^2$	0	-	-
MHA + Cysteine + Glutamate + Hemo	-	6.8×10^8	1.7×10^{10}	-
MHA + Cysteine + Glutamate + $FePO_4$	-	-	2×10^3	-
GCBA	-	-	2.4×10^9	7.0×10^8
Casmen + Isov + Hemo	-	-	-	7.2×10^8
BHIA + Isov + Hemo	-	-	-	4.8×10^8
Purple + Isov + Hemo	-	-	-	1.3×10^9
Mycoplasma + Isov + Hemo	-	-	-	3.0×10^8
Direct Microscope Count	3.7×10^9	1.7×10^9	-	1.0×10^9

[a] Inoculated with 10^2 through 10^9 LDO organisms in 10-fold increments.

[b] ? = Confluent growth -- no growth at next dilution.

VCN = 3 µg/ml Vancomycin, 7.5 µg/ml Colistin, 12.5 U/ml Nystatin.

from 7×10^9/ml to 8×10^2/ml and pH 6.25 rising to pH 6.95; and finally (e) a resting phase of unknown length, 8×10^2/ml surviving organisms and pH 6.95 rising to pH 7.04. Maximum population density was observed on the 6th day following inoculation of the medium; on the 9th day when the population of organisms began to decrease rapidly, along with a rapid increase in pH, the color of the medium changed from light yellow to dark brown. Appearance of the soluble, dark-brown pigment in the liquid medium correlated with the exponential death phase of LDO.

The Pontiac strain has a different colonial morphology than the other strains, namely: the colonies are white and butyrous in consistency rather than the dirty gray color and viscid consistency characteristic of other LDO; they produce little or no water-soluble brown pigment on MH agar supplemented with $FePO_4$. All LDO strains produce a water-soluble substance on MH agar which fluoresces under long-wave UV; the organisms are not acid fast and are weakly Gram-negative, even when counterstained for 10 times the prescribed time. In stained smears prepared from LDO grown on agar, typical organisms are large rods in long chains making up tangled masses; however, when wet preparations from biphasic liquid cultures are viewed under phase illumination, the organisms are rarely seen as chains and usually appear to be large diplobacteria (2-3 μm) with many pleomorphic forms, including small coccoidal, large vibrioform, large swollen club-shaped, and long thin fusiform bacteria. In the CDC cultures 2 colony types predominate, namely: pin point, punctate, type and a 2-3-mm, smooth, round type; many colonies intermediate in size were seen. After repeated subculture, the larger colony type became predominant.

Attempts are continuing to develop liquid and solid media which will permit more rapid visible growth of LDO from minimal inocula, determination o· LDO survival in laboratory suspending media and possible natural reservoirs, and evaluation of methods for storing and disinfecting LDO. Observation of morphologic characteristics will also be made in conjunction with these studies.

Part II. Response of experimental animals.

When studies were initiated, reports from CDC indicated that guinea pigs developed lethal infections after IP inoculation with Legionnaires' disease organisms, whereas Swiss mice and rabbits were nonresponsive. Fraser et al. (1), using Philadelphia strain L-1 grown in embryonated eggs followed by two passages on MH agar, found that 6 of 6 guinea pigs died in 2.8 ± 0.4 (SE) days after an IP dose of 2×10^8 CFU and 5 of 6 died in 4 ± 0.6 days after 5×10^7 CFU. In the absence of other data, IP infection of guinea pigs was considered to be the reference model for animal studies and for defining strain virulence.

Studies in guinea pigs with organisms grown on MH agar. Subcultures from the CDC-MH agar cultures grew very slowly. For initial studies working stocks were prepared by harvesting 7-day growth in NRS-TS and aliquots of the harvest suspension were stored at -70°C. For later studies the Washington strain was harvested at 5 days when colonies were barely visible; harvested organisms were washed twice in TS before resuspension in 50% fetal calf serum-tryptose-saline (FCS-TS) for storage at -70°C.

Responses of guinea pigs to IP challenge with the 7-day preparations are shown in Table II. Typical signs of acute illness and death (described by CDC investigators) were observed only in guinea pigs

challenged with the Washington strain. The spleen harvested from one of these guinea pigs 1-2 hr after death on day 4 contained ~2 x 10^8 CFU of LDO and 6 x 10^6 CFU of a Gram-positive contaminant. Because of its apparently higher virulence, the Washington strain was used for subsequent studies.

TABLE II. LETHAL RESPONSE OF GUINEA PIGS AND AKR/J MICE AFTER IP CHALLENGE WITH LDO GROWN ON MH AGAR

CHALLENGE STRAIN	GUINEA PIGS			AKR/J MICE		
	Dose (CFU x 10^7)	Dead/no. tested	Day of death	Dose (CFU x 10^7)	Dead/no. tested	Day of death
Washington	5	4/4	3,4,4,5	2	2/6* [a]	2,?
Philadelphia:						
L1	10	1/2	6	4	0/6*	
L2	10	0/2		4	0/6*	
L3	20	1/2	11	8	0/6*	
L4	25	0/2		10	0/6*	
Pontiac	5	1/2	14	2	0/6	
Flint	10	0/2		4	0/6	
Knoxville	3	0/2		1	0/6	
Vermont	1	0/2		0.4	0/6	

[a] *, signs of illness in all mice at 4-24 hr.

Attempts to enhance virulence by animal passage have been hampered by the presence of contaminants in the tissues of infected animals. Direct transfer of tissues was unsatisfactory because of the high incidence of contaminating bacteria; this was an unusual finding in infections with highly invasive bacteria, but was reported to be a common occurrence in guinea pigs infected with LDO (Fraser, CDC). A variety of bacterial species have been recovered but generally each guinea pig harbors a single type, suggesting that secondary invasion occurs in tissues traumatized by LDO infection. Preselec ion of uncontaminated tissues also was attempted, i.e., LDO spleen homogenates were held at -70°C while cultures of representative samples were incubated for 2 weeks on MH, blood (BA) and MacConkey (MAC) agars to determine the concentration of LDO and to insure the absence of contaminants. This procedure also proved to be unsatisfactory.

Guinea pigs, inoculated with the thawed homogenates from an apparently uncontaminated spleen, became ill; their tissues obtained on day 3 contained high concentrations of β-hemolytic, Gram-negative coccobacteria that grew equally well on BA and MH agar (Table III). Organisms typical of LDO were recovered only from the peritoneal washings at a ratio of 1 LDO CFU to 100 β-hemolytic colonies. The β-hemolytic organism was assumed to be of recipient origin because only LDO organisms were demonstrable in the donor homogenate and in a suspension (GP-MH) derived from MH agar plates that had been inoculated with a volume of homogenate equivalent to the transfer dose.

TABLE III. BACTERIAL COUNTS (CFU) IN SAMPLES RECOVERED FROM SICK GUINEA PIGS 3 DAYS AFTER CHALLENGE WITH THE WASHINGTON STRAIN

CHALLENGE INOCULUM (CFU)	GUINEA PIG NO.	SAMPLE	CFU ON AGAR	
			MH	Blood
6×10^7 LDO grown on MH agar	1	Peritoneal wash	7×10^8	0
		Spleen	5×10^8	0
		Blood	3×10^5	0
1×10^8 LDO in spleen homogenate from guinea pig #1	2	Peritoneal wash	9×10^8	9×10^8
		Spleen	3×10^7	2×10^7
		Blood	2×10^5	5×10^4

Recently, inoculation of embryonated eggs proved to be a better method than culture on artificial media for detection of contaminating bacteria. Approximately 10^6 LDO CFU from the GP-MH suspension were inoculated into yolk sacs of 5-6-day embryonated eggs. Three days later the yolk and its membranes were harvested from a viable egg, and homogenates were plated on BA, MAC and MH agar. Minute atypical (0.5 mm) colonies surrounded by a 1.0 mm zone of greenish discoloration were seen growing on BA after 11 days. At about the same time these atypical colonies also grew out on MH agar. Colony counts indicated that approximately equal concentrations of LDO and the BA-type were present in the yolk-membrane homogenate. The BA colonies contained Gram-variable, coccobacillary forms and some Gram-negative, filamentous forms; organisms from BA colonies reacted with fluorescein-tagged goat anti-LDO serum to produce 4+ fluorescence. In subcultures on BA, colonies grew more rapidly achieving their maximum size in 2-4 days, filamentous forms were no longer present and immunofluorescent activity was absent, indicating that LDO organisms

were eliminated by passage on BA. Attempts to eliminate the small colony form from MH cultures of LDO were unsuccessful. Unlike the mixed population in the membrane homogenates, the isolated BA-type was avirulent for guinea pigs. In the presence of high concentrations of LDO it is doubtful that a low concentration of a BA-type organism would be detected by our standard tests for contamination. For cultures derived from animal tissues it may be necessary to include passage in embryonated eggs as a final test for purity.

In an attempt to establish fatal infection of guinea pigs with a reduced challenge dose, inoculation by the intratracheal or intrathoracic route was examined. Challenge doses (0.5 ml) containing 10^3 CFU from the 5-day working stock of Washington strain were introduced into the trachea of 2 guinea pigs and into the thoracic cavity of a second pair. Control animals received 0.5 ml diluent. The first pair was sacrificed when the animals became moribund on days 6 and 7; LDO organisms were not detected in cultures of the lungs, blood, spleen and liver, but 10^3 - 10^6 enterobacteria or micrococci/ml were recovered in tissue homogenates; the highest concentration of contaminant was present in the lungs. One of the intrathoracic pair became sick and was sacrificed on day 8; at the same time one of the intratracheal control pigs was also necropsied. The surface of the lung from the intrathoracic guinea pig contained small nodules and hemorrhagic areas; cultures of lung homogenate had a low level of contaminants and 10^6 LDO/ml. Fewer LDO were present in spleen and liver with essentially none in the blood. The lung of the intratracheal control animal appeared to be healthy but contained 10^5 Klebsiella/ml and an equal number of an unidentified species. These findings suggested that intrathoracic challenge could be used to initiate infection with a relatively low dose of organisms and should minimize complications introduced by secondary invaders.

Studies in mice with organisms grown on MH agar. Although the outbred Swiss mouse was fully resistant to LDO (McDade, CDC), studies were conducted to examine resistance of the inbred AKR/J strain and to compare its resistance with that of nude and haired heterozygous Swiss mice. AKR/J mice were challenged by IP, IV or SC inoculation with each of the 9 strains of LDO, and the Swiss mice by IP inoculation with Philadelphia L-1 and L-2 and the Washington strains. All challenge suspensions had approximately the same turbidity but dosage ranged from 0.4 - 10 x 10^7 CFU. Mice in SC-challenge groups remained healthy and showed no reaction to LDO. Responses to IV challenge were identical to those shown in Table I for IP challenge of AKR/J mice: all mice in the Washington or Philadelphia groups rapidly developed signs of illness and appeared to be very sick 24-hr postchallenge; Philadelphia challenge groups fully recovered by day 2, but Washington groups showed overt signs of illness through day 4 with some deaths on days 2 and 3. Unlike AKR/J mice, nude and haired Swiss mice were unaffected by challenge. Subsequent trials were limited to IP challenge of AKR/J mice with the Washington strain.

Response to LDO was reminiscent of the toxic reactions observed after inoculation of mice with high doses of other Gram-negative bacteria. Inoculation with 10^6 viable organisms of the first passage cultures (7-day growth) of the Washington strain consistently initiated signs of illness within 3-6 hr, but generally a much higher dosage was required to cause death. Addition of 5% bacteriological mucin to the challenge suspension failed to affect its activity, but heating for 30 min at 65°C killed the LDO organisms and destroyed their capacity to induce illness. The LD_{50} has not been defined because the number of CFU in an effective dose was affected by growth time of cultures and by serial passage on MH agar (Table IV).

TABLE IV. CFU AND MOUSE VIRULENCE TESTS FOR 3 PREPARATIONS OF THE WASHINGTON STRAIN DERIVED FROM GROWTH ON MH AGAR

PREPARATION			IP CHALLENGE DOSE (CFU x 10^7)	AKR/J RESPONSE [a]	
No.	Serial passages on MH agar	Growth time (days)		Dead/no. tested	Day of death (mean ± SE)
1	1	7	5	3/6	2.7 ± 0.3
			2	2/6	2.5
			0.5	0/6	
2	1	5	0.9	10/10	1.4 ± 0.2
			0.09	5/10	3.8 ± 1.1
3	4	3	80	6/6	1.3 ± 0.2
			8	0/6	

[a] All mice displayed overt signs of illness for 48 hr.

Mice that survived sublethal challenge rapidly eliminated the challenge organism. Viable counts of spleen, peritoneal wash and blood at 4 hr, 1, 2 and 3 days after IP challenge with 5 x 10^6 CFU indicated that organisms were eliminated at the rate of 2 logs/day. Less than 15% of the challenge dose was recovered at 4 hr when 10% of the dose was still present in the peritoneal cavity. On day 1 when the animals still displayed signs of illness, the highest concentration of organisms (1.5 x 10^4 CFU) was found in the spleen; on day 2, after recovery, the highest concentration was found in the blood (4 x 10^2 CFU/ml); and on day 3, less than 10 CFU were found. Organisms disappeared more slowly, however, in survivors from groups in which death occurred. After challenge with 2 x 10^7 CFU, illness persisted

for 3-4 days; on day 3, 2 x 10^4 CFU were recovered from the spleen, 5 x 10^2 CFU from the peritoneal cavity and 8 CFU/ml from the blood. Unlike tissue samples from infected guinea pigs, samples from mice showed no evidence of secondary invasion by other bacteria.

We have found that an IP inoculation of 1-5 x 10^7 CFU LDO of the Washington strain (5th day growth) will kill AKR/J mice within 24 hr. The same inoculum given to Swiss mice, while making the animals sick (ruffled fur) does not result in death. Furthermore, we have found that pretreatment of AKR/J mice with goat-immune serum containing antibodies elicited by a different LDO strain protects the challenged AKR/J mice from sickness or death. If we remove a soluble antigen from the surface of LDO with NaOH and concentrate it by NH_4SO_4 precipitation and then use this as an immunogen, we can protect AKR/J mice from an ordinarily lethal challenge of LDO. Pretreatment of AKR/J mice with heat-killed LDO also protects the animals from a subsequent lethal challenge. Studies are presently underway to elucidate the "processing" of avirulent and virulent "strains" of LDO by both Swiss and AKR/J mice, and to define the biochemical composition of the protective soluble antigen.

Investigation of the acute onset of the toxic-like response of the AKR/J mouse may contribute evidence to support the hypothesis proposed by Friedman et al. (2) that the multiple organ involvement seen early in human illness is caused by a bacterial toxin.

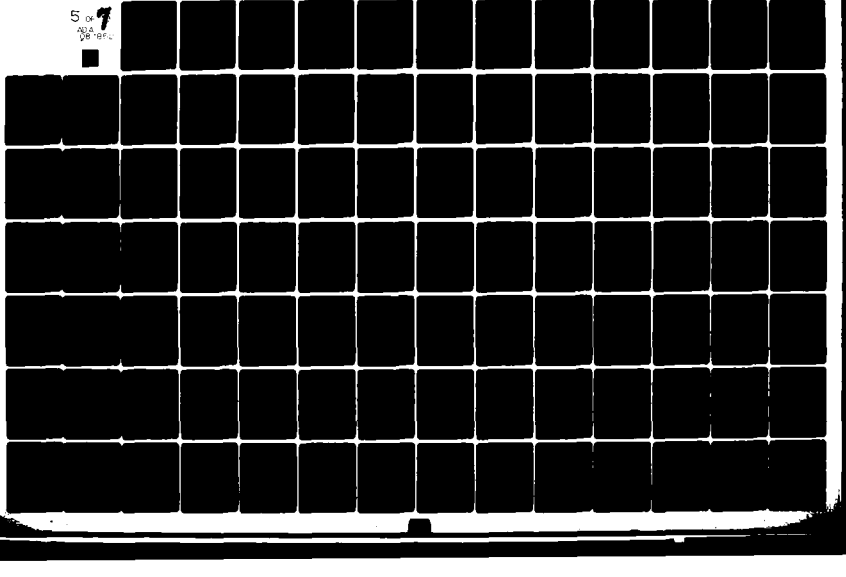
Organisms grown in embryonated eggs. In an attempt to increase virulence of the other strains of LDO, culture of the organisms in embryonated eggs was initiated. For preliminary tests frozen seed stocks prepared in October 1977 from the CDC MH cultures served as the source of egg inocula. Some strains, particularly the Washington strain, caused death of embryos within 2-3 days, possibly analogous to the toxic effect in mice. Whole yolk including the membrane was harvested before death of the embryo; the mixture was homogenized, diluted and stored at -70°C. Impression smears of a section of the membrane were stained with Giménez stain and examined for type of growth, approximate concentrations of organisms and the presence of contaminants. Quantitative counts of diluted homogenate on MH agar were used to estimate the dose for intrathoracic inoculation of guinea pigs.

In the yolk membranes, organisms from most strains were highly variable in size, shape and staining properties. Pairs of Giménez-positive slender rods (0.3 - 0.5 μm x 0.7 - 1.5 μm), joined end-to-end, predominated in heavily infected membranes and were present in microcolonies, large aggregates or as individuals. In less heavily infected membranes large and small aggregates of faintly-stained azure rods similar in size and shape to the Giménez-positive individuals were often the most numerous. Colony counts on MH agar were unexpectedly low when compared to the concentration in stained membranes. For most strains, ability to grow on MH agar was markedly reduced after freezing at -70°C. Except for the Washington strain, these yolk-grown preparations appeared to be avirulent for guinea pigs.

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Yolk-passage seed cultures furnished by Dr. J. McDade (CDC) were used as inocula in subsequent studies to prepare USAMRIID seed and working stocks of the 4 Philadelphia strains. Diluted homogenates of 1st passage yolk membrane harvested at 3 days were stored at -70°C as USAMRIID seed. Working stocks were prepared from pooled homogenates of 2nd passage yolk membranes harvested 5 days after egg inoculation.

Daily examination of membranes from eggs inoculated with strain L-1 suggested that for 48 hr postinoculation only organisms introduced in the inoculum were observable; large aggregates of Giménez-positive LDO organisms present at 24 hr were replaced by numerous small aggregates of deteriorating, Giménez-positive rods within 48 hr. By day 4 large numbers of azure-staining rods, remarkably uniform in size and shape, were present inside membrane cells and a few Giménez-positive individuals of similar size and shape were found extra- and intracellularly; 24 hr later Giménez-positive organisms were very numerous, uniform in appearance and predominantly extracellular. Both the 3-day first passage and 5- or 6-day 2nd passage preparations caused lethal infections in guinea pigs, but virulence was unrelated to the ability of the organisms to grow when subcultured on MH agar (Table V).

TABLE V. COLONIAL GROWTH (CFU) ON MODIFIED MH AGAR AND GUINEA PIG VIRULENCE OF EGG-GROWN PREPARATIONS OF PHILADELPHIA STRAIN L-1 [ORIGINAL INOCULA FOR EGG PASSAGE: CDC MH CULTURE OR CDC FROZEN YOLK MEMBRANE (YM)]

MEMBRANE PREPARATION		GROWTH TIME (days)	FREEZE-THAW CYCLES	GUINEA PIG TEST	
CDC inoculum	No. of egg passages			(CFU x 10^6)	Dead/No. tested (day of death)
MH	2	5	1	80	0/2
YM	1	3	0	3	2/2 (2,5)
	2	6	0	4.5	3/3 (4,4,4)
	2	5	1	NG [a]	1/2 (4)
			2	NG	3/3 (4,4,10)

[a] No growth.

Inability to culture working stock suspensions on artificial media leaves us with few criteria for characterizing the preparations. Attempts to separate organisms from tissue debris in homogenates of washed membranes have not been successful. When the L-1 preparation containing uniformly distributed diploid individuals was centrifuged over

a cushion of 30-90% Percoll, the organisms sedimented in aggregates surrounded by a thin layer of egg-derived material and failed to dissociate upon resuspension in diluent. Procedures similar to the Breed method for evaluating total organism count in dairy products were employed to estimate total count in the working stock suspensions (Table VI). If it is assumed that these estimates of total count were considerably higher than the viable count, the egg-derived working stocks of the Philadelphia strains were remarkably virulent for guinea pigs (Table VII). The lower virulence of corresponding preparations from MH cultures suggests that the artificial media presently in use selects for a population of attenuated LDO.

TABLE VI. WORKING STOCKS OF PHILADELPHIA STRAINS L-1, L-2, L-3 AND L-4 (5-DAY GROWTH FROM 2ND EGG PASSAGE OF CDC EGG MEMBRANE PREPARATIONS)

STRAIN	NUMBER OF MEMBRANES HARVESTED	HARVEST SUSPENSION			TOTAL COUNT/ MEMBRANE ($\times 10^9$)
		Total count LDO/ml ($\times 10^7$)	Giménez positive (%)	Aggregated LDO (% total)	
L-1	29	7	> 90	< 0.1	1.2
L-2	23	5	> 90	< 0.1	1.1
L-3	12	2	47	26	1.0
L-4	25	3	43	43	0.6

Examination of impression smears of spleen, liver, kidney and peritoneal cells from moribund animals challenged with egg-derived preparations revealed the presence of enormous numbers of Giménez-positive diploid rods. Spleens had the highest concentration of extracellular forms while the peritoneal wash contained only intracellular organisms. Most organisms gave strong immunofluorescent reactions with anti-LDO serum; spleen and liver contained rods of variable size while kidney contained tiny coccobacteria, smaller in size than antiserum-treated Rickettsia rickettsii (L. R. Bagley, Virology Division). Tissue homogenates failed to produce growth on MH agar. Our inability to culture LDO from egg inocula or moribund animals suggests that optimal requirements for growth of fully virulent organisms have yet to be defined.

TABLE VII. VIRULENCE TESTS IN GUINEA PIGS: WORKING STOCK PREPARATIONS GROWN IN EMBRYONATED EGGS.

STRAIN	IP CHALLENGE DOSE x 10^6 (Giménez-positive, total count)	GUINEA PIG RESPONSE	
		Dead/tested	Day of death
L-1 (frozen)	70	3/3	4, 4, 10
	7	1/2	4
	0.7	1/2	6
	0.07	0/2	
L-2 (frozen)	50	2/3	3, 4
	5	2/2	2, 5
	0.5	2/2	5, 5
	0.05	2/2	5, 6
L-3 (before freezing)	10	3/3	3, 3, 4
(frozen)	10	1/3	4
L-4 (before freezing)	14	2/3	5, 5
(frozen)	14	1/2	5

Publications:

None

LITERATURE CITED

1. Fraser, D. W., I. K. Wachsmuth, C. Bopp, J. C. Feeley, and T. F. Tsai. 1978. Antibiotic treatment of guinea pigs infected with agent of legionnaires' disease. Lancet. 1:175-178.

2. Friedman, H. M. 1978. Legionnaires' disease in nonlegionnaires. A report of five cases. Ann. Intern. Med. 88:294-302.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6411	78 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
78 06 20	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	025
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 78-7,2,	1, 3, 6		

11. TITLE (Precede with Security Classification Code): (U) Leukocyte adherence inhibition and macrophage migration inhibition to microbial antigens

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 04	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	0.6	30.0
	CURRENT 79	1.0	177.0

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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) Leukocytes; (U) Macrophages; (U) Cell-mediated immunity; (U) Leukocyte transformation

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

23 (U) Examine the leukocyte adherence inhibition (LIA) test as a means of detecting specific cell-mediated immunity (CMI) to microbial antigens of military medical significance in man and experimental animals and correlate with the more conventional macrophage migration factor (MIF) and lymphocyte transformation tests. Utilize LIA and MIF tests to study CMI against infectious microorganisms.

24 (U) Examine LIA response of experimental animals with known immune status to tularemia and/or purified protein derivative, i.e., tuberculosis. Perform simultaneous LAI and MIF assays on animals to validate LAI results. The seond phase will be to expand LAI and MIF studies to humans in order to examine the temporal course of CMI induction.

25 (U) 78 04 - 78 09 - Optimal conditions of the LAI assay were determined. Preliminary observations suggest that preincubation of immune peripheral blood leukocytes with specific antigen prior to conducting the LAI assay may provide more reliable results than when there is no preincubation.

*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

* U.S. GPO: 1974—546-469/8691

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BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 025: Studies of Leukocyte Adherence Inhibition and Macrophage Migration Inhibition to Microbial Agents

Background:

The in vitro production of mediators of cell-mediated immunity (CMI) or lymphokines by sensitized thymus-derived lymphocytes (T-cells) in response to stimulation by specific antigen is the basis for several commonly used measurements of CMI (1,2). However, most in vitro assays for CMI are generally complex, laborious, and require a varying amount of blood and complicated ancillary equipment. In contrast, a recently-described procedure, the leukocyte adherence inhibition assay (LAI), is a rapid and simple in vitro assay of CMI which requires a minimum of laboratory equipment (3). It has been used in human and experimental animal studies to detect host tumor immunity and/or the presence of serum "blocking factors" with a high degree of specificity (3-5). The basis for the LAI assay is the inhibition by specific antigen of the mononuclear cells' propensity to adhere to glass surfaces (2). This inhibition of adherence can be measured by several methods utilizing relatively small amounts of blood. It is our intent to examine the adaptability and reliability of LAI to the study of CMI to microbial antigens of military medical significance.

The production of macrophage migration inhibition factor (MIF) by T-cells is the basis for the MIF test,a generally accepted assay for CMI. The well-characterized specificity of the test makes it a very important correlate with which to compare other procedures such as LAI (1). For that reason the MIF test will be used initially to evaluate the LAI assay results.

Progress:

Studies were initiated to determine the optimal conditions for the LAI assay. Peripheral blood mononuclear cells were harvested by density gradient centrifugation and divided into 2 aliquots with RPMI-1640 plus 15% human serum albumin (HSA). Specific antigen was added to one of the cell suspensions and an equal volume of saline was added to the other. Both samples were then incubated for 30 min at 37^{o}C. At the end of this period, 0.5 ml of the cell suspension was added to glass tubes and the tubes were incubated horizontally. The tubes were lifted vertically at predetermined time intervals and the number of cells remaining in the suspension were counted. The results of a positive LAI assay are shown in Table I.

TABLE I. LAI ASSAY OF A GUINEA PIG IMMUNIZED WITH TULAREMIA AND FREUND'S COMPLETE ADJUVANT.

	NONADHERENT CELLS/mm^3	
Incubation Time	Saline	Saline Tularemia, 2 µg/ml
Preincubation	5605	5605
2 minutes	5246	4492
15	2099	1844
30	1749	2139
45	2355	2053
60	3121	2280
75	2944	2456
90	3346	2634
105	3269	2772
120	3558	2720
135	3654	2391
150	3840	2335
165	3890	2416
180	3647	2529

By analysis of covariance, there is a significant ($P < 0.01$) adherence stimulation, not adherence inhibition as reported by others. When the cell suspensions plus antigen are not preincubated, however, there is a trend towards adherence inhibition, but the overall results of the assay are unpredictable and unreliable. The preliminary observations of adherence stimulation will now have to be repeated and more time-course studies will be done to determine the time of maximum adherence stimulation. In addition, future LAI assay results will be correlated with the more conventional indirect MIF test or lymphocyte transformation assay in order to predict its validity.

Studies were initiated in collaboration with MAJ Donald Harrington (Work Unit A841 00 013), Animal Assessment Division, and Dr. N. R. Di Luzio, Tulane University Medical School, to evaluate the immunomodulating potential of glucan, a β-1,3-glucosidic polymer of glucopyranose units. Glucan has been shown to enhance the rejection or inhibit the growth of spontaneous and/or experimentally-induced tumors in man, mice and rats. In addition, increased HI antibody titers have been reported in experimental animals immunized with sheep RBC and glucan. Studies conducted in Dr. Di Luzio's laboratory also suggest that glucan may be a potential immunological adjuvant, since laboratory animals inoculated with glucan plus either killed Staphylococcus aureus or Candida albicans were significantly more resistant to virulent challenge than vaccinated controls. Studies in this work unit were initiated to assess glucan (a) as an adjuvant for use with marginally antigenic inactivated virus vaccines and (b) as an immunomodulator for stimulating nonspecific host resistance against selected viral infections.

An initial pilot study was done to examine the adjuvanticity of glucan when used with marginally antigenic inactivated viral vaccines in mice. Eighteen groups of 16 mice/group were inoculated SC, IP and IV with glucan (1 or 6 mg/mouse) plus inactivated VEE virus vaccine either SC or IP to determine the effect of dose and route of inoculation of glucan on resistance of mice to virus challenge. All mice were challenged IP on day 14 with 700 $MIPLD_{50}$ of VEE (Trinidad) virus and observed 21 days for mortality. Mice inoculated IV with glucan (1 mg/mouse) plus 0.3 ml vaccine IP were afforded significantly ($P < 0.01$) greater resistance to homologous virus challenge compared to vaccine controls; the survival rates for the 2 immunization groups of mice were 85 and 38%, respectively. Glucan did not significantly alter protection in the other immunization groups of mice. It should be noted that none of the mice given 5 mg (0.5 ml) of glucan plus VEE vaccine (0.3 ml) IV survived the initial inoculation, probably because of circulatory overload and/or pulmonary trapping of the glucan particles.

An experiment was also conducted to assess the ability of glucan, when given without vaccine, to protect mice against an otherwise lethal challenge dose of VEE virus. Results of this experiment are shown in Table II. Glucan (1 mg/mouse), inoculated IV on days -3 and 0, effectively enhanced resistance of mice to challenge on day 0. These preliminary data suggest that glucan may stimulate nonspecific host resistance against certain viral infections.

TABLE II. SUMMARY OF GLUCAN-INDUCED, NONSPECIFIC RESISTANCE IN MICE TO VEE VIRUS CHALLENGE

DAY OF IV GLUCAN INOCULATION (1 mg/mouse)	SURVIVAL/TOTAL CHALLENGED (% SURVIVAL)[a] Virus Challenge Group (dilution)		
	10^{-3}	10^{-4}	10^{-5}
-3, 0	1/17[b] (6)	4/15 (27)*	6/17 (35)*
0, 3	0/19 (0)	1/16 (6)	4/16 (25)
1, 4	1/20 (5)	1/20 (5)	1/20 (5)
Saline controls	1/20 (5)	0/20 (0)	1/20 (5)

[a] Mice were challenged IP on day 0 with 7,700 (10^{-3}) to 77 (10^{-5}) $MIPLD_{50}$ VEE (Trindad) virus and observed 21 days for mortality.
[b] Fischer's exact test was used to compare mortality data of saline controls with other treatment groups.
*$P < 0.05$

Additional studies were conducted to further assess glucan's ability to potentiate host resistance to infectious diseases. Three groups of 4 monkeys each were vaccinated on days 0 and 28 with a marginal dose of inactivated VEE virus vaccine, alone or combined with either 10 or 5 mg/kg of glucan. As can be seen in Table III, SN antibody titers in the group

of monkeys given vaccine plus 10 mg/kg of glucan were significantly ($P < 0.05$) higher by day 28 than titers in control monkeys. Following booster inoculation given on day 28, antibody titers in monkeys given vaccine combined with 10 mg/kg of glucan remained significantly higher for at least 63 days compared to titers of monkeys given vaccine alone. On day 56, for example, antibody titers in the glucan-vaccine group were 45-fold higher than those of controls. Secondary antibody responses in monkeys given 5 mg/kg of glucan plus vaccine were also significantly enhanced between days 42 and 56 compared with vaccine controls. Additional studies are in progress to investigate the adjuvant activity of glucan with other marginally antigenic inactivated vaccines in monkeys.

TABLE III. SUMMARY OF RESULTS OF THE ABILITY OF GLUCAN TO ENHANCE THE PRIMARY AND SECONDARY HUMORAL ANTIBODY RESPONSE IN CYNOMOLGUS MONKEYS VACCINATED WITH INACTIVATED VEE VACCINE

	GEOMETRIC MEAN SN TITER BY GLUCAN DOSE		
DAY	0	5 mg/kg	10 mg/kg
7	16	54	91
14	8	32	54
21	8	32	64
28	4	13	108*
Booster			
33	38	54	181*
35	152	362	1448*
42	54	512*	1772*
49	54	512*	1024*
56	91	861*	4096*
63	64	215	1218*

*$P < 0.05$ compared to controls.

Publications:

None

LITERATURE CITED

1. Lawrence, H. S., 1971. Factors and activities produced in vitro by lymphocytes, In: In vitro Methods in Cell-Mediated Immunity (B. R. Bloom and R. P. Glade, eds.) Academic Press, New York 95-180.

2. Powell, A. E., A. M. Sloss, and R. M. Smith. 1978. Leukocyte-adherence inhibition:a specific assay of cell-mediated immunity dependent on lymphokine-mediated collaboration between T lymphocytes. J. Immunol. 12:1957-1966.

3. Halladay, W. J. and S. Miller. 1972. Leukocyte adherence inhibition: a simple test for cell-mediated tumour immunity and serum blocking factors. Int. J. Cancer 9:477-483.

4. Flores, M., J. H. Marti, N. Grosser, J. K. MacFarlane and D. M. P. Thomson. 1977. An overview: antitumor immunity in breast cancer assayed by tube leukocyte adherence inhibition. Cancer 39:494-505.

5. Hellström, I, K. E. Hellström, G. Van Belle, and G. A. Warner. 1977. Leukocyte-mediated reactivity to human tumors as detected by the leukocyte adherence inhibition test. I. Demonstration of tumor type-specific reactions. Am. J. Clin. Pathol. 68:706-714.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0J6413	78 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
78 07 12	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	026
b. CONTRIBUTING				
c. [illegible]	STOG 78-7,2,	1, 3, 6		

11. TITLE (Procode with 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	CONT	DA	C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	0.6	32.0
FISCAL YEAR CURRENT 79	1.0	92.4

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
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NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Procode 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 (Furnish individual paragraphs identified by number. Procode text of each with Security Classification Code.)

23 (U) Determine cell-surface antigen expression in Rift Valley fever virus and arenavirus infections so as to understand molecular biological action during the disease process. The technique will be applied to vaccine development for viruses of military importance.

24 (U) Develop direct or indirect methods for quantitation of cell-surface viral antigens in cell culture systems and compare to other systems presently in use.

25 (U) 78 07 - 78 09 - Necessary equipment was ordered and most is now on hand. Radioisotope counters were ordered. The rabbit colony was established so that immunoreagents are currently being produced. Preliminary studies with VEE and Pichinde viruses are now under way.

*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

* U.S. GPO: 1976—546-543/6691

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 026: Cell Surface Expression of Viral Antigens during the Infectious Process

Background:

The presence of virus-specific surface antigens on infected cells has been demonstrated for a large number of viruses which include: Chikungunya, VEE, EEE, Sindbis, Semliki Forest, JBE, Pichinde and LCM. These virus-specific antigenic changes in host membranes have been detected by a number of different procedures: immunofluorescence, immunoradiolabeling, immuno-electron microscopy and assays employing cytotoxic 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 surface of the cell, altered or unmasked host proteins or a combination of both.

This work unit was recently activated to study the nature and role of infected cells during the evolution of viral infections. These antigens will be quantitatively analyzed using an immunoprecipitation technique in which the cell surface is first radiolabeled and then reacted with specific antisera; the precipitate will be analyzed by SDS-PAGE. The agents on which this study will focus include Rift Valley fever virus (new strains of which have proved fatal to man in Egypt) and the arenaviruses (where there exist anomalies in the observed postinfection immune responses to these virus as measured by classical serologic tests and for which no vaccine has as yet been developed).

Progress:

All pertinent literature has been reviewed and the equipment necessary for this project has been procured. These studies are now in preliminary stages, with the screening of appropriate cell lines, development of antisera and setting up of equipment.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6415	78 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
78 08 11	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	027
b. CONTRIBUTING				
c. /0/0/0/0/0/0/	STOG 78-7,2,	1, 3, 6		

11. TITLE (Precede with Security Classification Code) (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	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	0.8	8.3
FISCAL YEAR CURRENT 79	0.5	91.2

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21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Immunology; (U) Endogenous pyrogen; (U) Radioimmunoassay

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

23 (U) Produce and purify antibodies to human endogenous pyrogen (EP). Using 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) 78 08 - 78 09 - Intracerebroventricular injection of EP into rats has been valuated and established as an assay method for fever induction. It is effective and allows work with smaller quantities of EP than alternative rabbit assays. In preparation for experiments with human EP, crude on-hand stocks of rabbit EP have been utilized to gain familiarity with handling pyrogen preparations. Butanol and methanol extractions, and Sephadex G-50 gel filtration of this material gave the partial purifications expected.

Rabbit pyrogen, partially purified by butanol and methanol extractions, was injected into a goat for production of antibodies to rabbit EP.

* Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 027: Production and Use of Endogenous Pyrogen Antibodies in Early Detection of Infections of Military Importance

Background:

Endogenous pyrogen (EP) is part of the defense mechanism a host animal utilizes to ward off bacterial or viral infection. It is made in polymorphonucleocytes and monocytes in response to such stimuli as phagocytosis or bacterial endotoxin, and causes fever in the infected animal by interaction with the hypothalamus. Highly purified preparations of EP suggest that it also interacts with phagocytic cells, causing release of inflammation-related constituents (1).

Because EP induces fever in animals, it must be present before the temperature increases. This suggests that its detection may be an early indication of infection. Hence, this study was undertaken to develop a radioimmunoassay for EP.

Human EP is a protein which has been observed in crude preparations to have an apparent MW of 15,000 daltons, although some of its activity is associated with a 38,000-dalton species. These have a pI of 7 and 5, respectively. It has been purified with substantial loss of material by a combination of conventional and immunoabsorption techniques (2). An attempt will be made to improve the overall yield of pyrogen by modifying these methods.

Antibodies to human EP have been produced (3) in rabbits, 7 mon after initial inoculation. These appeared mainly in the IgG fraction of the antisera and were purified by a combination of immunoabsorption and Sephadex 4B gel filtration. Antibodies prepared by modification of this procedure will be used in the radioimmunoassay for EP, and to aid purification of the pyrogen.

Progress:

Intracerebroventricular injection of EP into rats has been evaluated and established as an assay method for fever induction. It is effective and allows work with smaller quantities of EP than alternative rabbit assays.

In preparation for experiments with human EP, crude on-hand stocks of rabbit EP have been utilized to gain familiarity with handling pyrogen preparations. Butanol and methanol extractions, and Sephadex G-50 gel filtration of this material gave the partial purifications expected.

Rabbit EP, partially purified by butanol and methanol extractions, was injected into a goat for production of antibodies to rabbit EP.

Publications:

None

LITERATURE CITED

1. Klempner, M. S., C. A. Dinarello, and J. I. Gallin. 1978. Human leukocytic pyrogen induces release of specific granule contents from human neutrophils. J. Clin. Invest. 61:1330-1336.

2. Dinarello, C. A., L. Renfer, and S. M. Wolff. 1977. Human leukocytic pyrogen: purification and development of a radioimmunoassay. Proc. Natl. Acad. Sci. USA 74:4624-4627.

3. Dinarello, C. A., L. Renfer, and S. M. Wolff. 1977. The production of antibody against human leukocytic pyrogen. J. Clin. Invest. 60:465-472.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6414	78 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. DISS'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 08 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	131
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 78-7,2, 1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(U) Rift Valley fever virus infection: 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	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	0.3	5.0
FISCAL YEAR CURRENT 79	1.0	60.3

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 (Precede EACH with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Immunology; (U) Rift Valley fever virus; (U) Laboratory animals; (U) Bunyaviruses

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

23 (U) Define antigenic and other laboratory characteristics of recent Rift Valley fever virus (RVFV) 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 understand better Bunyaviruses, most of which could be considered of importance as potential BW offensive weapons against U. S. troops.

24 (U) Compare different virus isolates for pathogenicity for several laboratory hosts and for their ability to induce neutralizing antibody to other RVFV strains; study pathogenesis of RVFV infection in several hosts, including genetically defined numbers of the same species.

25 (U) 78 08 - 78 09 - Two recent and two previous RVFV isolates were compared. The 1977 Egyptian isolate (ZZ501) was 10,000-fold more lethal for rats than the other isolates, although they all had similar pathogenicity for mice, cotton rats, hamsters, guinea pigs and gerbils. Convalescent sera from infections with all 4 strains neutralized ZZ501 and the currently used inactivated vaccine protected mice equally well against ZZ501 or parent (Entebbe strain) virus challenge. ZZ501 induced 3 patterns of infection which seem to resemble those seen in humans: nonlethal (guinea pig), acute fulminant (mouse, hamster, rat) and encephalitic (gerbil, cotton rat). Inbred rat strains had differing patterns of infection and resistance to fulminant disease was inherited in a dominant fashion in F-1 animals.

*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. A91C 00 131: Rift Valley Fever Virus Infection: Genetic and Cellular Effects

Background:

Rift Valley fever virus (RVFV) has posed a threat as a potential BW agent a pathogen of domestic animals, and a natural infection capable of incapacitating large numbers of nonimmune persons in an epidemic/epizootic setting. Its potential for disease in the endemic forest cycle is unknown. In 1975 a new clinical spectrum of disease was reported from South Africa: hemorrhagic fever and encephalitis. Then in 1977 RVF invaded new territory by entering Egypt. Human cases numbered in the tens of thousands and were usually the classical, acute, undifferentiated febrile illness; but an estimated 1% of the cases were associated with hemorrhagic fever or encephalitis. Disease activity decreased during the winter, but recurrence has been confirmed this year posing a continuing problem in Egypt and a potential threat to the Middle East. The work reported here was performed over a 6-mon period as part of a program to respond to the need to update our understanding of RVF in light of the evolving clinical spectrum of human disease, changing global epidemiology, and newer concepts of the nature of cellular and genetic determinants of viral infections.

Progress:

Investigation of the Egyptian RVFV Strains. Viremic serum from a fatal hemorrhagic fever patient was obtained from Dr. James M. Meegan, NAMRU-3, Cairo, and was designated ZZ501. The first passage was prepared in certified fetal rhesus lung cells ($FRhL_1$) and transmitted to the Salk facility for possible use as a vaccine seed. The following studies were performed with the $FRhL_2$ passage (ZZ501 $FRhL_2$).

Mice inoculated with the Entebbe strain inactivated RVFV vaccine in current use were shown to be immune to ZZ501 challenge in a quantitatively similar fashion as to Entebbe challenge. This supports the use of Entebbe vaccine to protect laboratory personnel or other at-risk persons from the Egyptian virus strain. Furthermore, cross-neutralization (N) tests with convalescent rat or guinea pig sera could not distinguish Entebbe or ZZ501 strains of RVFV.

The virus was examined for lethality and infectivity in several species (Table 1) and these results will be discussed further.

TABLE I. SUBCUTANEOUS INOCULATION OF LABORATORY ANIMALS WITH RVFV (ZZ501 $FRhL_2$)

ANIMAL	LOG PFU INJECTED	NO. SURVIVORS/TOTAL	NO. ANTIBODY / NO. TESTED
ICR Swiss mouse	1	10/10	0/10
	3	4/14	2/ 4
	5	0/ 5	-
Sprague-Dawley rat	1	6/15	3/ 6
	3	3/25	2/ 2
	5	1/10	1/ 1
Syrian hamster	1	0/10	-
	3	0/ 5	-
	5	0/ 5	-
Cotton rat (Sigmodon hispidus)	1	5/ 5	5/ 5
	3	5/ 5	5/ 5
	5	5/ 5	5/ 5
	7	4/ 9	-
Gerbil	5	2/ 5	2/ 2
	7	2/ 5	2/ 2
Guinea pig	1	5/ 5	0/ 5
	3	5/ 5	1/ 5
	5	5/ 5	5/ 5
	7	5/ 5	-

Comparison of RVFV Strains. Three virus strains were chosen for comparison to ZZ501 (Table II). Two were low passage seed from South Africa obtained in 1951 (SA51) and in 1975 (SA75) and the 3rd was a high mouse passage strain used to prepare the vaccine (Entebbe). Virus pools in FRhL cells and immune sera have been generated. All strains cross-react by the N test although detailed studies are not yet complete. Plaque morphology is similar although ZZ501 has the clearest plaques in our Vero cell system. All grow to similar titer in most cell lines studied (1-5 x 10^7 PFU/ml) except SA51 which reaches somewhat higher levels (1-2 x 10^8 PFU/ml). Molecular properties are similar for all 4 strains.

TABLE II. ORIGIN OF RVFV STRAINS USED

STRAIN	AREA	ISOLATED FROM	PASSAGE BEFORE RECEIVED	PASSAGE AFTER RECEIVED	REMARKS
ZZ501	Egypt	Human, hemorrhagic fever	None	$FRhL_2$	Clearest plaques; lethal for rats
Entebbe	Uganda	Sheep	180 mouse	$FRhL_1$	Current vaccine seed
SA51	South Africa	Sheep	SM_1 $lamb_2$	$FRhL_2$	Grows to highest titer
SA75	South Africa	Human, uncomplicated case	None	$FRhL_3$	

The 4 strains were compared quantitatively for their pathogenicity for several laboratory hosts based on results with ZZ501 (Table I). In most cases the outcome of infection was the same (Table III); however, ZZ501 was significantly more pathogenic for outbred Sprague-Dawley rats. This was confirmed using inbred Wistar Furth (WF) rats.

TABLE III. COMPARISON OF RVFV STRAINS IN LABORATORY ANIMALS AFTER SC INOCULATION

ANIMAL	LOG PFU INJECTED	NO. SURVIVORS/TOTAL			
		ZZ501	Entebbe	SA51	SA75
Mouse	1	10/10	2/10	4/10	6/10
	3	4/14	0/14	1/14	3/12
Rat	1	6/15	9/10	5/ 5	5/ 5
	3	3/25	8/ 8	5/ 5	10/10
	5	1/21	13/16	14/17	9/11
	7	-	11/16	10/14	13/15
Hamster	1	0/10	0/ 5	0/ 5	0/ 5
Cotton rat	5	5/ 5	5/ 5	6/ 6	5/ 6
	7	11/19	9/11	10/15	10/10
Guinea pig	5	10/10	5/ 5	-	5/ 5
	7	12/12	5/ 5	5/ 5	5/ 5
	8	-	-	5/ 5	-

Pathogenesis. Table I describes the outcome of SC infection of several laboratory animal species with RVFV. Mice, Sprague-Dawley rats, and hamsters were susceptible to infection with low doses of virus, and infection was usually lethal. Cotton rats were easily infected (all 5 animals given 10 PFU developed antibody) but only succumbed to large doses of virus. Guinea pigs, however, were resistant both to infection (10^3 PFU only resulted in antibody in 1 of 5 recipients) and to the lethal effects of RVFV. In serial sacrifice studies (Table IV) outcome of infection was shown to correlate with viremia and type of pathologic lesions. Highly susceptible animals died within 2-6 days with progressive viremia and extensive liver necrosis. Cotton rats and gerbils, which died later (7-21 days) with clinical evidence of encephalitis, had modest transient viremias. Guinea pigs were clinically resistant to RVF and had virtually no detectable viremia. This suggests that mice, Sprague-Dawley rats, and hamsters develop a primary viremia which regularly leads to hepatocytic infection, liver necrosis, and extremely high secondary viremias. Cotton rats and gerbils, on the other hand, occasionally have brain involvement during their primary viremia and succumb to encephalitis. The encephalitis occurs late, brain virus titers are low, and serum N antibody is present, a constellation of findings which suggest an immunopathologic mechanism. An interesting model was provided by the ribavirin-treated mouse; early death from hepatic necrosis can be prevented but the animal may succumb later to encephalitis.

TABLE IV. VIREMIA AND OUTCOME OF RVFV INFECTION

ANIMAL	INOCULUM[a]	DAY[b]				OUTCOME
		1	2	3	4	
Mouse	3.0	2.6 (2/5)	5.1 (3/5)	7.0 (3/5)	3.8 (2/5)	Fulminant hepatitis
Sprague-Dawley rat	3.0	2.7 (1/3)	5.6 (3/3)	6.3 (2/3)	5.1 (2/2)	Fulminant hepatitis
Hamster	2.0	3.2 (3/3)	$\geq$8.0 (3/3)	-	-	Fulminant hepatitis
Gerbil	7.0	3.6 (3/3)	1.8 (2/3)	2.2 (1/3)	<1.4 (0/3)	Encephalitis
Cotton rat	7.0	4.5 (3/3)	1.0 (1/3)	<1.4 (0/3)	<1.4 (0/3)	Encephalitis
Guinea pig	7.0	1.6 (1/3)	<0.7 (0/3)	<0.7 (0/3)	<0.7 (0/3)	No disease

[a] Log_{10} PFU ZZ501 $FRhL_2$ injected SC on day 0.

[b] Log_{10} geometric mean of positive samples.
( ) No. viremic/No. tested; 3-5 animals sacrificed on day noted.

More detailed studies were undertaken in the mouse system. Some sort of barrier to SC infection develops in the mouse between 4-6 weeks of age. One PFU is lethal for newborn, 2-4-, and 5-6-week-old mice if given by the IP or IC route. When virus is injected SC, 1 PFU kills newborn or 2-4 week-old mice but 5-6 week-old animals require 100 PFU. Nude athymic mice are somewhat more resistant to SC infection than their heterozygous counterparts. Since nude mice are known to have activated macrophages, we examined virus clearance and growth in resident peritoneal cells. Virus clearance was not accelerated, but in preliminary experiments replication in macrophages from nude mice was reduced. Glucan, a macrophage activator, was found to provide some protection to mice infected with RVFV. A genetic basis for resistance may also operate. Offspring of Swiss mice which survived RVF resisted subsequent challenge.

The most striking example of the role of host genetics in determining the outcome of RVF was provided by the inbred rat studies (Table V). Some strains were clinically unaffected by 5×10^5 PFU of RVFV. Others (Brown Norway [BN] and WF) died of total hepatic necrosis within 3 days of inoculation. The LD_{50} for WF and BN rats was < 5 PFU. The F_1 hybrids of resistant and susceptible strains (Le x WF and Le x BN) were resistant to 5×10^5 PFU challenge, suggesting that lethal infection with RVFV may be the result of a recessive gene.

TABLE V. INFECTION OF INBRED RAT[a] STRAINS WITH RVFV (ZZ501 $FRhL_2$)

RAT STRAIN	AgB	VIRUS DOSE (PFU)	NO. SURVIVORS/INOCULATED	DISEASE
Lewis	1	5×10^5	5/ 5	No disease
		5×10^3	10/10	
ACI	4	5×10^5	2/ 5	Encephalitis
		5×10^3	9/10	
F344	1	5×10^5	17/20	Mixed
		5×10^3	13/14	
Wistar-Furth	2	5×10^5	0/ 5	Fulminant
		5×10^3	0/10	
Brown Norway	3	5×10^5	0/ 5	Fulminant
		5×10^3	0/ 5	
Buffalo	6	5×10^5	0/10	No disease
		5×10^3	0/ 5	
Maxx	3	5×10^5	2/ 5	Encephalitis
		5×10^3	2/ 5	

[a] 10-15 week-old female rats.

The full spectrum of human disease may be reproduced in the appropriate rat strain, since Maxx and ACI rats develop encephalitis. Their disease resembles the human condition in that it occurs after the acute stage and is associated with the presence of serum antibody and not viremia. Only about half of these 2 strains developed encephalitis, so we attempted to develop a more uniform model. Using a higher virus dose, passing virus in the rat blood or brain, or using the IP route of inoculation failed to improve the model in ACI rats. IC inoculation resulted in fulminant disease. Using younger Maxx rats resulted in the appearance of fulminant disease, but in initial experiments younger male ACI rats were more susceptible to encephalitis without suffering the early death from hepatic necrosis.

Collaborative studies with other investigators are underway to investigate the use of selected adjuvants to enhance the early appearance and persistence of antibody in primates and sheep, to optimize the RVFV vaccination schedule, define the principles of ribavirin use in RVFV infections, analyze the specificity of the immune response for individual virion antigens, and develop a primate hemorrhagic fever model.

Presentation:

Peters, C. J. Rift Valley fever in Egypt. Presented, Global Epidemiology Working Group, Washington, DC, 26 January 1978.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6429	78 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
78 08 08	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	138
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2	1, 3, 6		

11. TITLE (Precede with Security Classification Code)

(U) Efficacy of zinc treatment in experimental endotoxemia and bacterial sepsis

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
76 02	78 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 78	0.8	66.0
FISCAL YEAR CURRENT 79	0	0

19. RESPONSIBLE DOD ORGANIZATION

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

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SOCIAL SECURITY ACCOUNT NUMBER:

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) Prophylaxis; (U) Zinc; (U) Therapy; (U) Endotoxemia; (U) Gram-negative sepsis

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

23 (U) Objectives were expanded to include the determination of mechanisms involved in the hypozincemia of bacterial infections and endotoxicosis. Provide information relating to the pathogenesis of infectious disease and for evaluating the usefulness of plasma Zn alterations in the early detection of infectious diseases of importance in BW defense.

24 (U) Time course of plasma Zn depression and hepatic Zn accumulation in various cytosol fractions is determined following the induction of various bacterial infections and inflammatory conditions.

25 (U) 77 10 - 78 09 - Hypozincemia was induced in rats by Salmonella typhimurium and LVS Francisella tularensis infections, endotoxin and various phlogistic agents. Plasma Zn depression, regardless of the initiating stimulus, is dependent on new hepatic mRNA synthesis and subsequent synthesis of a unique protein, identified as metallothionein (MT), which possesses high binding capacity for Zn. In a relatively mild infection (LVS), the measured half-life for MT (19 hr) is consistent with the idea that MT-Zn represents a highly labile pool of sequestered Zn. Results of other studies, performed to complete initial objectives, revealed that IP administration of S. typhimurium endotoxin induced an early and persistent hypertaurinemia and a generalized hypoaminoacidemia at 5 hr; taurine, liberated from formed elements of the blood, appears to be responsible. Physiologic and metabolic consequences of hypertaurinemia are unknown.

Objectives have been met. The work unit is completed.

Publications: Proc. Soc. Exp. Biol. Med. 156:334-339, 1977; Am. J. Physiol. 234:E399-E406, 1978; Fed. Proc. 37:890, 1978; J. Cell Physiol. 95:115-124, 1978; Can. J. Microbiol. 24:834-838, 1978.
Proc. Soc. Exp. Biol. Med. in press, 1978.

*Available to contractors upon originator's approval

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

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BODY OF REPORT

Project No. 3A061101A91C: Medical Defense Against Biological Agents (U)

Work Unit No. A91C 00 138: Efficacy of Zinc Treatment in Experimental Endotoxemia and Bacterial Sepsis

Background:

Zinc is an essential trace element involved in diverse biological functions. Bacterial infection as well as inflammation and endotoxicosis in rats are known to produce numerous physiologic and metabolic alterations, including the redistribution of Zn from plasma to liver. Previous work from this research effort has demonstrated the prophylactic efficacy of parenteral Zn (PZn) administration in endotoxemia (1) and the diverse (sometimes beneficial, sometimes detrimental) effects of PZn on the course of various bacterial infections (2). In addition, preliminary results (3) indicated that endotoxin induced marked alterations in plasma amino acid homeostasis which could be prevented by PZn.

These findings prompted the subsequent extension of research objectives to obtain information on the mechanism involved in the altered plasma Zn homeostasis which occurs during endotoxicosis, sepsis, and after the administration of various phlogistic agents. Emphasis in these studies was placed on identification of the major ligand(s) responsible for the apparent sequestration of Zn in the liver and factor(s) controlling their synthesis. In addition, endotoxin-induced changes in the concentration of plasma free amino acids were further defined since such alterations could be involved in pathophysiologic mechanisms during endotoxemia.

Progress:

Hepatic sequestration of Zn. A key role for hepatic Zn-binding proteins, metallothioneins, has been established for the hypozincemia which occurs in rats during endotoxemia and bacterial infections (4), and after the administration of phlogistic agents (5). In addition, recent evidence indicates that metallothioneins are also involved in the redistribution of Zn from plasma to liver during hypersensitivity reactions induced with a protein antigen, bovine serum albumin (BSA). Induction of hepatic synthesis of metallothioneins thus appears to be an integral part of the mechanism responsible for the redistribution of Zn from plasma to liver in these conditions. Due to their high number of half-cystinyl residues, metallothioneins possess a rather unique ability to sequester various bivalent metals such as Zn, Cd, and Hg as mercaptide complexes. The induction of metallothioneins is not dependent on an intact pituitary-adrenal axis and control of their synthesis appears to be at the transcriptional level. The reader is referred to a recent publication (4) for additional information on the methods developed and employed for the isolation and characterization of these proteins.

Many diverse chemical agents which include disulfiram, isoproterenol, turpentine, regitine, indomethacin, and heavy metals such as Cd, Zn, and Hg have been found to induce hypozincemia and initiate the synthesis of hepatic metallothioneins in the rat. All of these substances produce inflammation (often severe tissue necrosis) when administered in pharmacologic amounts. These results suggest that the stimulus for the synthesis of metallothioneins may be part of the response to tissue necrosis/inflammation. Additional support for this contention arises from recent findings that hypersensitivity reactions (produced in rats sensitized and challenged with BSA) also induce hypozincemia and hepatic metallothionein accumulation.

Plasma amino acid homeostasis. Studies were performed in rats to determine the effect of endotoxin administration on individual plasma free amino acid concentrations since altered amino acid homeostasis could be involved in pathophysiologic mechanisms during endotoxemia. Intraperitoneal administration of Salmonella typhimurium endotoxin (1.0 mg/100 gm body weight) induced an early and persistent hypertaurinemia, hyperglucagonemia, as well as transient hyperinsulinemia and a generalized hypoaminoacidemia during a 5-hr experimental period.

Depressions in plasma concentrations of most amino acids appeared to be attributable to endotoxin-induced elevations in peripheral glucagon and ureagenesis. Arginine nearly disappeared from plasma within 3 hr and was not detectable by 5 hr. Marked alteration in arginine concentration could be due, in part, to hyperglucagonemia and the liberation of intracellular arginase from various tissues and its enhanced activity in peripheral blood.

Overt hepatocellular damage was detectable by 3 hr and could contribute to altered plasma glucose and amino acid homeostasis during the latter stages of endotoxicosis.

Hypertaurinemia was demonstrable with a wide range of endotoxin doses (0.05-1 mg/100 gm body weight). Early hypertaurinemia may result as a consequence of destruction of formed elements of the blood (polymorphonuclear leukocytes and platelets are particularly rich in taurine), whereas the sustained elevation may involve more complex mechanisms. Decreased renal clearance does not appear to be involved in altered plasma taurine homeostasis. The physiologic and metabolic consequences of hypertaurinemia during endotoxemia are unknown but may involve certain aspects of temperature regulation, carbohydrate metabolism and behavioral alterations commonly observed in rats during endotoxemia.

Two manuscripts entitled "Induction of hypozincemia and hepatic metallothionein synthesis in hypersensitivity reactions" and "Alterations in plasma amino acid homeostasis during experimental endotoxemia" have been submitted for publication.

Objectives have been met. The work unit is completed.

Presentation:

Sobocinski, P. Z., W. J. Canterbury, Jr., C. A. Mapes, R. E. Dinterman, E. C. Hauer, and F. B. Abeles. Hypozincemia of inflammation: sequestration of zinc by hepatic metallothioneins. Presented, Annual Meeting, FASEB, Atlantic City, NJ, 9-14 Apr 1978 (Fed. Proc. 37:890, 1978).

Publications:

1. Sobocinski, P. Z., W. J. Canterbury, Jr., and M. C. Powanda. 1977. Differential effect of parenteral zinc on the course of various bacterial infections. Proc. Soc. Exp. Biol. Med. 156:334-339.

2. Mapes, C. A., P. T. Bailey, C. F. Matson, E. C. Hauer, and P. Z. Sobocinski. 1978. In vitro and in vivo actions of zinc ion affecting cellular substances which influence host metabolic responses to inflamation. J. Cell. Physiol. 95:115-124.

3. Sobocinski, P. Z., W. J. Canterbury, Jr., C. A. Mapes, and R. E. Dinterman. 1978. Involvement of hepatic metallothioneins in hypozincemia associated with bacterial infection. Am. J. Physiol. 234:E399-E406.

4. Walker, R. I., S. L. Snyder, P. Z. Sobocinski, K. F. McCarthy, and J. E. Egan. 1978. Possible association of granulocyte mobilization to the peritoneal cavity with $ZnCl_2$-induced protection against endotoxin. Can. J. Microbiol. 24:834-838.

LITERATURE CITED

1. Sobocinski, P. Z., M. C. Powanda, W. J. Canterbury, S. V. Machotka, R. I. Walker, and S. L. Snyder. 1977. Role of zinc in the abatement of hepatocellular damage and mortality incidence in endotoxemic rats. Infect. Immun. 15:950-957.

2. Sobocinski, P. Z., W. J. Canterbury, Jr., and M. C. Powanda. 1977. Differential effect of parenteral zinc on the course of various bacterial infections. Proc. Soc. Exp. Biol. Med. 156:334-339.

3. Sobocinski, P. Z., M. C. Powanda, and W. J. Canterbury. 1976. Effect of zinc pretreatment on endotoxin-induced mortality and hyperaminoacidemia in rats. Fed. Proc. 35:360.

4. Sobocinski, P. Z., W. J. Canterbury, Jr., C. A. Mapes, and R. E. Dinterman. 1978. Involvement of hepatic metallothioneins in hypozincemia associated with bacterial infection. Am. J. Physiol. 234:E399-E406.

5. Sobocinski, P. Z., W. J. Canterbury, Jr., C. A. Mapes, R. E. Dinterman, E. C. Hauer, and F. B. Abeles. 1978. Hypozincemia of inflammation: sequestration of zinc by hepatic metallothioneins. Fed. Proc. 37:890.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6415	77 12 13	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
77 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	61101A	3A161101A91C	00	140
b. CONTRIBUTING				
c. [illegible]	STOG 78-7.2, 1, 3, 6			

11. TITLE (Precede with Security Classification Code)

(U) Effects of antioxidants upon the inactivation of lipid-containing viruses

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	77 12	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 78	0.4	15.0
FISCAL YEAR CURRENT 79	0	0

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

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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) Flaviviruses; (U) Prophylaxis; (U) Alphaviruses; (U) Arenaviruses; (U) Laboratory animals

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

23 (U) Investigate feasibility and the ability of antioxidants to inactivate highly virulent lipid-continuing viruses in vivo and in vitro which may be natural disease or BW threats to U. S. military forces.

24 (U) The in vivo effect is accomplished by feeding the antioxidant to laboratory animals followed by challenge with virulent virus. Many dangerous viruses for man contain lipid. Any success in reducing virulence by antioxidant treatment will be beneficial to the military.

25 (U) 77 06 - 77 12 - Data suggest that rats can be protected from the lethal effects of Venezuelan equine encephalomyelitis virus infection by the prefeeding of the animals with the antioxidant, butylated hydroxytoluene.

Work will continue under a mission-funded work unit. This ILIR work unit is terminated.

*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 65 (FOR ARMY USE) ARE OBSOLETE

* U.S. GPO: 1974—540-843/960

BODY OF REPORT

Project No. 3A161101A91C: In-house Laboratory Independent Research (U)

Work Unit No. A91C 00 140: Effects of Antioxidants upon the Inactivation of Lipid-contining Viruses

Background:

The main components of biological membranes are phospholipids and proteins. In 1974 Eletr et al. (1) showed that the perturbations which antioxidants such as butylated hydroxytoluene (BHT) induced in the lipid components of biological membranes caused measurable effects on membrane function. Snipes et al. (2) subsequently showed that BHT has the ability to inactivate lipid-containing viruses in vitro. Their viral studies were very interesting for the following reasons: BHT is one of several antioxidants that are commonly added to foods to maintain freshness and prevent spoilage by oxidation. The effectiveness of BHT as an antioxidant for certain foods is probably due in good part to its low water solubility and its antioxidant activity is most effective. Cupp et al. (3) demonstrated that in vitro BHT disrupted the bacteriophage PM2 virion releasing ^{32}P-labeled nucleic acids and phospholipid materials.

This finding coupled with well documented mammalian membrane perturbation by BHT allowed for the postulation of at least 2 distinct mechanisms by which antioxidants such as BHT or butylated hydroxy-anisole (BHA) could bring about the inactivation of lipid-containing viruses. The first obviously was the disruption of the intact virion by the direct effect of BHT upon the virus itself. The second was that the incorporation of the antioxidant into lipoproteins of mammalian cells may alter the entrance of lipid-containing viruses into those perturbed membranes. It is of interest to note that Snipes' group had already considered that in vivo, the partition coefficient of BHT between the virus and its surroundings might well influence the degree of inactivation. In vivo the lipid-containing viruses would be exposed to the antioxidants for periods of time much longer than those used in the in vitro experiments. It appears feasible, based on the experiments of Snipes and Cupp, for the in vitro model system to be pursued in animals.

Progress:

Data suggest that rats can be protected from the lethal effects of Venezuelan equine encephalomyelitis virus infection by the prefeeding of the animals with the antioxidant, BHT.

Work will continue under a mission-funded work unit. This ILIR work unit is terminated.

Publications:

None.

LITERATURE CITED

1. Eletr, S., M. A. Williams, T. Watkins, and A. D. Keith. 1974. Perturbations of the dynamics of lipid alkyl chains in membrane systems: effect on the activity of membrane-bound enzymes. Biochim. Biophys. Acta 339:190-201.

2. Snipes, W., S. Person, A. Keith, and J. Cupp. 1975. Butylated hydroxytoluene inactivates lipid-containing viruses. Science 188:64-66.

3. Cupp, J., P. Wanda, A. Keith, and W. Snipes. 1975. Inactivation of lipid-containing bacteriophage PM2 by butylated hydroxytoluene. Antimicrob. Agents Chemother. 8:698-706.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OG6428	78 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
77 10 01	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	141
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 78-7.2.	1, 3, 6		

11. TITLE (Precede with Security Classification Code): (U) Rapid detection of immune complexes in infectious diseases 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	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	0.4	20.0
FISCAL YEAR CURRENT 79	0.5	58.5

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) Antibody; (U) Immune complexes; (U) Isotachophoresis; (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 rapidly 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) 77 10 - 78 09 - Following our previous demonstration that soluble immune complexes could be demonstrated by analytical isotachophoresis, our efforts were directed toward a closer look at human immunoglobulins. Dr. Richard Wistar of the Naval Medical Research Center, Bethesda, provided us with isolated subclasses of the 4 human IgG myeloma proteins. We have been able to show that each subclass has its own unique effective mobility. This allowed us to clearly demonstrate for the first time that this method could be used for identification and isolation of the 4 subclasses of human IgG.

Publications: J. Chromatogr. 162: in press, 1979.
J. Immunol. Methods, in press, 1978.

*Available to contractors upon originator's approval

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

* U.S. GPO: 1974—540-687/5601

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BODY OF REPORT

Project No. 3A061101A91C: In-house Laboratory Independent Research (U)

Work Unit No. A91C 00 141: 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 a 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 inoic 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 in which 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:

Basically 2 unique studies have been undertaken and completed. The first dealt with the demonstration of soluble immune complexes which had an effective mobility intermediate to the homologous antigen and antibody and will be published early in 1979 in the Journal of Chromatography.

The second study described a new method for demonstrating the 4 individual subclasses of human IgG immunoglobulins by means of analytical isotachophoresis; it will be published shortly in the Journal of Immunological Methods. Individual subclasses of IgG purified from sera of multiple myeloma patients were demonstrated for the first time to have distinct effective mobility patterns. When a mixture of purified IgG with each of the 4 subclasses is analyzed, a characteristic mobility pattern with 4 unique peaks is obtained. It was found that an IgG of an unknown subclass could be identified by the superimposition of the

peak of the unknown upon one of the 4 well-identified peaks. This method using microgram quantities of purified protein in microliter volumes can be performed in 15 min.

Presentation:

Hedlund, K. W., and D. Nichelson. 1978. Identification of soluble immune complexes by analytical isotachophoresis. Presented, Annual Meeting, ASM, Las Vegas, NV, 14-19 May 1978. (Abstracts of Meeting - 1978, p. 59).

Publications:

1. Hedlund, K. W., and D. E. Nichelson. 1979. A demonstration of soluble immune complexes by analytical isotachophoresis. J. Chromatogr. 162: 76-80.

2. Hedlund, K. W., R. Wistar, and D. E. Nichelson. 1979. The identification of the subclasses of human IgG by analytical isotachophoresis. J. Immunol. Meth. 25:43-48.

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 0J6412	78 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
78 04 28	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	142
b. CONTRIBUTING				
c. //////////	STOG 78-7.2. 1, 3, 6			

11. TITLE (Precede with Security Classification Code): (U) Development of radioimmune assay procedures for quantitation of viral antibodies and antigens

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 02	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		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 78	0.5	10.0
	CURRENT 79	0.5	25.4

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 (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Staphylococcus aureus protein A; (U) Radioimmunoassay; (U) Vaccines; (U) Antibodies; (U) Antigens

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 radioimmune assay (RIA) for quantitation of viral antigens and antibodies. Such a procedure would be of inestimable value for measuring antibody responses in immunized personnel, so as to know how much protection there is against a disease of military importance.

24 (U) Prepare concentrated radiolabeled virus antigens. Add S. aureus protein A (SaCI) to precipitate labeled material. Test supernatants for residual (unbound) antigen and construct a titration curve. Optimum conditions for each disease virus will be determined. Methods will be compared to conventional serologic assays.

25 (U) 78 04 - 78 09 - RIA methods for measuring antibodies to alphaviruses were developed, using SaCI and tritiated virus antigens. Sera from approximately 80 personnel inoculated with VEE, EEE, and WEE vaccines were tested by RIA; results were compared with conventional plaque reduction neutralization tests (PRNT). The RIA test was more rapid, sensitive, reproducible, and precise. It is recommended that RIA replace the PRNT for routine testing of sera for alphavirus antigens. The validity of the method is being tested using coded sera. The RIA is being adapted to measure antibodies to the viruses of Rift Valley, yellow and Lassa fevers. Preliminary efforts to develop competitive binding assays for measuring concentrations of antigens in inactivated alphavirus vaccine lots have been successful. Further progress depends on acquisition of monospecific antibodies raised against individual virus proteins, and on the production of radiolabeled viruses with higher specific radioactivities.

Publications: Arthropod-Borne Virus Information Exchange 34:180-182, 1978.
J. Clin. Microbiol. 8:54-60, 1978.

*Available to contractors upon originator's approval

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

* U.S. GPO 1978—640-861/6681

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BODY OF REPORT

Project No. 3A161101A91C: In-House Laboratory Independent Research

Work Unit No. A91C 00 142: Development of Radioimmune Assay Procedures for Quantitation of Viral Antibodies and Antigens

Background:

Protein A is a molecule present in high concentration on the surface of certain Staphylococcus aureus strains (1). It rapidly binds most mammalian IgG molecules (2) through an interaction with the Fc region. Formalin-fixed staphylococci retain protein A on their surfaces (3), and may be used as solid phase adsorbents in radioimmunoassay (RIA) procedures to separate virus-antibody complexes from unbound radiolabeled virus by low-speed centrifugation. Protein A-bearing Staphylococcus (SaCI) thus substitutes for the secondary antibody (anti-IgG) in an RIA, and offers several advantages. Since protein A combines with most mammalian IgG molecules, it is not necessary to prepare individual precipitating anti-IgG antibodies for each species tested. The reaction between IgG and SaCI is very rapid (4); 1- to 10-min incubation times are sufficient, compared to the 12-18 hr required for the secondary antibody technique. The SaCI reagent is also easily and economically prepared (4). The first objective of this project is the development of RIA using SaCI to measure antibodies against alphaviruses in human and other mammalian sera. This objective was met during the past year.

Progress:

A detailed account of the development of a RIA to measure antibodies to alphaviruses has been published (1).

The following general procedure was developed to measure alphavirus antibodies in immune sera, using SaCI: 2-fold serial dilutions of sera were presented in RIA buffer, which consisted of HBSS containing 0.5% bovine serum albumin and 10 mM HEPES (N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid) buffer, pH 7.6. To each 0.45-ml volume of serum dilution, 50 µl of ^{3}H-labeled virus (diluted in RIA buffer to an appropriate concentration, usually 8.1 $\log_{10}$ PFU/ml) was added. Labeled virus and serum were incubated for 1 hr at 37 C. Following incubation, 100 µl of each mixture were removed for assay and replaced with 100 µl of SaCI. Each tube was briefly mixed using a Vortex shaker and centrifuged at 2,600 rpm for 10 min. A 100-µl sample of each supernatant was then assayed for residual (unbound) ^{3}H-virus activity; the proportion of virus bound to SaCI was calculated. Under the conditions stated, <5% of the labeled VEE and WEE was bound nonspecifically. However, for labeled EEE, greater proportions often bound nonspecifically. This nonspecific adsorption could be inhibited by the addition of 0.2 M potassium phosphate buffer (pH 7.6) to the RIA buffer. In block titrations were EEE was included, this 0.2 M phosphate buffer was substitute for the standard buffer, with no effect on the VEE and WEE RIA endpoints.

RIA titer was defined as the dilution of serum expected to effect 50% binding of ^{3}H-labeled virus to SaCI This value was obtained by probit analysis of all data points for each curve. Although probit analyses were routinely performed by computer, close approximations could also be obtained by plotting data points on probit paper.

Using this procedure, it was observed that as serum was diluted out, less ^{3}H-virus was bound, and a sigmoid curve was generated. An example of a typical titration curve for a hyperimmune guinea pig serum is tabulated in Table I.

TABLE I. PRECIPITATION OF ^{3}H-VEE ANTIBODY COMPLEXES FROM HYPERIMMUNE GUINEA PIG SERUM DILUTIONS USING SaCI and 8.1 $\log_{10}$ PFU of H-VEE

RECIPROCAL SERUM DILUTION	% OF ^{3}H-VEE BOUND
1600	93.6
3200	93.8
6400	92.9
12800	81.0
25600	62.5
51200	47.6
102400	35.5
204000	26.9

The 50% binding endpoint, calculated for this curve, was determined to be 1:44,000. The 95% confidence interval for this point ranged from 1:38,000 to 1:50,000, less than a 2-fold dilution, and very precise in comparison with the variation inherent in conventional serology. For comparison, the plaque reduction neutralization (PRN) titer for this serum was 1:5120.

The RIA titer was a function of both the antibody and virus concentrations employed. When less virus was used, higher endpoints were obtained. (Table II).

TABLE II. RIA TITERS OF HYPERIMMUNE GUINEA PIG SERUM, AS A FUNCTION OF ^{3}H-VEE CONCENTRATIONS EMPLOYED IN THE SaCl PRECIPITATION PROCEDURE

CONCENTRATION OF ^{3}H-VEE (Log 10 PFU/ml)	RECIPROCAL RIA TITER CALCULATED
9.2	4750
8.9	9450
8.6	18900
8.3	38500
8.0	77000

Since serum titer was an inverse function of antigen concentration, it was necessary to standardize all test viruses to a uniform concentration using independent criteria such as PFU, or electron microscope particle counts. Alphavirus preparations were routinely diluted to 8.1 logs virions/ml, since this represented the maximum dilution of labeled virus containing enough counts to work with conveniently.

Using appropriately diluted viruses, specificities of hyperimmune guinea pig sera (raised against VEE, WEE, and EEE viruses) were tested in a block titration. The combination of viruses with their homologous antisera clearly resulted in greater binding than the heterologous combinations. In fact little or no cross-reactivity was observed (Table III).

TABLE III. SEPARATION OF VIRUS-ANTIBODY COMPLEXES FROM MIXTURES OF ^{3}H-LABELED ALPHAVIRUSES AND HOMOLOGOUS OR HETERLOGOUS HYPERIMMUNE GUINEA PIG SERA

ANTISERUM	RECIPROCAL DILUTION	% ^{3}H-LABELED VIRUS BOUND[a]		
		VEE	WEE	EEE
VEE	100	85.9	7.0	11.2
	1,000	82.6	2.4	5.1
	10,000	49.1	4.4	2.1
WEE	100	6.3	87.0	9.2
	1,000	6.3	77.2	2.0
	10,000	5.3	39.7	0.1
EEE	100	2.1	0.4	92.5
	1,000	1.4	0.1	52.8
	10,000	0.9	0.3	11.8
Nonimmune	100	3.6	5.9	5.7
	1,000	1.4	4.9	3.7
	10,000	1.7	4.8	3.9

[a] ^{3}H-labeled virus preparations were adjusted to contain 8.1 $\log_{10}$ PFU/ml. The RIA diluent in this experiment contained 0.2 M potassium phosphate buffer.

A more rigorous test of specificity is shown in Table IV, which lists RIA endpoints for single-injection cotton rat sera raised to various subtypes of VEE virus. The RIA titer for each serum against its homologous virus is underlined and in all cases is clearly higher than the titers against VEE viruses from heterologous subtypes. This suggests that the RIA might be useful for differentiating antibodies to epidemiologically important VE virus subtypes in sera collected in the field. Using conventional serology, it is often difficult to distinguish epizootic VEE, strains IAB, from enzootic strains, ID and IE. Yet from the Table it is apparent that the test clearly differentiated epizootic from enzootic antibodies.

TABLE IV. TITRATION OF COTTON RAT ANTISERA AGAINST VEE SUBTYPES

ANTISERUM TYPE	50% RIA TITER					
	IAB	ID	IE	II	III	IV
IAB	7717	1420	1348	1237	446	142
ID	1385	5670	3102	540	230	80
IE	925	1310	3427	470	609	275
II	202	185	701	2404	277	146
III	462	330	1631	270	8861	327
IV	80	80	80	80	80	1518

A potential problem was identified, when low dilutions of immune sera were tested, less ^{3}H-labeled virus was bound than was bound by intermediate serum concentrations (Table V). This prozone is the result of a saturation of receptors for IgG, when low serum dilutions are tested.

TABLE V. INHIBITION OF BINDING ^{3}H-VEE/ANTIBODY COMPLEXES IN LOW DILUTIONS OF IMMUNE SERUM

RECIPROCAL SERUM DILUTION	% OF ^{3}H-VEE BOUND
2	5
4	6
8	6
16	9
32	22
64	81
128	84
256	83
512	70
1024	51
2048	31
4096	20

As shown in Table VI, the addition of purified IgG to a dilution of serum which bound 78% of the labeled virus, resulted in a significant inhibition of binding, when IgG concentrations exceeded 0.25 mg/ml. Likewise, normal human serum inhibited binding when its concentration exceeded 3%. This limitation may be offset in part by the addition of more protein A, but presently it is not feasible to test dilutions of serum lower than 1:40.

TABLE VI. INHIBITION OF BINDING ^{3}H-VEE-ANTIBODY[a] COMPLEXES TO S. AUREUS IMMUNOADSORBENT BY IgG OR NONIMMUNE SERUM

INHIBITOR	IgG CONCN (mg/ml)[b]	CORRESPONDING SERUM DILUTION	% ^{3}H-VEE ANTIBODY BOUND	
			SaCl 1X	SaCl 2X
Nonimmune serum	1.71	1:8	0.9	1.5
	0.85	1:16	1.5	6.9
	0.43	1:32	34.4	76.3
	0.21	1:64	62.7	84.6
	0.10	1:128	79.5	79.5
	0.05	1:256	75.4	78.9
IgG	2.00		0.8	1.1
	1.00		2.0	7.6
	0.50		2.1	15.8
	0.25		51.1	81.0
	0.12		76.2	84.4
	0.06		78.4	NT[c]
	0.03		77.5	NT
None			78.6	80.1

[a] ^{3}H-VEE-antibody complexes were formed by incubating (37 C, 1 hr) a 1:5,000 dilution of serum no. 2 with 8.1 $\log_{10}$ PFU of ^{3}H-VEE.
[b] Determined by radial immunodiffusion.
[c] NT, Not tested.

This limitation presents no real problem, however, since the RIA is more sensitive than conventional serologic tests. When paired sera from 73 individuals were screened for seroconversions to VEE, a close correlation was obtained between results of the RIA and PRN tests, Table VII. All preinoculation sera were negative using both tests; 63 postinoculation sera were positive by both tests. Eight individuals failed to seroconvert by either test. Two sera were negative by PRN, and equivocal by RIA (equivocal sera bound 20-50% of labeled virus). No sera were scored positive by PRN and negative by RIA. The converse was also true. In other words, no false positives or false negatives were observed. It was concluded that there was a very close association between seroconversions detected by the conventional PRN and RIA tests.

Sera from USAMRIID personnel were routinely monitored for VEE, WEE, and EEE antibodies by RIA and PRN tests. In a comparison of 50-60 sera titrated for each virus antibody, close correlation was observed (r 0.85) between titers obtained by both methods. The RIA titers were 3-6-fold higher than PRN titers, but RIA titers could be adjusted by changing the concentration of radiolabeled virus employed.

TABLE VII. COMPARISONS OF PRN AND RIA TESTS FOR DETECTING SEROCONVERSIONS[a] AMONG INDIVIDUALS INOCULATED WITH VEE VACCINE STRAIN TC-83

RESULTS OF TESTS		NO. OF POSTINOCULATION SERA IN CATEGORY
PRN[b]	RIA[c]	
+	+	63
−	−	8
−	±	2
+	−	0
−	+	0

[a] All 73 preinoculation sera were scored negative by both PRN and RIA tests.
[b] All sera were tested at 1:80 dilution only.
[c] + = ≥ 80% plaque reduction in serum dilution 1:10.
+ = ≥ 50% ^{3}H-VEE bound; − = <20% bound; ± = ≥20% bound ≤50%.

The utility of the RIA procedure for measuring antibodies in other mammalian sera was also examined. (Drs. R. W. Dickerman and W. F. Scherer generously provided many of the VEE-immune sera.) All mammalian sera tested, and known to be positive for VEE antibodies, were positive using the SaCI, RIA procedure, except for serum from Didelphis, the American opossum. Mammalian species for which the test was useful included: monkey, guinea pig, hamster, white and cotton rats, mouse, dog, sheep, burro, horse, cow, pig, goat and several species of bats. The test unfortunately was not effective in detecting antibodies in any of 8 avian species tested, including chicken, quail, ibis, heron, starling and magpie. These results conform to our expectations based on reports in the literature which state that protein A reacts, albeit to various degrees, with all IgG molecules from all mammalian species except Didelphis, and does not react with avian IgG.

Preliminary data suggest that competitive binding assays, based on the SaCI methodology, can be developed to measure antigen concentrations in inactivated vaccine preparations. To obtain valid results, however, monspecific sera raised against individual viral proteins must be employed. Further development of competitive binding assays will depend on the acquisition of these antisera, and on production of radiolabeled viruses with higher specific activities.

Publications:

1. Jahrling, P. B. 1978. Development of a radioimmune assay for antibodies to alphaviruses using staphylococcal protein A. Arthropod-Borne Virus Information Exchange. 34: 180-182.

2. Jahrling, P. B., R. A. Hesse, and J. F. Metzger. 1978. Radioimmunoassay for quantitation of antibodies to alphaviruses with staphylococcal protein A. J. Clin. Microbiol. 8:54-60.

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1. Myhre, E. B., and G. Kronvall. 1977. Heterogeneity or nonimmune immunoglobulin Fc reactivity among gram-positive cocci: description of three major types of receptors for human immunoglobulin G. Infect. Immun. 17:475-482.

2. Kronvall, G., and R. C. Williams, Jr. 1969. Differences in anti-protein A activity among IgG subgroups. J. Immunol. 103:828-833.

3. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. 115:1617-1624.

4. Cullen, S. E., and B. D. Schwartz. 1976. An improved method for isolation of H-2 and Ia alloantigens with immunoprecipitation induced by protein A-bearing staphylococci. J. Immunol. 117:136-142.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OH6423	78 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
78 08 08	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

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a. PRIMARY	61101A	3A161101A91C	00	143
b. CONTRIBUTING				
c. ////////	STOG 78-7.2.	1. 3. 6		

11. TITLE (Precede with Security Classification Code)*

(U) Defects in cellular immunity after VEE vaccination and repair with transfer factor

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	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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1.0	35.0
FISCAL YEAR CURRENT 79	1.0	34.1

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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) Vaccines; (U) Encephalitis equine (VEE); Transfer factor; (U) Enzyme immunoassay (ELISA)

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

23 (U) Compare immunization with attenuated VEE (TC-83) with inactivated TC-83 (C-84) vaccines in regard to cellular immune responses of guinea pigs challenged with strain 68U201 VEE virus. Assess effect of dialyzable leukocyte extracts containing transfer factor on these immune responses. The study is needed before any large-scale trials are carried out in man on the C-84 vaccine. The goal is a more effective vaccine with greater safety for protection of military personnel against VEE used as a BW agent.
24 (U) Inoculate guinea pigs with graded doses of C-84 vaccine along with appropriate controls. Determine cellular and humoral immunity before and after challenge. Several weeks later inoculate with C-84 and repeat testing. Inoculate vaccinated guinea pigs with subfractions of human transfer factor.
25 (U) 77 10 - 78 09 - A guinea pig model was developed to investigate a possible C-84 VEE vaccine induced predisposition to loss of VEE immunity. Additional studies were initiated to demonstrate the restorative or enhancing capabilities of human transfer factor given together with the C-84 vaccine. Results show no predisposition by C-84 vaccination to loss of VEE immunity. However, human transfer factor boosted viral antibody titers as measured by plaque neutralization tests or by enzyme-linked immunosorbent assay (ELISA). ELISA was shown to be a specific and sensitive assay for Legionnaires' disease and tularemia as well as VEE and WEE organisms. The assay also shows promise for early detection of cell-associated antigens.

Publications: Fed. Proc. 37:1558, 1978.
Clin. Immunol. Immunopathol., in press, 1979.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A061101A91C: In-house Laboratory Independent Research (U)

Work Unit No. A91C 00 143: Defects in Cellular Immunity After VEE and Repair with Human Transfer Factor

Background:

Initial clinical trials of a candidate inactivated VEE vaccine (C-84) in human volunteers showed that certain individuals, having prior exposure to both inactivated and attenuated precursors of C-84, responded with low to moderate neutralizing antibody titers; however, their peripheral lymphocytes remained unresponsive to in vitro stimulation with specific antigen. Previous studies in mice had provided direct evidence for the possibility of a vaccine-induced, specific clonal destruction or depletion model by showing that reduction of T-cells but not B-cells occurred during VEE infection (1). Also, extensive lymphoreticular tissue destruction had been observed in guinea pigs after VEE infection (2). A guinea pig model was developed to investigate further the clonal depletion hypothesis with additional studies to investigate the possibility of restoration or augmentation of lymphocyte responsiveness by human transfer factor (TF).

This TF is a nontoxic material which has been successfully used in humans to restore or transfer cell-mediated immunity (CMI) to a variety of microbial antigens and as an adjuvant for humoral immunity in rats (3) and rabbits (4).

Progress:

Measurement of virus-specific cellular immunity in guinea pigs by a lymphocyte transformation (LT) assay showed that the attenuated VEE vaccine (TC-83) produced more rapid and prolonged responses than either a low dose (0.1 ml) or a high dose (0.5 ml) of inactivated C-84 vaccine. In this study each of the 3 treatments produced a high level of cellular immunity by week 4. The 3 groups plus an untreated control group were then infected SC with 10^7 live VEE strain 68U201 organisms. Twelve weeks after challenge, increased VEE-specific LT responses were shown in high-dose, C-84 treated and control animals; however, responses were low or absent in TC-83 treated and low-dose C-84 treated animals. A boost of all groups with C-84 at week 16 resulted in increased LT responses in all except the low dose C-84 group. Since this group contained only 2 animals at this point, a repeat experiment was initiated with 2 larger groups of animals, one vaccinated with low-dose C-84, the other with high-dose C-84. Both groups were challenged as before, 4 weeks after vaccination. Unlike the earlier experiment, LT results showed a low to absent response in the low-dose C-84 animals that continued through at least 4 weeks after vaccination. Also, in contrast

to earlier experiments, a C-84 boost of these animals produced an increased LT response. These results do not support a clonal destruction hypothesis. Of the several variables, such as vaccine dose, timing of challenge, and challenge strain, remaining to be tested to more completely rule out such a hypothesis, the most important seemed to be vaccine dose. Consequently, 4 groups of guinea pigs were vaccinated with serial 3-fold dilutions of C-84; they were challenged at 4 weeks with 68U201; no adverse reactions were observed following challenge. Within the limits of present data it appears that vaccination with C-84 does not predispose guinea pigs to a more severe infection with VEE.

In recognition of the desirability of boosting guinea pig cellular and humoral responses to VEE, experiments were initiated to quantitate the adjuvant activities of human TF in these animals. A limited series of experiments have been previously reported (3). The following 4 treatment groups were used: (a) Freund's complete adjuvant plus C-84, (b) TC-83, (c) C-84 alone, and (d) TF plus C-84. The results of weekly LT studies showed that within the ceiling defined by groups (a) and (b) and the floor defined by group (c) there was no significant increase in cellular immunity produced by a reasonable dose of VEE-specific human TF. However, a several-fold boost in humoral antibody response, as measured by the plaque neutralization test, was demonstrated comparing groups (c) and (d). Table I shows mean O.D. data for the 4 animal plasma tested at each serum dilution. The PRN_{80} titers for each animal were $\leq$1:10.

TABLE I. ENZYME-LINKED IMMUNOSORBENT ASSAY OF 4 GUINEA PIGS GIVEN VEE COMPARED TO THOSE GIVEN VEE PLUS HUMAN TF.

DILUTION	MEAN OD_{400} $\pm$ SE			
	2 weeks		4 weeks	
	VEE	VEE + TF	VEE	VEE + TF
1:80	1.06 $\pm$ 0.06	1.06 $\pm$ 0.07	0.98 $\pm$ 0.04	1.41 $\pm$ 0.08
1:160	0.87 $\pm$ 0.02	0.89 $\pm$ 0.03	0.87 $\pm$ 0.02	1.25 $\pm$ 0.09
1:320	0.91 $\pm$ 0.03	0.86 $\pm$ 0.02	0.72 $\pm$ 0.03	1.10 $\pm$ 0.09

The desirability of a more rapid and less cumbersome alternative to the plaque neutralization test for VEE-specific antibody led to development of a microadaptation of the enzyme-linked immunosorbent assay (ELISA). The specificity, sensitivity and adaptability of this assay for antibody was demonstrated by the following studies. Reagents for specificity testing were generated by vaccination of 2

groups of guinea pigs with WEE or TC-83 respectively. At 36 days the WEE group was boosted with WEE-strain 72V4768 and the TC-83 group with Trinidad. One week later, all animals were bled and plasma was stored frozen in aliquots. The respective antigens were provided as virus-rich bands from sucrose density gradients by Dr. Jahrling. Viruses were UV-inactivated and total protein was determined. Viral antigen solutions containing equivalent amounts of protein were used to coat ELISA plates and serial 2-fold dilutions of homologous and heterologous plasma samples were assayed. For both viruses, the homologous combinations of antigen and plasma produced a positive titer 7-8 tube dilutions higher than heterologous combinations, demonstrating a satisfactory level of specificity for the assay. Experiments involving EEE virus were confounded by an apparent inability to bind virus to ELISA plates. The rapid adaptability of ELISA for bacterial and viral antibody determinations is impressive. In a period of several weeks, sensitive and specific antibody determinations were developed for Legionnaires' disease and tularemia. Although preliminary comparative experiments show that ELISA results are on the same order of sensitivity as radioimmunoassay (RIA) results, a more complete series of comparisons should be done. A specific comparison of ELISA and RIA with hemagglutinin (HA) and/or complement fixation (CF) assays is necessary. Table II shows results obtained on a series of human sera tested by plaque neutralization, RIA, and ELISA assays. Table III presents results from a similar experiment involving a guinea pig plasma that had a titer of 1:5120 by RIA.

TABLE II. WEE AND VEE ANTIBODY LEVELS IN HUMAN SERA

SERUM	RECIPROCAL TITER					
	Plaque Neut.		RIA		ELISA	
	WEE	VEE	WEE	VEE	WEE	VEE
1	640	640	564	926	1280	320
2	80	640	212	544	320	320
3	320	40	< 40	76	5120	1280
4	320	2560	752	2986	2560	2560
5	1280	< 40	839	40	1280	320

TABLE III. GUINEA PIG ANTI-VEE ANTIBODY MEASURED BY ELISA

DILUTION	OD_{400}			
	Negative plasma		Positive plasma[a]	
	Duplicate	Mean	Duplicate	Mean
1:80	0.21 0.23	0.22	0.88 0.90	0.89
1:160	0.18 0.18	0.18	0.85 0.78	0.82
1:320	0.16 0.14	0.15	0.70 0.74	0.72
1:640	0.14 0.14	0.14	0.62 0.64	0.63
1:1280	0.16 0.16	0.16	0.51 0.52	0.51
1:2560	0.16 0.18	0.17	0.44 0.42	0.43

[a] RIA titer of this plasma was 1:5120.

Presentation:

Andron, L. A. Enzyme-linked immunosorbent assay of antibody levels in guinea pigs given antigen plus transfer factor. Presented, Annual Meeting, American Association of Immunologists, Atlanta, GA, 4-8 Jun 1978. (Fed. Proc. 37:1558, 1978.)

Publication:

Ascher, M. S., and L. A. Andron. 1979. Transfer factor *in vitro*: nonspecificity of components that enhance lymphocyte proliferation to antigen. Clin. Immunol. Immunopathol. 12: in press.

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3. Anderson, A. O., M. S. Ascher, and L. A. Andron. 1977. Adjuvant properties in human dialysable leukocyte extracts (DLE) containing transfer factor. pp. 692-694. In Regulatory Mechanisms in Lymphocyte Activation, (D. O. Lucas, ed.). Academic Press, New York.

4. Roda, A., E. Roda, G. Pizza, and C. Boucheix. 1978. Human transfer factor produced *in vitro* increases antibody titers in rabbits. N. Engl. J. Med. 298:629.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6424	78 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
78 08 08	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	144
b. CONTRIBUTING				
c. ////////	STOG 78-7,2, 1, 3, 6			

11. TITLE (Precede 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	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
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e. KIND OF AWARD:
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18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	0.5	49.0
FISCAL YEAR CURRENT 79	0.5	31.6

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH 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 (Furnish individual 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 criterias 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) 77 10 - 78 09 - Experimental conditions for collection and in vitro culture of rat peritoneal macrophages were evaluated. Preparations acceptable for studies on the interaction of macrophages with F. tularensis were derived from sodium caseinate-induced rat peritoneal exudate cells cultured in vitro in media 199 plus 10% rat serum. Feeding of virulent (SCHU S4) or avirulent (LVS) strains of F. tularensis to cultivated macrophages resulted in unremarkable ingestion. In the presence of specific immune sera more bacteria were phagocytized. SCHU S4 but not LVS grew within normal macrophages. Both strains were killed by macrophages harvested from immunized rats. F. tularensis was shown to contain a mannose-rich capsule which is extruded with NaCl. Decapsulated bacteria were poorly ingested by macrophages even in the presence of antibody. Decapsulated SCHU S4 proliferated within macrophages. These observations demonstrate that antibody derived from LVS vaccination is directed toward capsular material and that resistance of F. tularensis to destruction by phagocytes does not reside with the bacterial capsule.

Publications: J. Cell Biol. 75:197a, 1977.
J. Reticuloendothel. Soc. 24:115-138, 1978.
In: Liposomes in Biology and Pathology, in press, 1978.

* Available to contractors upon originator's approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, NOV 68 AND 1498B, MAR 69, FOR ARMY USE ARE OBSOLETE. * U.S. GPO 1974—

BODY OF REPORT

Project No. 3A061101A91C: In-house Laboratory Independent Research (U)

Work Unit No. A91C 00 144: Mechanisms and Determinants of Microbial Pathogenicity

Background:

Although "professional" phagocytes are suitably equipped to ingest, kill and digest microbial intruders, certain microorganisms have devised a variety of mechanisms for circumventing the antimicrobial action of reticuloendothelial (RE) cells. Such virulent microorganisms invade host tissues, replicate, and cause disease. Successful colonization requires the death or neutralization of one or more functions of phagocytes. Bacteria, such as group A streptococci, as they multiply in tissues, release soluble materials that are toxic for phagocytes (1). Other bacteria exert their cytotoxic actions on the phagocyte after phagocytosis has occurred. Virulent staphylococci can avoid the attention of phagocytic cells by suppressing the inflammatory response, resulting in an inability of the host to focus neutrophils into sites of infections (2). Virulence of rickettsiae, anthrax bacilli, gonococci and Mycoplasma is expressed as an ability to avoid phagocytosis. These and other important pathogenic microorganisms appear to bear on their surface substances that inhibit phagocytosis. Resistance to phagocytosis is sometimes due to a component of the bacterial cell wall, and sometimes to a capsule enclosing the wall secreted by the bacterium. Mycoplasma hominis and Francisella tularensis (3), when added to neutrophils in vitro remain extracellular. Intracellular bacteria which enter phagocytic cells can only establish a successful infection if they resist killing. If the ingested microorganism can resist intracellular digestion processes, it has the opportunity to survive and multiply. Some microorganisms, such as Salmonella typhi have accomplished this by inhibiting the chain of metabolic events which lead to maximum bactericidal activity; although the bacteria are ingested, they are not all killed. Other microorganisms prevent fusion of lysosomes with phagocytic vacuoles, while many other successful infectious microorganisms resist killing and digestion within phagolysosomes (4).

The life span of the neutrophil is so brief that it is rarely an important site for microbial growth. In contrast, the longer lived macrophage is a more important site. At an infectious locus, incoming macrophages ingest extracellular microbes as well as dying neutrophils which contain viable microorganisms. A great variety of pathogenic organisms have the ability to survive and grow in macrophages; they have, in fact, learned to "coax" this phagocyte to protect, nourish, and disseminate them to distant sites within the host (5). Such a facultative intracellular parasite is F. tularensis. Its virulent strains (SCHU S4) infects and grows within macrophages. In contrast, the vaccine strain of F. tularensis (LVS) is

readily destroyed by RE cells. The availability and pathogenic characteristics of these strains offer a unique opportunity to understand the mechanisms which permit virulent microorganisms to survive and grow intracellularly.

This work unit, however, is not limited to the study of F. tularensis but intends to contribute fundamental information as to the cellular biology of available pathogenic microorganisms of military medical importance. This information is required to minimize the impact of infectious diseases on military and support personnel by identifying mechanisms and determinants of microbial pathogenesis and assure development of optimal prophylaxis and therapy.

Characterization of the biochemistry, structure and function of phagocytes and their organelles in normal and diseased animals is an essential element in clarifying mechanisms of microorganism induced pathophysiology. Such studies may disclose how microorganisms, by altering normal cellular physiology, can mitigate host defense mechanisms, promote cellular dysfunction and enhance host susceptibility to infectious diseases.

Effective future control of infectious organisms will most probably require a knowledge of the mechanisms of infection and factors that make microorganisms pathogenic. Little is known about this significant aspect of microbiology; our understanding and continued defense against evolving microbes requires that modern biochemical and immunological techniques be brought to bear on these problems. Studies on microbial killing and digestion in phagocytes are in their infancy, but it is important to conceive and characterize the ways in which microorganisms can avoid being killed and digested, not only for their basic biological interest, but because of their application to development of vaccines, understanding of persistent and latent infections, design of new therapeutic agents and our ability to deal with any strange new pestilences that periodically arise and threaten us.

Progress:

Standardization of experimental methods

Microorganisms. Stock cultures of F. tularensis strains SCHU S4 (S4), LVS, 503 and 425 were prepared, aliquoted into 5-ml vaccine bottles, quantitated and stored at 60°C. The concentration of organisms obtained varied from a low of 1.55×10^{10} viable bacteria/ml for S4 to 3.04×10^{10} viable counts/ml for strain 425. A 30-40% loss in viable counts of all strains was found following a single freeze (-20°C) thaw cycle. The relative virulence of each strain for rats was determined by IP injection of rats (Table I). S4 proved to be uniformly lethal to rats with as few as 10^3 organisms. Both 425 and 503 strains killed rats but higher doses were required. LVS was uniformly avirulent at the concentrations employed.

TABLE I. RELATIVE IP VIRULENCE OF 4 STRAINS OF F. TULARENSIS FOR RATS

STRAIN/DOSE	NO. DEATHS BY DAYS										NO. DEATHS/6
	1	2	3	4	5	6	7	8	9	10	
LVS/10^7	0	0	0	0	0	0	0	0	0	0	0
10^6	0	0	0	0	0	0	0	0	0	0	0
10^5	0	0	0	0	0	0	0	0	0	0	0
425/10^7	0	1	3	1	0	0	0	0	0	0	5
10^6	0	1	3	0	0	0	0	0	0	0	4
10^5	0	0	0	1	1	1	0	0	0	0	3
503/10^7	0	2	2	1	1	-	-	-	-	-	6
10^6	0	0	3	1	1	0	0	0	0	0	5
10^5	0	0	0	0	1	1	0	0	0	0	2
S4/10^7	0	2	2	1	1	-	-	-	-	-	6
10^6	0	2	0	1	3	-	-	-	-	-	6
10^5	0	0	3	0	1	2	-	-	-	-	6
10^4	0	0	0	1	0	2	1	1	-	-	6
10^3	0	0	0	0	1	1	2	2	-	-	6

Macrophage isolation and culture. Experimental conditions for collection and for the in vitro culture of rat peritoneal macrophages were evaluated. In contrast to mice, rats were found to have few resident peritoneal macrophages; it was necessary to induce the accumulation of macrophages in the peritoneum by prior injection of an irritant. Administration of 7 ml of thioglycolate into the peritoneal cavity 5 days prior to explantation yielded 50 x 10^6 cells/350-gm rat, of which 42.8% were mononuclear phagocytes (MNP) (Table II).

TABLE II. COLLECTION AND PLATING DATA FROM THIOGLYCOLATE STIMULATED (7 ml, day -4) PERITONEAL EXUDATES FROM 352-gm RATS

	VALUES				
	MNP	LYMPH.	PMN	EO	OTHERS
Cellular composition of peritoneal exudates					
($\times 10^6$)	21.4	15.0	0.9	10.0	1.6
(%)	43.8	30.7	1.8	20.5	3.3
No. cells seeded on t75 flasks					
($\times 10^6$)	16.5	12.2	0.7	8.1	1.3
No. nonadhering cells recovered after 2 hr					
($\times 10^6$)	2.1	6.6	0.3	3.4	0.8
No. cells remaining attached to flask					
($\times 10^6$)	14.5	5.5	0.4	4.7	0.5
(%)	56.6	21.5	1.6	18.4	2.0

In contrast to thioglycolate, sodium caseinate-induced exudates resulted in a higher percent of MNP (56%). Culturing of exudates in vitro on plastic tissue culture flasks for 2 hr and washing the resulting monolayer effectively removed most nonadhering cells. Mononuclear phagocytes comprise in excess of 80% of the monolayer while PMN contribute to > 10% of the population. Since the latter cells are short-lived and ingested by mononuclear phagocytes, 93% of the cells composing the monolayer after overnight culture are MNP (Table III). These preparations were found acceptable for in vitro studies on the interaction of mononuclear phagocytes with microorganisms, drugs, antigens and/or adjuvants.

TABLE III. COLLECTION AND PLATING DATA FROM SODIUM CASEINATE-STIMULATED (15 ml, day -4) PERITONEAL EXUDATES IN 350-gm RATS

	VALUES				
	MNP	LYMPH.	PMN	TO	OTHERS
Cellular composition of peritoneal exudates					
($\times 10^6$)	33.6	9.2	9.1	6.6	1.3
(%)	56.3	15.5	15.3	11.0	2.2
No. cells seeded on t75 flasks					
($\times 10^6$)	25.7	6.8	6.7	5.2	1.0
No. nonadhering cells recovered after 2 hr					
($\times 10^6$)	4.9	6.2	4.1	4.6	.5
No. cells remaining attached to flask					
($\times 10^6$)	20.8	0.6	2.6	0.6	0.5
(%)	82.9	2.4	10.4	2.4	2.0

A subjective evaluation was undertaken to determine the most satisfactory media for maintenance of macrophages in in vitro culture for up to 48 hr. Tissue culture media 199, NCTC 135 and RPMI 1640 were used in conjunction with various concentrations of rat, rabbit, fetal calf, bovine, human, horse and monkey sera. Of all the possible combinations only media 199 with rat serum concentrations of 10-30% supported peritoneal macrophages in cultures from 1-10 days with little loss of cells from the monolayer. Cell viability after 48 hr in culture was > 98%. The monolayer was found to secrete no less than 500 µg lysozyme/10^6 macrophages/24 hr. This is an equivalent of 26 x 10^6 lysozyme molecules/macrophage/24 hr or 300 molecules/sec. These values testify to the functional viability of macrophages cultured under present conditions.

Microbicidal capacity of cultured macrophages. Quantitative procedures for evaluating bactericidal capacity of cultivated macrophages were adopted which employ gravimetric methods and a balance sheet concept to decrease experimental errors. Table IV shows results of infecting macrophage monolayers with either LVS or S4 strains of F. tularensis at a

ratio of 1 macrophage/1000 bacteria. The quantity of either strain ingested by macrophages during a 2-hr period was unremarkable, but as anticipated in the presence of specific immune sera substantially more bacteria were phagocytized. The virulent, S4, but not the avirulent LVS, strain, grew within the macrophages during an 18-hr culture period.

TABLE IV. PHAGOCYTOSIS AND FATE OF F. TULARENSIS INTERACTING WITH RAT PERITONEAL MACROPHAGE IN IN VITRO CULTURE.

MACROPHAGE	SPECIFIC TITER	STRAIN	NO. BACTERIA/MACROPHAGE	
			0 hr	18 hr
Normal	< 0 >	LVS	0.2	<0.1
	1:80	LVS	3.5	0.8
	1:2800	LVS	8.4	0.4
	< 0 >	SCHU S4	0.4	3.5
	1:80	SCHU S4	24.9	126.8
	1:80	SCHU S4	10.9	63.7
	1:2800	SCHU S4	16.3	82.7
Immune	0	SCHU S4	0.1	<0.1
	1:80	SCHU S4	18.4	2.3
Normal	0	SCHU S4 (decapsulated)	0.2	2.3
	1:80	SCHU S4 (decapsulated)	0.6	2.2
	1:2800	SCHU S4 (decapsulated)	0.9	3.3

Macrophages obtained from the peritoneal cavity of rats 2 weeks after LVS immunization were used to access the microbicidal capacity of "immune" macrophages. In contrast to normal cells, immune macrophages were capable of inhibiting the intracellular growth of the virulent strain of F. tularensis, SCHU S4. The absence of immune serum diminished initial ingestion of organisms but did not affect the capacity of the phagocyte to kill virulent F. tularensis.

The interactions between F. tularensis and rat peritoneal macrophages appear to provide an opportune model for elucidating bacterial characteristics which confer resistance against the microbicidal action of macrophages. Furthermore, the experimental model provides an opportunity to characterize cellular biochemical modification which enhance macrophage resistance to F. tularensis infection following immunization.

Modification of macrophage/F. tularensis interactions due to bacterial surface properties. The surface of F. tularensis appears to be characterized by the presence of a capsule, which can be isolated by treatment with 10% NaCl for up to one week at 4°C. We have found that an, as yet, unidentified material can be removed from the 4 available strains of F. tularensis, by incubation in 1-15% NaCl. The bacteria proved to be particularly resistant to this treatment, remaining viable and virulent for rats after exposure to 15% NaCl for up to 10 days.

The 4 available strains of F. tularensis were treated with 10% NaCl for 1-3 days at 4°C to remove capsular material. Decapsulated bacteria fed to rat macrophage in vitro were not avidly injested. The addition of immune sera did not increase the number of injested decapsulated bacteria (Table IV). This observation demonstrates that antibody derived from LVS vaccination is directed toward capsular material. Decapsulated bacteria ingested in the presence or absence of antibody are not killed by the macrophage but proliferate within it. It appears that resistance of F. tularensis to destruction by phagocytes does not reside with the bacterial capsule.

Capsular and cell wall materials isolated by NaCl treatment were dialyzed, lyophilized and analyzed by gas chromatography for fatty acid (FA) composition. No differences were observed among the capsular or cell wall material isolated from the 4 strains. Further specific characterization of chromatographic peaks was not attempted in light of negative results. It does not appear that FA composition of either the cell wall or capsular material can account for virulence of the S4 strain.

The surface properties of F. tularensis were further characterized by binding sites with fluorescein-labeled lectins. It was shown that binding of concanavalin A (Con A) by the 4 strains decreased in a manner which corresponded to the relative virulence of each strain. Con A is a protein which demonstrates specific binding with mannose residues. Little if any Con A bound to bacterial cell wall following decapsulation of strains with NaCl. These data are, therefore, consistent with the findings of Hood (%) who demonstrated a greater concentration of mannose residues on the outer coats of virulent S4 then on the cell wall.

Cell fractionation studies

Enzyme assays were developed and optimized in order to use the techniques of cell fractionation for probing macrophage-microbe interactions. Enzymes investigated were candidate markers for the plasma membrane, mitochondria, endoplasmic reticulum and lysosomes of cells intended for use in future studies. Enzymes were characterized and optimized for activity, pH, buffer, ionic requirements and linearity of reaction. Cells analyzed included

peritoneal macrophages from the mouse, rat and guinea pig and rhesus monkey alveolar and Vero. Following initial isopycnic centrifugation trials of homogenates of rat and guinea pig macrophages and Vero cells the enzymes listed in Table V were chosen as appropriate markers for subcellular organelles.

TABLE V. ASSAY CONDITIONS FOR PROPOSED MARKER ENZYMES OF CELLULAR ORGANELLES

ORGANELLE	ENZYME	BUFFER	pH	PROTEIN REQUIRED (µg)
RAT MACROPHAGES				
Plasma membrane	5'-Nucleotidase	Tris	8.6	20
	Alkaline phosphatase	Glycine	9.0	5
Mitochondria	Cytochrome oxidase	Citrate	5.5	25
	Malate dehydrogenase	Phosphate	7.6	20
Endoplasmic reticulum	Sulfatase C	Imidazole	7.0	10
Lysosome	α-Mannosidase	Acetate	4.6	5
	N-Ac-β-Glucosaminidase	Citrate	4.5	5
	α-Galactosidase	Acetate	5.0	5
GUINEA PIG MACROPHAGES				
Plasma membrane	5'-Nucleotidase	Tris	8.6	10
	Alkaline phosphatase	AMP	8.6	5
Mitochondria	Cytochrome oxidase	Citrate	6.0	25
Endoplasmic reticulum	α-Glucosidase	Clycine	9.0	5
Lysosome	N-Ac-β-Glucosaminidase	Citrate	4.0	5
	α-Galactosidase	Acetate	4.6	5
VERO CELLS				
Plasma membrane	5'-Nucleotidase	Tris	8.6	5
Mitochondria	Malate dehydrogenase	Phosphate	7.6	20
Endoplasmic reticulum	α-Glucosidase	AMP	8.6	5
	Alkaline phosphatase	AMP	8.6	5
Lysosome	N-Ac-β-Glucosaminidase	Citrate	4.0	5
	α-Galactosidase	Citrate	4.0	5

Presentation:

Canonico, P. G., H. Beaufay and M. Nyssens-Jadin. 1977. Analytical fractionation of mouse peritoneal macrophages: physical and biochemical properties of subcellular organelles from resident (unstimulated) and cultivated cells. Presented, Annual Meeting, American Society for Cell Biology, San Diego, CA, 15-18 Nov 77 (J. Cell Biol. 75:197a, 1977).

Publications:

1. Canonico, P. G., H. Beaufay, and M. Nyssens-Jadin. 1978. Analytical fractionation of mouse peritoneal macrophages: physical and biochemical properties of subcellular organelles from resident (unstimulated) and cultivated cells. J. Reticuloendothel. Soc. 24:115-138.

2. Canonico, P. G., A. T. McManus, and M. C. Powanda. Biochemistry and function of polymorphonuclear leukocytes in the infected and traumatized host. In Lysosomes in Biology and Pathology, Vol. 6. (J. T. Dingle and P. Jacques, eds.). North Holland Co., New York (In press).

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APPENDIX A

TSP-20

AUTOMATED BIOCHEMICAL ANALYSIS

Biochemical Quantitative Techniques - Karen A. Bostian, M.S.
Amino Acid Analyses - Richard E. Dinterman, B.S.
Trace Metal Analyses - Edward C. Hauer, B.S.

Robert W. Wannemacher, Jr., Ph.D.
Coordinator
Physical Sciences Division

Biochemical Quantitative Techniques

Objective:

Develop, or find and utilize, reliable methods to quantitate concentrations of various metabolites in fluids including blood, urine, feces and tissue extracts from both man and laboratory animals utilizing AutoAnalyzer systems (Technicon).

Progress and Summary:

Since the last annual report 66,569 samples were analyzed. The percentage distribution of FY 1978 samples by division is:

Physical Sciences	72.0	Bacteriology	0.4
Pathology	23.9	Aerobiology	0.3
Medical	2.7	Virology	0.2
Animal Assessment	0.5		

Processing was as follows: 30.3% were analyzed by completely automated systems requiring only reagent and standard preparations (these were Lowry proteins and most were done for either Dr. Middlebrook or Dr. Leppla, Pathology Division); 19.2% were for specific protein analysis. Most of these were run on samples from a group of 213 human controls and from volunteers in a dengue vaccine study. Also, rat albumin and transferrin were run on a study conducted by Dr. Mapes in which effects of species and diet were studied; 13.5% required special reagent preparation, standardization, dilutions and equipment set-ups, i.e., analyses for lactate, phosphorous, creatinine, urea nitrogen and ammonia; 10.7% were for either cholesterol, triglycerides or free fatty acids and required manual extractions prior to assay; 8.1% were tested for β-hydroxybutyrate; 6.1% required Somogyi filtrates and were analyzed for glucose; 5.4% were tested for Fe concentration using a colorimetric system; 3.9% were assayed for nitrogen using an automated Kjeldahl technique; 2.1% were analyzed for SGOT or ornithine carbamoyl-transferase for LTC Sobocinski; 0.7% were for TIBC analysis.

Technical Advances:

1. The creatinine methodology has been converted to an AutoAnalyzer II system which is more efficient than the type I system previously used.

2. The β-hydroxybutyrate system is used to analyze urine using a blanking procedure.

3. An automated ornithine carbamoyl-transferase system was put to use.

Amino Acid Analyses:

Objective:

Develop and utilize rapid, reliable methods for quantitation of amino acids in various biological fluids using the Beckman 121MB Amino Acid Analyzer.

Progress and Summary:

Types of Analyses	Sample Type	Number	Requestor (Division)
Physiologic (33 amino acids)	Human serum	374	Mr Higbee & Dr. Wannemacher (PS)
	Monkey blood	285	Dr. Wannemacher & R. E. Dinterman (PS)
	Rat serum	179	LTC Sobocinski (PS)
	LEM extract	6	LTC Sobocinski (PS)
	Platelet extract	9	Dr. Mapes (PS)
	Brain extract	49	LTC Sobocinski (PS)
Hydrolysate (18 amino acids)	Sequencer extract	235	CPT Cades (Path)
	Protein hydrolysate	32	Dr. Leppla (Path)
		26	Ms. Johnson (Path)
		24	Mr. Thompson (PS)
		20	Dr. Spero (Path)
		6	Dr. Wannemacher & R. E. Dinterman (PS)
Special Test Taurine	Rat serum	588	LTC Sobocinski (PS)
	Platelet extract	14	Dr. Mapes (PS)
	Brain extract	34	LTC Sobocinski (PS)
	Rat urine	24	LTC Sobocinski (PS)
	Liver perfusate	36	LTC Sobocinski (PS)
3-Methylhistidine	Human urine	155	Mr. Higbee & Dr. Wannemacher (PS)
	Rat urine (hydrolyzed, rotoevaporated and reconstituted)	89	Dr. Wannemacher & R. E. Dinterman
Hydroxyproline and 3-Methylhistidine	Monkey urine (hydrolyzed, rotoevaporated and reconstituted)	72	Dr. Wannemacher & R. E. Dinterman (PS)

The installation of 2 Beckman analyzers resulted in an increased output of all tests from this support program. FY 1978 physiologic analyses were up 17%, hydrolysates 100%, and special tests 1,000%, compared to FY 1977 production.

Trace Metal Analyses:

Objective:

To provide rapid and reliable methods for analysis of trace metals in various biological samples.

Progress and Summary:

A total of 25,076 analyses of serum, plasma, urine, feces, tissue, buffer, medium and associated extracts were done for Zn, Cu, Fe, Ca, Mg, Cd, Li, Se and ceruloplasmin. All but serum and plasma require extensive manual dilution/dissolution.

Several Wang calculator programs were written this reporting period for investigators in Physical Sciences, Pathology, and Bacteriology Divisions. Available programs include statistical analysis, linear regression (Scatchard analysis), and several methods for plotting data, none of which were available from Wang Laboratories.

Technical Advances:

A method for analyzing trace metals in as little as 40 μl of sample has been adapted from the literature. This permits the analysis of only 2 ng Zn or Cu.

Publication:

Hauer, E. C., and M. V. Kaminski, Jr. 1978. Trace metal profile of parenteral nutrition solutions. Am. J. Clin. Nutr. 31:264-268.

TSP-21

AUTOMATIC DATA PROCESSING SUPPORT FOR USAMRIID

Glen A. Higbee, M.S.
Administrative Division
Computer Science Office

Objective:

Automatic data processing (ADP) technical support consists of maintaining the capability of utilizing computers and calculators to process repetitive data generated by work units within USAMRIID.

Progress and Summary:

The remote job entry (RJE) batch terminal at USAMRIID continues to serve a major role in providing ADP support. In addition to allowing access to the CDC 3500 computer at WRAIR and the Univac 1108 computer at National Bureau of Standards (NBS), RJE capabilities will be available in the fall of 1978 on the IBM 360/50 at Fort Detrick. Support from NBS has decreased significantly and may be terminated to all non-NBS agencies by October 1978. Much of the work load currently processed at NBS will be transferred to the Fort Detrick computer.

Current technical support tasks include the following:

1. Immunization. This entire system has been expanded, redesigned and rewritten under Work Unit A841 00 031. Most of this system is now operational and will soon be transferred to TSP-21 for routine operation and maintenance.

2. Library. The generation of periodic division listings, annual library master listings, and monthly update transaction listings to validate USAMRIID library activity during each month has continued to be an effective aid in saving personnel time and in reducing errors.

3. Paper tape. Equipment is on order that will allow the paper tape to be read with a paper tape reader connected to a CRT terminal. With a paper tape reader as part of a CRT terminal system, the reader can be removed from the current RJE batch terminal, resulting in a lease cost savings of about $2,000 annually.

4. Registry of infectious organisms. A master file of all infectious organisms currently used at USAMRIID is maintained to reflect current usage. The computer master file is updated, sorted and printing monthly by organism, division, location of use and registry number. Each month, 17 of these reports are forwarded to the Safety Officer for appropriate distribution.

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5. Label printing. Approximately 49,400 identification labels were produced during the past year from 14 requests. Usually a request for label generation can be satisfied in one day.

6. Calculators. Programming support has been provided to requesting divisions for solutions to problems within the capabilities of programmable calculators.

7. Electrolytes. This program computes intra- and extracellular water and electrolyte concentrations for numerous tissues and muscle types. Data from 8 monkeys have been processed during the past year.

8. Statistical programs. Some of the statistical data analysis routines that are frequently used include: probit analysis, independent comparisons between multiple proportions, simulation analysis and modeling, analysis of variance (1-way, k-way, and for repeated measurements), analysis of covariance, Kendall's correlation, Kolmogorov-Smirnov 1-sample and 2-sample tests, Wilson nonparametric analysis of variance, Kruskal-Wallis multisample tests, Wilcoxon rank-sum test (independent and paired), Fisher's exact test, and Forcythe polynomial regression.

TSP-22

RESEARCH SEROLOGY AND VIROLOGIC SUPPORT SERVICES

Joseph A. Mangiafico, M.P.H., Bacteriology Division (Part A)
Helen H. Ramsburg, A.B., Virology Division (Part B, through 31 Dec 77)

Part A. Research Serology

Objectives:

Conduct basic research for development, standardization and evaluation of new serological procedures, or the modification of established procedures, in support of USAMRIID requirements. Provide serological support service for all studies conducted in volunteers and/or involving efficacy, safety and epidemiological assessment of vaccines in humans. Provide centralized services for serological evaluation of antigen and/or antibody content in biological specimens derived from approved work units.

Progress and Summary:

Research Serology conducted approximately 12,795 serological tests in support of USAMRIID work projects. Types and numbers of tests performed are shown in Table I.

TABLE I. TYPES AND NUMBER OF TESTS PERFORMED FOR USAMRIID DIVISIONS IN PAST YEAR

DIVISION	TYPE AND NUMBER OF TESTS						TOTALS BY DIVISION
	Aggl.	CF	HA	HI	Oudin	NT	
Aerobiology	562	38	12	167			779
Animal Assessment			12	441	117	12	582
Animal Resources			839	1410			2249
Bacteriology	400	119				309	828
Medical	503		113	1390		2331	4337
Pathology					985	741	1726
Physical Sciences	76						76
Virology			108	1335		18	1461
Other	5	7	129	591	25		757
TOTALS	1,546	164	1,213	5,334	1,127	3,411	12,795

TABLE II. REMOVAL OF NONSPECIFIC INHIBITORS BY PHOSPHOLIPASE C

TREATMENT	PHOSPHOLIPASE C (μg/0.1 ml)	C	RECIPROCAL VEE HI TITER BY HR					
			0.5	1	2	3	4	24
Serum #1								
Undiluted	2,500		80	80	160	160	160	<10[a]
10^{-1}	250		80	80	80	80	40	<10[a]
10^{-2}	25		160	80	80	80	40	<10
10^{-3}	2.5		320	320	160	80	40	<10
10^{-4}	0.25		640	1280	1280	1280	640	160
10^{-5}	0.025		640	1280	1280	1280	1280	320
Untreated	--		640	640	640	640	1280	640
Kaolin, 25%	--		<10	<10	<10	<10	<10	<10
PRN_{80}		<10						
Serum #2								
Undiluted	2,500		160	160	160	80	80	10[a]
10^{-1}	250		80	80	80	40	40	10[a]
10^{-2}	25		80	80	40	40	40	± - 160[b]
10^{-3}	2.5		80	160	40	40	40	± - 160[b]
10^{-4}	0.25		320	160	160	320	160	± - 160[b]
10^{-5}	0.025		640	640	320	640	320	± - 320[b]
Untreated			320	160	160	320	160	160
Kaolin, 25%			10	10	10	10	10	10
PRN_{80}		80						

[a] Nonspecific agglutination
[b] Rough pattern

Major support services included characterization of 41 viral strains of EEE supplied by Dr. Walder. The viral strains were tested by the kinetic micro-HI technique to determine antigenic relationships.

Research initiated this year consisted of a preliminary study to determine the effectiveness of phospholipase C in removing or destroying nonspecific lipid inhibitors of HI from human sera and the approximate concentration of the enzyme needed along with length of incubation. Phospholipase C, derived from Clostridium welchii, was purchased commercially and received as a dry material. The label activity was stated to be 8.4 U/mg dry weight. Concentrations used were based upon the label claims of the manufacturer. No additional estimates of activity or purity were made. For this study 2 human sera were selected, #1 was negative and #2 was positive (1:80) to VEE virus by PRN_{80}. Both sera were preabsorbed with goose RBC for removal of nonspecific agglutinins, and pretested by HI to determine that each had nonspecific inhibitors present. Serial 10-fold dilutions of phospholipase C (ranging in concentration from 2500 in the undiluted to 0.025 μg/0.1 ml in the 10^{-5} dilution) were made in 0.05 M Tris buffered saline, pH 7.6 with 0.02 M $CaCl_2$. Each serum was treated with an equal volume of the 6 dilutions of the enzyme for the 6 incubation periods at 37°C. Controls consisted of untreated (unabsorbed) and 25% kaoline-absorbed sera for each of the incubation periods. At the end of each period, treated sera and controls were diluted to 1:10 with cold borate saline, pH 9.0, to stop or substantially reduce enzyme activity. Sera were transferred to microtiter plates for serial 2-fold dilutions in cold borate saline with bovine serum albumin; 4 HA units of VEE antigen were added to each well. Plates were held overnight at 4°C; cells were then added and plates read to determine HI endpoints. Results for both sera are shown in Table II. For the negative serum, best results were obtained with a concentration of 25-2.5 μg/0.1 ml of phospholipase C, when incubated for 24 hr at 37°C. Results with the VEE-positive serum were not as clear as those seen in the negative serum. Again, optimum enzyme concentration was between 25 and 2.5 μg/0.1 ml. Sera which were treated with these concentrations of enzyme demonstrated acceptable titers at 4 hr of incubation. At 24 hr, incubation patterns were rough and hard to read, due to nonspecific agglutination, the cause of which has not been determined.

Research was also initiated this year to enhance the HA titer of the Rift Valley fever (RVF) antigen. This antigen was prepared by Merrell-National Laboratories by sucrose-acetone extraction of suckling mouse livers and inactivated with 0.3% β-propiolactone. When reconstituted, the antigen yielded only 8-16 HA units.

Recently, enhanced HA was reported for viruses of the Bunyaviridae with increased salt concentrations in diluents (1). HA and HI tests were carried out according to the method of Clarke and Casals (2) using goose erythrocytes in phosphate buffers, adjusted to final NaCl molarities of 0.15, 0.25 and 0.4. Antigens were used at final pH values of 5.8, 6.0, 6.2 and 6.4. Increased salt concentrations showed no enhancement of RVF HA titers.

LITERATURE CITED

1. Beaty, B. J., R. E. Shope, and D. H. Clarke. 1977. Salt-dependent hemagglutination with Bunyaviridae antigens. J. Clin. Microbiol. 5:548-550.

2. Clarke, D. H., and J. Casals. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg. 7:561-573.

Part B - Virologic Support Services

Objective:

To conduct research for the development, standardization, and evaluation of new serological procedures or the modification of established serological procedures in support of specific USAMRIID requirements, and to provide virological support services for all studies carried out in volunteers and/or involving efficacy, safety and epidemiological assessment of vaccines in humans.

Progress and Summary:

Standard plaque reduction (PR) serum neutralization (SN) tests (1) performed to measure neutralizing antibody formed in response to the formalin-inactivated TC-83 (attenuated VEE, C-84), EEE and WEE vaccines are summarized in Table I.

TABLE I. SUMMARY OF GROUP A ARBOVIRUS PR-SN TESTS PERFORMED FIRST QUARTER FY 1978

INVESTIGATOR (Division)	SPECIMEN TESTED	NO. SAMPLES TESTED			
		VEE		EEE	WEE
		Trinidad	TC-83		
Eddy (Virology)	Human			6	6
Edelman (Virology)	Human			7	
Oster (Rickettsiology)	Human	15	15	15	15
Ramsburg (Virology	Burro Human	10 10			

PR tests performed to measure the level of neutralizing antibodies formed against VEE subgroup (2) strains MF-8, V-198, 3880, Mena II, Fe-3-7c, Mucambo (MUC) and Pixuna (PIX) in response to vaccination with the C-84 vaccine are presented in Table II.

TABLE II. SUMMARY OF VEE SUBGROUP PR-SN TESTS PERFORMED FIRST QUARTER FY 1978

INVESTIGATOR (Division)	SPECIMEN TESTED	NO. SAMPLES TESTED						
		MF-8 (I-B)	V-198 (I-C)	3880 (I-D)	Mena II (I-E)	Fe-3-7c (II)	MUC (III)	PIX (IV)
Edelman (Virology)	Human	2	2	3		1		
Oster (Rickettsiology)	Human	15	15	15	15	15	15	15

Tests to determine the optimal system for virus preparation and optimal tissue culture systems for measuring PR-SN antibody present in serum from 2 species of mammals were continued. The tests are presented in Table I.

Virological support services performed consisted of completion of the testing of 3 nonroutine specimens for the presence of viral agents (Table III).

TABLE III. SUMMARY OF VIOLOGICAL SUPPORT SERVICES PERFORMED FIRST QUARTER FY 1978

INVESTIGATOR (Division)	SPECIMEN TESTED	NO. HOST SYSTEMS EMPLOYED			
		Tissue cultures	Embryonated eggs	Agar plates	Microscopic slides
German (Medical)	Nonroutine:				
	Passage	4 Vero			
	CPE	54 CEC			
		25 CEC			
		15 Vero			
	Plaqueability	42 HeLa			
		79 Vero			
		36 CEC			
	Pockability		50 CAM[a]		
	Isolation and characterization		16 yolk sacs	34	92
	Pathology		11 embryos		

[a] Chorioallantoic membranes.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1973. Annual Progress Report, FY 1973, pp. 460-462, Fort Detrick, MD.

2. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1976. Annual Progress Report, FY 1976, pp. 437-440, Fort Detrick, MD.

TSP-23

TECHNICAL SUPPORT FOR THE CELL CULTURE PREPARATION

John P. Kondig
Virology Division

Objective:

To provide a centralized facility for the preparation or acquisition of cell cultures, media reagents and related materials, including viral vaccine substrates, for use by investigators of the Institute.

Progress and Summary:

During the past year the cell culture preparation and supply laboratory has produced ≃ 6,000 L of tissue culture medium and balanced salt solutions. It has also provided a total of 30 cell lines, strains, or primary cells in the various quantities listed in Table I.

TABLE I. CELL CULTURES PRODUCED - QUANTITY BY DIVISION AND INVESTIGATOR

DIVISION INVESTIGATOR	NO. PREPARED						
	Roller bottles	T-75 flasks[a]	T-25 flasks[a]	6-well plates	Tubes	24-well plates	Media[b] (liters)
Aerobiology							
Dominik				1,530			30.0
Kastello							9.0
Kishimoto	24				98	2	23.2
Jemski					252		8.0
Animal Assessment							
Harrington	59	30		10,160			209.7
Stephens						1,164	54.0
Reynolds							25.0
Bacteriology							
Mangiafico				6,155			90.1
McGann							60.0
Canonico							9.9
Howell							41.0
Ascher							9.0
Pathology							
Middlebrook	369	643				8,754	441.0
Anderson							26.6
Leppla		9				16	21.2
Morcock							8.0

TABLE I. CELL CULTURES PRODUCED - QUANTITY BY DIVISION AND INVESTIGATOR (Continued)

DIVISION INVESTIGATOR	NO. PREPARED						
	Roller bottles	T-75 flasks[a]	T-25 flasks[a]	6-well plates	Tubes	24-well plates	Media[b] (liters)
Rickettsiology							
Johnson	450		4,390				45.4
Kenyon	381	104	1,805				62.8
Anderson	195		1,030				27.0
Virology							
Cole	73	8,127	4,345	1,600	30		266.7
Eddy	34	605	487	1,150		377	58.3
Gangemi	1,350	454	89	1,880			112.0
Jahrling	554	163	800	13,790	36	182	243.7
Levitt		2	50	5,335			72.3
Luscri		31	1,560				42.0
Ramsburg	7	50	85	1,085			
Rosato	53		2,360	920	2,360		44.0
French	36	393	2,485	4,316		195	166.1
Rice	285	173	291	1,810			48.3
Gonder		81	110	380			5.8
Peters	4	381	187	2,530		460	141.3
Seed	2,096	4,714					
Total seed	3,974	12,161	20,374	53,235	3,302	6,091	2,360.4
Total + seed	6,070	16,875					

[a] Or equivalent.
[b] Media, serum, salt solutions, etc.

TSP-24

LABORATORY ANIMAL SUPPORT
Harry Rozmiarek, LTC, VC
Animal Resources Division

Objective:

To provide comprehensive laboratory animal support to include (a) animal health care, (b) complete surgical service for laboratory animals, and (c) a source of animal blood and tissues.

Progress:

A total of 102,778 laboratory animals were obtained from 15 commercial and 4 noncommercial sources. Numbers of animal requisitions, animal deliveries, and animals issued to each division are presented in Tables I and II.

TABLE I. ANIMAL REQUISITIONS/ANIMAL DELIVERIES PROCESSED AND RECEIVED

DIVISION	NO. BY QUARTER				
	FY 77-4	FY 78-1	FY 78-2	FY 78-3	Totals
Aerobiology	19/ 9	17/ 25	15/ 21	23/ 32	74/ 87
Animal Assessment	19/ 14	14/ 19	20/ 39	17/ 19	70/ 98
Animal Resources	13/ 19	2/ 2	0/ 0	2/ 2	17 23
Bacteriology	15/ 46	17/ 37	34/ 46	18/ 29	84/ 158
Pathology	8/ 16	7/ 15	19/ 29	38/ 51	72/ 111
Physical Sciences	34/ 79	44/ 77	45/ 89	39/ 68	162/ 213
Rickettsiology	12/ 11	20/ 26	34/ 35	58/ 59	124/ 131
Virology	30/ 51	19/ 40	53/ 77	64/ 78	166/ 246
Totals	150/256	140/241	220/336	259/338	769/1,167

TABLE II. ANIMALS ISSUED

DIVISION	NO. BY SPECIES						
	Mice	Rats	Hamsters	Guinea pigs	Rabbits	Monkeys	Gerbils
Aerobiology	5,806	650	312	339	6	73	
Animal Assessment	13,803	614	1,694	441	134	209	
Animal Resources	15	65	14	14		1	
Bacteriology	6,539	1,338		1,040	38	49	
Pathology	7,847	549		425	15	26	
Physical Sciences	560	16,809	27	692	7	86	
Rickettsiology	30,351	114	20	844	10	51	20
Virology	7,081	1,015	947	1,320	35	111	50

Quality control evaluation of newly received animals and observation of issued animals provided evidence of a variety of health problems in laboratory animals used at USAMRIID. Investigation in this area has been identified as a research effort and results can be found under Work Unit A841 00 040.

Nonhuman primates were skin tested quarterly for tuberculosis. In April 1978 a single animal was found to have what appeared to be a positive skin test but no lesions suggestive of tuberculosis were found upon necropsy. The room that the suspect animal came from was placed under quarantine and the remaining animals in that room were skin tested at 2-week intervals for 60 days with negative results. A subsequent quarterly test revealed no positive skin tests. Occasional cases of fatal gastric dilatation have been encountered in nonhuman primates both in the breeding colony and in the holding area. The etiology of this condition is currently unknown.

A total of 2,169 serologic screening tests were performed for evidence of specific antibodies to various agents to allow selection of suitable animals for individual studies. Results are presented in Table III.

TABLE III. RESULTS OF SEROLOGIC SCREENING[a] OF NONHUMAN PRIMATES

RECIPROCAL TITER	NO. POSITIVE								
	SEA	SEB	SEC	DEN-1	DEN-2	WN	JE	SLE	YF
10	20	23	15	17	17	14	9	22	6
20	15	13	20	8	26	36	35	23	21
40	4	3	11	2	5	8	15	3	21
80	9	5	13	5	5	2	5	4	8
160	4	2	8	1	2	5	1	2	2
320			11			3		2	
640	3	1				4			
> 1280	2		11			4			
Total N	---	141	---		241	241	241	241	241
% Positive	13	19	36	13	22	31	26	23	24

[a]WN = West Nile; JE = Japanese encephalitis virus; SLE = St. Louis encephalitis virus; YF = yellow fever virus.

The population of farm animas currently stands at 25 burros, 1 pony, 42 sheep, 28 goats and 7 chickens. Births include 8 burros, 19 goats and 10 sheep. Opening of new pasture areas have enabled the burros to be separated from the sheep and goats during moderate weather. Corynebacterium pseudotuberculosis was incriminated in several cases of SC abcesses in goats. Vaccination of all new born and newly arrived goats has not reduced the incidence of this disease. All sheep and goats had negative titers for Brucella abortus; and all Equidae were negative for equine infectious anemia. A total of 25 burros, 19 monkeys, 1 pony, 42 sheep, 29 goats and 7 chickens were being maintained as blood and serum donors. The volume of

blood collected by species for each organization is presented in Table IV.

TABLE IV. VOLUME OF BLOOD SUPPLIED

ORGANIZATION	NO. POSITIVE							
	Monkey	Sheep	Goat	Goose	Chicken	Pony	Burro	Rabbit
Aerobiology	270							
Anim. Assess.	620	40						
Anim. Res.	600	5		5			5	
Bacteriology	235	21,905	2,855	5	5	5	5	5
Pathology	2,887	65			240			
Phys. Sci.		25,250	908		10			
Rickettsiology	3,885	430						
Virology	170	1,190	400	10	10	10	60	
USAMBRDL							6,600	
FCRC							30	
TOTALS	8,667	48,885	4,163	20	265	15	6,700	5

Radiological support included 119 radiographs taken of a variety of animal species. Laboratory animal surgical support supplied to the Institute included the types and numbers of surgical procedures listed in Table V.

TABLE V. SURGICAL PROCEDURES PERFORMED ON LABORATORY ANIMALS

ANIMAL	PROCEDURE	NO. PERFORMED
PRIMATES		
	Catheterizations	
	Femoral artery	57
	Femoral vein	153
	Carotid artery	100
	Jugular vein	98
	Portal vein	4
	Hepatic vein	4
	Mesenteric vein	4
	Inguinal hernia and testicle removal	1
	Decatheterizations	
	Jugular vein	9
	Carotid artery	9
	Ocular procedure	4
	Wound suture	58
	Prolapsed uterus	1
	Left ventricle	6
	Biopsy	7
	Pin fracture	2
	Rectal prolapse	1
	Amputation	43
	Dental procedures	3
	Tracheotomy	1
	Skull implant removal	1

RATS	Catheterizations	
	Jugular vein	184
SHEEP	Tail dock	13
	Castrations	11
	Amputation	1
BURRO	Wound suture	1
GOAT	Remove abscess	1
RABBIT	Laparotomy	1
TOTAL		609

Thirty racks of stainless steel, nonhuman primate cages (4 cages per rack) and 40 racks of rodent cages with automatic watering systems were procured as replacements for cages which did not meet Animal Welfare standards. Additional orders have been placed for 15 guinea pig racks and 108 nonhuman primate racks. When these racks are received all the old substandard cages will be eliminated. Automatic watering systems have been requested for all animal rooms in Animal Resources Division, Suites 3, 4 and 5. The systems will have completely interchangeable connections with quick releases so that the rooms can be converted easily to use for different species. Caging will then meet requirements of the Animal Welfare Law and the American Association for Accreditation of Laboratory Animal Care.

TSP-25

BACTERIAL CULTURE SERVICE AND MEDIA SUPPLY

Wallace G. Fee (Part A)
William E. Kline (Part B)
Bacteriology Division

Part A - Bacterial Culture Service

Objective:

To provide a centralized facility to maintain a stock culture collection of bacterial strains with complete records of source and cultural history and to supply well characterized bacterial suspensions for use in approved work units within the Institute.

Background:

The Culture Service insures that strains of bacteria utilized in research at USAMRIID will be stored and maintained under optimal conditions with complete cultural history records, and uniform, well-characterized working cultures, readily available to investigators.

Progress and Summary:

Services provided USAMRIID investigators are listed in Table I.

TABLE I. INVESTIGATORS FOR WHOM SERVICES WERE PROVIDED DURING FY 1978

INVESTIGATOR	ORGANISM	STRAIN	NO. SAMPLES
CPT Little	*Streptococcus pneumoniae*	Type I, A-5	164
	Francisella tularensis	LVS	2
Dr. Neufeld	*S. pneumoniae*	Type I, A-5	56
	F. tularensis	LVS	2
MAJ Peters	*Serratia marcescens*	ATCC #13880	10
Dr. Mapes	*F. tularensis*	LVS	32
	Salmonella enteritidis ser. *typhimurium*	USAMRIID	4
Mr. Rill	*S. pneumoniae*	Type I, A-5	15
Dr. Wannemacher	*S. pneumoniae*	Type I, A-5	63
	S. enteritidis ser. *typhimurium*	USAMRIID	16
MAJ J. Anderson	*S. pneumoniae*	Type I, A-5	17
	Pseudomonas stutzeri	ATCC #17588	2

TABLE I. INVESTIGATORS FOR WHOM SERVICES WERE PROVIDED DURING FY 1978 (Continued)

INVESTIGATOR	ORGANISM	STRAIN	NO. SAMPLES
Dr. Abeles	S. pneumoniae	Type I, A-5	52
	F. tularensis	LVS	14
	Staphylococcus aureus	ATCC #14455	15
	S. enteritidis ser. typhimurium	USAMRIID	1
	Escherichia coli	ATCC #11775	4
CPT Wing	S. enteritidis	USAMRIID	3
Mr. Beall	S. pneumoniae	Type I, A-5	44
	F. tularensis	LVS	25
Mrs. Pace	S. pneumoniae	Type I, A-5	14
LTC Sobocinski	Staphylococcus epidermidis	ATCC #14990	1
	S. aureus	ATCC #14458	4
	S. enteritidis ser. typhimurium	USAMRIID	5
	E. coli	ATCC #11775	3
	S. pneumoniae	Type I, A-5	2
	F. tularensis	LVS	2
LTC Hedlund	S. aureus	ATCC #14458	1
	E. coli	ATCC #11775	1
	Lactobacillus acidophilys	USAMRIID	1
Mr. Thompson	S. enteritidis ser. typhimurium	USAMRIID	4
	S. pneumoniae	Type I, A-5	2
CPT Critz	F. tularensis	LVS	38
LT Anderson	E. coli	ATCC #11775	1
CPT Messinger	S. pneumoniae	Type I, A-5	2
MAJ De Sa Pereira	S. enteritidis ser. typhimurium	USAMRIID	2
LT Miller	F. tularensis	LVS	9
	S. pneumoniae	Type I, A-5	2
	E. coli	ATCC #11775	2
	S. aureus	ATCC #14458	2
Mr. Matson	F. tularensis	LVS	19
	S. enteritidis ser typhimurium	USAMRIID	1
MAJ Hall	E. coli	ATCC #11775	1
LT McCarthy	F. tularensis	LVS	1
CPT Kastello	E. coli	ATCC #11775	1

TABLE I. INVESTIGATORS FOR WHOM SERVICES WERE PROVIDED DURING FY 1978 (Continued)

INVESTIGATOR	ORGANISM	STRAIN	NO. SAMPLES
Dr. Morcock	F. tularensis	LVS	6
	E. coli	ATCC #11775	1
Dr. Jemski	F. tularensis	LVS	1

Part B - Media Supply

Objective:

To provide centralized facilities for preparation of standardized lots of culture media, solutions and reagents for approved research within the Institute and for clinical laboratories at Fort Detrick, Fort Ritchie and Camp David.

Progress and Summary:

The following media, reagents, etc., were prepared by Media Section during FY 78 for investigators in Aerobiology, Animal Assessment, Bacteriology, Pathology, Physical Sciences, Rickettsiology and Virology Divisions as well as the Clinical Laboratories at Fort Ritchie and Camp David.

PLATES OR TUBES	NO.
Agar (special)	750
Blood agar base w/sheep blood	16,172
Blood agar base plain	4,425
Bacto agar plates	100
BI plates	1,160
Brain heart infusion	6,766
Brain heart agar	96
Chocolate agar	3,675
Cysteine tryptic agar	396
Citrate sulfate	144
Diffusion agar	100
Eosine methylene blue agar	12
G. C. medium	50
Glucose cysteine blood agar	12,460
Gelatin phosphate diluent	1,860
Gelatin saline diluent	4,624
Heart infusion broth	3,145
Lactose broth	70
Lysozyme agar	170
Legionnaires' agar	4,550

BULK MEDIA	LITERS
Agarose	20.0
Brain heart infusion	37.0
Borate saline buffer	3.0
Casein acid digest	3.0
Cysteine broth	1.0
Dextrose gelatin veronol	4.0
Gelatin saline diluent	2.0
Glycine buffer	0.5
GN broth	0.5
Kaolin	4.0
Legionnaires' medium	6.0
Normal saline	3.0
NTE - PG medium	4.0
Phosphate buffered saline	72.0
Protein A medium	13.0
K phosphate solution	1.0
Na caseinate	1.0
Na carbonate	5.0
Na bicarbonate solution	1.0
Sucrose phosphate glutamate buffer	11.0

PLATES OR TUBES	NO.
MacConkeys' agar	1,964
Mannitol salt agar	720
Modified casein partial hydrolysate broth	147
Mueller-Hinton agar	1,610
Nutrient agar	623
Nutrient agar w/starch	80
Phosphate buffered saline	180
Salmonella-Shigella agar	405
Selenite broth	50
Sabouraud's dextrose agar	360
Sucrose phosphate glutamate buffer	1,397
Sodium citrate solution	422
Thayer Martin	490
Thioglycollate medium	2,228
Tryptose agar	100
Tryptose saline diluent	4,026
Trypticase soy agar	3,516
Trypticase soy broth	1,080
Water, sterile distilled	706
XLD agar	40
TOTAL	80,869

BULK MEDIA	LITERS
Thioglycollate	1.0
Thorne's medium	2.0
Tryptose saline	13.0
Tryptose soy broth	20.0
Veronol buffer	10.0
Water (glass distilled) distilled	53.0
Water (glass distilled)	15,071.0
TOTAL	15,362.0

APPENDIX B
VOLUNTEER STUDIES
(See Work Unit A841 00 001)

PROTOCOL TITLE AND NO. (No. Volunteers[a])	COMMENTS AND RESULTS
Acceptability Study of Venezuelan Equi e Encephalomyelitis Vaccine, Inactivated, Dried, MNLBR 109, C-84-1 (IND 914) Protocol 77-1 (7 MRVS)	Addendum 1: Two immunizations produced adequate responses at 6 months. Addendum 2: Serologic responses are not yet available.
Proposal for the Clinical Evaluation of a Two Vaccination Schedule of Inactivated Rocky Mountain Spotted Fever Vaccine (IND 862) (Diluted 1:3) Protocol 77-2 (10 MRVS)	No local or systemic reactions were observed. Serologic responses were positive in all but one volunteer, although not as high as in the natural disease.
Evaluation of WR 171,669 in the Treatment of Multi-Drug Resistant P. falciparum Malaria Protocol 77T-1 (3 MRVS + 1)	In follow-up, no recurrence of malaria occurred. (Previously reported in Work Unit A841 00 021)
Rejuvenation, Preservation and Characterization of the African (Uganda I) Strain of Plasmodium falciparum Protocol 77T-3 (2 MRVS)	In follow-up, no recurrence of malaria occurred. One individual developed mononucleosis with mild liver dysfunction; evaluation of this problem is underway. (Previously reported in Work Unit A841 00 021)
Evaluation of the Human Response to the Administration of Rift Valley Fever Vaccine, Formalin-Inactivated, Tissue Culture Origin, NDBR 103, Lots 1-6 (IND-365) Protocol 78-1 (13 MRVS + 7)	Serologic responses for lots 1-3 were comparable to lot 6. Results for lots 4 and 5 are not yet complete, since cell contamination occurred.
Evaluation of Human Response to the Administration of Dengue Virus Vaccine (Type 2) Live, Attenuated (IND 1257) Protocol 78-2 (6)	Mild symptoms (fatigue and headache) occurred in 4 of 6. Leukopenia occurred (5 of 6) but returned to normal values. Serologic responses were elevated in 5 of 6 (one volunteer had evidence of prior infection with dengue-2).

Transfer of Cell-Mediated Immunity to Microbial Antigens with Dialysable Leukocyte Extracts (Transfer Factor)	The project was started. Clinical and serologic evaluation is not yet complete.
Protocol 78-3 (18 MRVS + 1)	
Proposal for the Clinical Evaluation of a Two Dose Schedule of Inactivated Rocky Mountain Spotted Fever Vaccine, Undiluted (IND 862)	No adverse reactions occurred. Titers are not yet available.
Protocol 78-4 (10 MRVS)	

[a]Volunteers are professional staff members, unless indicated to be MRVS (Medical Research Volunteer Subject).

APPENDIX C
U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
PROFESSIONAL STAFF MEETINGS
FY 1978

DATE	LECTURER	TITLE OF PRESENTATION
16 Sep	Dr. Harold A. Neufeld, PhD, Physical Sciences Division	Mechanisms Governing the Inflammation Induced Inhibition of Starvation Ketosis
	MAJ James H. Anderson, Jr., MD, Physical Sciences Division	Alterations of Insulin Physiology in Infection
	Dr. Robert W. Wannemacher, Jr., PhD, Physical Sciences Division	Protein Sparing Therapy During Sepsis in the Rhesus Monkey
	LTC Philip Z. Sobocinski, PhD, Physical Sciences Division	Metallothioneins: Unique Hepatic Proteins Involved in the Hypozincemia of Infectious Diseases
20 Oct	Dr. Francis E. Cole, Jr., PhD, Virology Division	Status of Dengue Vaccine Development
	Ms. Helen H. Ramsburg, MA, Virology Division	Variation in VEE Neutralization Antibody Titers with Various Virus Sources and Cell Cultures
	Dr. Neil H. Levitt, PhD, Virology Division	The Interaction of Human Peripheral Leukocytes with Group A Arboviruses
	Dr. Peter B. Jahrling, PhD, Virology Division	Opsonization of Alphaviruses
1 Dec	Dr. Robert A. Altenbern, PhD, Pathology Division	Modulation of Staphylococcal Enterotoxin B Production by Cell Membrane Alteration
	MAJ William C. Hall, DVM Dr. John D. White, PhD, Pathology Division	Pathogenesis of Vascular Lesions in Rickettsial Spotted Fevers
	MAJ Arthur O. Anderson, MD, Pathology Division	The Mechanism of Lymphocyte Homing

DATE	LECTURER	TITLE OF PRESENTATION
19 Jan	CPT James S. Little, PhD, Bacteriology Division	The Synthesis, Transport, and Secretion of Plasma Proteins by the Livers of Control and Streptococcus pneumoniae-Infected Rats
	MAJ Harold P. Hawley, MD, Bacteriology Division	Parameters for Immunity to Francisella tularensis
	MAJ Michael S. Ascher, MD, Bacteriology Division	Regulatory Mechanisms in Delayed Hypersensitivity
	Dr. Peter G. Canonico, PhD, Bacteriology Division	Mussels, Waffles, and Brussels Sprouts: The Culinary Habits of a Migratory Civil Servant
16 Feb	CPT Dennis E. Jones, DVM, Animal Assessment Division	Lethal Pichinde Virus Infection in Guinea Pigs: Evaluation of Selected Antiviral Drugs on the Course of Infection
	CPT Reginald R. Tschorn, DVM, Animal Assessment Division	The Effect of Whole-Body ^{60}Co Irradiation on an Acute Klebsiella pneumoniae Rat Model
	CPT Jack A. Reynolds, DVM, Animal Assessment Division	Glucan-Induced Alterations in Host Resistance to VEE
	Dr. Ching-Tong Liu, PhD, Animal Assessment Division	Body Fluid Compartments and Distribution of Tissue Water and Electrolytes in Monkeys with Yellow Fever
	Dr. Ching-Tong Liu, PhD, Animal Assessment Division	Cardiohepatic Responses to Intravenous Infusion of Leukocytic Endogenous Mediator in Conscious Rhesus Monkeys
16 Mar	Dr. Richard H. Kenyon, PhD, Rickettsiology Division	Immunochemical Studies with Non-Indigenous Tick-Borne Rickettsiae
	Dr. James W. Johnson, PhD, Rickettsiology Division	Plaque Formation by Strains of Spotted Fever Rickettsiae in Monolayer Cultures of Various Cell Types
	Dr. Ralph F. Wachter, PhD, Rickettsiology Division	Effect of Poly(ICLC) on the Immunogenicity of the Soluble Phase I Antigens of Coxiella Burnetii

DATE	LECTURER	TITLE OF PRESENTATION
16 Mar	CPT Janet C. Gonder, DVM, Rickettsiology Division	Cynomolgus Monkey Model for Q Fever Infection
27 Apr	Mr. Edgar W. Larson, BS, Aerobiology Division	Airborne Transmission of Japanese B Encephalitis Virus Infections
	LTC Richard Kishimoto, PhD, Aerobiology Division	Experimental Q Fever Infection in the Nude Mouse: Clinical and Immune Responses
	MAJ William C. Hall, DVM, Pathology Division	Experimental Q Fever Infection in the Nude Mouse: Pathology
	Dr. George H. Scott, PhD, Aerobiology Division	Respiratory Melioidosis in Mice and Hamsters: Immune Responses with Live and Killed Vaccines
	Dr. Joseph V. Jemski, PhD, Aerobiology Division	Interaction of the Rat with the LV and SCHU Strains of Francisella tularensis
24 May	LTC Harry Rozmiarek, DVM, Animal Resources Division	Laboratory Animal Medicine Training (Professional and Technician)
	CPT John G. Miller, DVM, Animal Resources Division	Rhesus Monkey Production and Availability: Progress and Current Status
	MAJ Richard D. Montrey, DVM, Animal Resources Division	Use of the WRAIR Primate Registry
	Dr. Carl T. Hansen, PhD, Veterinary Resources Branch National Institutes of Health	Genes, Animals and Medicine

APPENDIX D

U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FORMAL PRESENTATIONS AND BRIEFINGS BY STAFF

Date and Group or Individual	Participating Staff Member(s)	Title
2-7 Oct 77 28th Annual Session of the American Association for Laboratory Animal Science, Anaheim, California	CPT Clayton L. Hadick, VC	An Improved Technique for Long Term Intravenous Feeding in Unrestrained Rats.
6 Oct 77 AMA Working Group Conference on Trace Elements in Intravenous Feedings, Arlington, Virginia	William R. Beisel, M.D.	Pathological Factors Affecting Requirements for Trace Elements.
9-14 Oct 77 American Physiological Society, Hollywood, Florida	C. T. Liu, Ph.D.	Effects of Intestinal Infusion of Staphylococcal Enterotoxin B (SEB)
12-14 Oct 77 17th Interscience Conference on Antimicrobial Agents & Chemotherapy Meeting, New York, New York	William R. Beisel, M.D.	Chaired a Symposium on "Metabolic Responses to Infection."
		Metabolic Responses to Infection--An Overview.
	Robert W. Wannemacher, Jr., Ph.D.	The Unique Role of Amino Acid Metabolism During Infectious Illness.
	CPT Michael C. Powanda, MSC	The Usefulness of Animal Model Infections in Determining Mechanisms of Host Metabolic Responses.

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Nov 77 American College of Veterinary Pathologists, Toronto, Canada	MAJ William C. Hall, VC	Pathogenetic Studies of the Spotted Fever Rickettsiae.
8-11 Nov 77 American Society of Tropical Medicine and Hygiene Meeting, Denver, Colorado	LTC Michael S. Ascher, MC	Further Immunologic Evaluation of Inactivated Venezuelan Equine Encephalomyelitis Vaccine in Man.
	Peter B. Jahrling, Ph.D.	Characterization of Virulent Venezuelan Encephalitis Viruses Isolated from throat Wash Fluids Obtained from TC-83 Vaccine Reactors.
	COL Gerald A. Eddy, VC	Alterations in Machupo Virus Resulting from Selective Passages.
15-18 Nov 77 American Society for Cell Biology, San Diego, California	Peter G. Canonico, Ph.D.	Analytical Fractionation of Mouse Peritoneal Macrophages: Physical and Biochemical Properties of Subcellular Organelles from Resident (Unstimulated) and Cultivated Cells.
16 Nov 77 US Army Institute of Surgical Research, Ft Sam Houston, Texas	William R. Beisel, M.D.	Clinical Rounds and Presentation. Clinical Aspects of Host Metabolism during Generalized Infection.
17-18 Nov 77 Symposium on Metabolic Aspects of the Critically Ill Patient, San Antonio, Texas	William R. Beisel, M.D.	Gluconeogenesis, Ureagenesis, and Ketogenesis During Sepsis.
2 Dec 77 The Johns Hopkins University School of Medicine, Baltimore, Maryland	MAJ Arthur O. Anderson, MC	Cells and Tissues of the Immune System, Lecture 8 of Principles of Immunology.

4 Dec 77 National Institute of Allergy and Infectious Diseases, Bethesda, Maryland	Richard H. Kenyon, Ph.D.	Recent Studies of Rocky Mountain Spotted Fever.
4-12 Dec 77 Antwerp, Belgium	COL Gerald A. Eddy, VC	The Development of a Vaccine Against African Hemorrhagic Fever.
14 Dec 77 National Institutes of Health, Bethesda, Maryland	John L. Middlebrook, Ph.D.	Diphtheria Toxin Binding to Mammalian Cells: Demonstration of a Receptor.
23-27 Jan 78 Fifth NATO NBC Medical Working Party, Brussels, Belgium	COL Richard F. Barquist, MC	Status of Vaccine Development for BW Medical Defense.
30 Jan 78 Hood College Masters Program, Frederick, Maryland	MAJ Arthur O. Anderson, MC	T and B Cells in Lymphatic Tissues.
1-4 Feb 78 American Society for Parenteral and Enteral Nutrition, Houston, Texas	Robert W. Wannemacher, Jr., Ph.D.	Protein Sparing Therapy During Pneumococcal Sepsis in the Rhesus Monkey.
Feb 78 University of Maryland Dental School, Baltimore, Maryland	John D. White, Ph.D.	SEM in Study of Infectious Diseases.
17 Feb 78 University of Maryland Dental School, Baltimore, Maryland	Edgar W. Larson	Experimental Serobiology and the Transmission of Respiratory Infections.
24 Feb 78 The Johns Hopkins University School of Medicine, Baltimore, Maryland	MAJ Arthur O. Anderson, MC	Lymphocytes, Recirculation, Lymph Node Structure, T and B Cells, and Cell Cooperation.

15 Mar 78 Hood College Masters Course on Pathogenesis, Frederick, Maryland	MAJ Arthur O. Anderson, MC	Immunosurveillance of Tumor Immunity.
16 Mar 78 University of Maryland Dental School, Baltimore, Maryland	Harold A. Neufeld, Ph.D.	Effect of Infection and Inflammation on Ketone Body Metabolism.
23 Mar 78 Harvard University, Cambridge, Massachusetts	John L. Middlebrook, Ph.D.	Diphtheria Toxin Binding to Mammalian Cells: Demonstration of a Receptor.
29 Mar 78 Scholars Seminar, Hood College, Frederick, Maryland	Harold A. Neufeld, Ph.D.	Effect of Infection and Inflammation on Ketone Body Metabolism.
Apr 78 Western Maryland University, Westminster, Maryland	John D. White, Ph.D.	TEM and SEM in Research Applications.
7 Apr 78 University of Maryland Dental School, Baltimore, Maryland	Peter G. Canonico, Ph.D.	Interdependence of Hepatic and Neutrophil Biochemistry in the Infected Host.
9-14 Apr 78 American Societies for Experimental Biology, Atlantic City, New Jersey	William R. Beisel, M.D.	Energy Metabolism: Comparisons in Starvation and Sepsis.
	C. T. Liu, Ph.D.	Cardiohepatic Responses to Intravenous Infusion of Leukocytic Endogenous Mediator in Conscious Rhesus Monkeys.
		Effects of Intestinal Infusion of Solutions with Different Toxicities in Control and Staphylococcal Enterotoxin B (SEB)-Exposed Rabbits.

	CPT Reginald R. Tschorn, VC	Effect of Klebsiella pneumoniae after Whole-Body ^{60}Co-irradiation in Rats.
	MAJ Edward L. Stephen, VC	Experimental Infection of Mice and Squirrel Monkeys Using Swine Influenza Virus: Effect of Amantadine, Rimantadine, and Ribavirin.
	Robert W. Wannemacher, Jr., Ph.D.	Metabolic Fuel Utilization by Skeletal Muscle of Rhesus Monkeys During Pneumococcal Sepsis.
12 Apr 78 American Association of Pathologists, Atlantic City, New Jersey	MAJ Arthur O. Anderson, MC	Cytoskeletal Control of Lymphocyte Recirculation.
17 Apr 78 Beckman Instruments, Inc., Bethesda, Maryland	Robert W. Wannemacher, Jr., Ph.D.	Application of Amino Acid Analysis in Physiological Fluids.
19 Apr 78 Uniformed Services School of Medicine, Bethesda, Maryland	CPT Michael D. Kastello, VC	A Comparative Approach to Renal Physiology.
28 Apr 78 American Society of Clinical Nutrition, San Francisco, California	Robert W. Wannemacher, Jr., Ph.D.	Zinc, Iron, and Copper Metabolism During Intravenous Hyperalimentation in Septic and Non-Septic Monkeys.
14-19 May 78 American Society for Microbiology, Las Vegas, Nevada	William R. Beisel, M.D.	Multifaceted Role of Leukocytic Endogenous Mediators in Host Defense.

Rebecca B. Dorland	Receptors for Diphtheria Toxin on Cultured Mammalian Cells.
CPT Joseph D. Gangemi, MSC	Protection of Guinea Pigs Against Lethal Pichinde Virus Infection by Immunization with Pichinde Virus Subunits.
MAJ Donald G. Harrington, VC	Adjuvant Effects of Tilorone Hydrochloride (Analog 11,567) in Mice given Inactivated Venezuelan Equine Encephalitis Virus Vaccine.
LTC Kenneth W. Hedlund, MC	Identification of Soluble Immune Complexes by Analytical Isotachophoresis.
Anna D. Johnson	Purification and Characterization of Two Immunologically Distinct Staphylococcal Exfoliative Toxins.
CPT Dennis E. Jones, VC	Evaluation of the Antiviral Activity of Triacetyl Ribavirin, Rimantadine, and Poly(ICLC) in a Model Arenavirus Infection.
LTC Richard A. Kishimoto, MSC	Cynomolgus Monkey Model for Experimental Q Fever Infection.
Ralph W. Kuehne	Treatment of Tacaribe Virus Infection of Mice Using Various Antiviral Compounds.
Stephen H. Leppla, Ph.D.	Development of an Efficacious Glutaraldehyde Toxoid of Pseudomonas Aeruginosa Exotoxin A.

17-18 May 78 Texas Branch of the American Association for Laboratory Animal Science Annual Meeting, Austin, Texas	LTC Harry Rozmiarek, VC	Laboratory Animal Medicine Support in a Class 3 and 4 Biomedical Containment Facility.
23 May 78 Washington Pathology Society, Bethesda, Maryland	MAJ Arthur O. Anderson, MC	Lymphocyte Homing and Recirculation.
1 Jun 78 University of South Carolina School of Medicine, Columbia, South Carolina	CPT Joseph D. Gangemi, MSC	Biophysical and Biochemical Studies on Arenaviruses.
4-8 Jun 78 American Society of Biological Chemists and American Association of Immunologists Meeting, Atlanta, Georgia	CPT Leo Andron, MSC	Enzyme-Linked Immunoabsorbent Assay of Antibody Levels in Guinea Pigs Given Antigen Plus Transfer Factor.
	CPT Hardy M. Howell, MSC	Humoral Immune Response in Tularemia-Infected AKR/J Mice.
	Harold A. Neufeld, Ph.D.	The Role of Insulin in the Inhibition of Starvation Ketosis by Inflammatory Stress.
	Robert W. Wannemacher, Jr., Ph.D.	The Possible Role of Thyroid Hormone in Regulating the Rates of Protein Degradation in Skeletal Muscle.
5 Jun 78 Dengue Virus Vaccine Workshop, Walter Reed Army Institute of Research, Washington, DC	Dr. Francis E. Cole, Jr.	Progress with Dengue 1 Vaccine.

5-7 Jun 78 Nutrition Conference, National Institutes of Health, Bethesda, Maryland	William R. Beisel, M.D.	The Effects of Specific Diseases on Nutritional Status of the Aging Adult.
7 Jun 78 University of Glasgow School of Medicine, Glasgow, Scotland	MAJ Arthur O. Anderson, MC	Control of Lymphocyte Recirculation.
12 Jun 78 VI International Conference on Lymphatic Tissues and Germinal Centers in Immune Reactions, Damp/Kiel, Germany	MAJ Arthur O. Anderson, MC	Basic Mechanisms of Lymphocyte Recirculation in Lewis Rats.
13 Jun 78 State University of New York, Stonybrook, New York	Edgar W. Larson	Hazard Potential from Laboratory Activities; Aerosol Generation, Biohazard and Injury Control in the Biomedical Laboratory.
19-23 Jun 78 Weizman Institute, Israel	Harold A. Neufeld, Ph.D.	The Effect of Inflammatory Stress on Ketone Body Formation in the Rat.
19-23 Jun 78 Israel Institute of Biological Sciences, Israel	Harold A. Neufeld, Ph.D.	The Detection of Air-Borne Biologicals by Luminescent Procedures. Mission of USAMRIID.
25-28 Jun 78 Conference on the Assessment of Energy Metabolism in Health and Disease, sponsored by Ross Laboratories, Black Point Inn, Prouts Neck, Maine	William R. Beisel, M.D.	Relation of Fever to Energy Expenditure.
1 Jul 78 Merck, Sharp, and Dohme Research Laboratories, Rahway, New Jersey	Peter G. Canonico, Ph.D.	Biochemical Properties of Mouse Macrophage Subcellular Organelles.

16 Jul 78 Annual Meeting of American College of Veterinary Internal Medicine, Scientific Seminar, Dallas, Texas	MAJ Edward L. Stephen, VC	Aerosol Therapy for Bacterial Lung Disease.
23-28 Jul 78 Gordon Conference on Microbial Toxins, Andover, New Hampshire	John L. Middlebrook, Ph.D.	Diphtheria Toxin Receptors on Vero Cells.
4 Aug 78 Gordon Research Conference on Immunochemistry and Immunobiology, Plymouth, New Hampshire	MAJ Arthur O. Anderson, MC	Importance of Transmembrane Cytoskeletal Control of Cell Surface Receptors in Lymphocyte Homing and Emigration.
29 Aug 78 International Congress for Virology, The Hague, Netherlands	Peter B. Jahrling, Ph.D.	Summarizing USAMRIID's Arenavirus Program.
Sep 78 Armed Forces Institute of Pathology, Washington, DC	MAJ William C. Hall, VC	Respiratory Diseases of Nonhuman Primates.
6 Sep 78 University of Washington Medical School, Seattle, Washington	Edgar W. Larson	Hazard Potential from Laboratory Activities: Aerosol Generation. Biohazard and Injury Control in the Biomedical Laboratory.
13-14 Sep 78 National Capital Area Branch of the American Association of Laboratory Animal Medicine, Hunt Valley, Maryland	CPT Janet C. Gonder, VC	Left Fasicular Block in a Baby Rhesus Monkey.
	CPT Jack A. Reynolds, VC	A Rapid Procedure for Shortening Canine Teeth of Nonhuman Primates.
	LTC Harry Rozmiarek, VC	Quality Assurance in Laboratory Animal Research and Testing: Rodents and Rabbits.

	CPT John G. Miller, VC	What's Your Diagnosis? Acariasis in Calomys callosus, Caused by Demodex species.
	CPT James M. Bryant, VC	What's Your Diagnosis? Acariasis in C57 Black Mice.
19 Sep 78 George Washington University, Washington, DC	Judith Pace	Regulation of Fatty Acid Metabolism During Streptococcus Pneumonia in the Rat.
24-29 Sep 78 29th Annual Session of the American Association for Laboratory Animal Science, New York, New York	LTC Harry Rozmiarek, VC	Reverse Gnotobiotics: Containment of Infectious Microorganisms.

APPENDIX E

PUBLICATIONS OF U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FISCAL YEAR 1978

1. Alluisi, E. A., W. R. Beisel, B. B. Morgan, Jr., and L. S. Caldwell. 1979. Effects of sandfly fever on isometric strength, endurance and recovery. J. Motor Behavior, in press.

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GLOSSARY

ADP	automatic data processing
BHF	Bolivian hemorrhagic fever
BUN	blood urea nitrogen
CBC	complete blood count
CEC	chick embryo cell (culture)
CPE	cytopathic effect
DEC	duck embryo cell (culture)
ED_{50}	median effective dose(s)
EEE	Eastern equine encephalitis (virus)
FA	fatty acid(s)
HA	hemagglutinins, hemagglutination
HI	hemagglutination inhibition
ID	intradermal(ly)
ID_{50}	median infectious dose(s)
IM	intramuscular(ly)
IN	intranasal
IP	intraperitoneal(ly)
IV	intravenous(ly)
JE	Japanese encephalitis
LD_{50}	median lethal dose(s)
MA	microagglutination, microagglutinin
MMD	mass median diameter
$MIPLD_{50}$	median infectious intraperitoneal lethal dose(s)
mRNA	messenger RNA

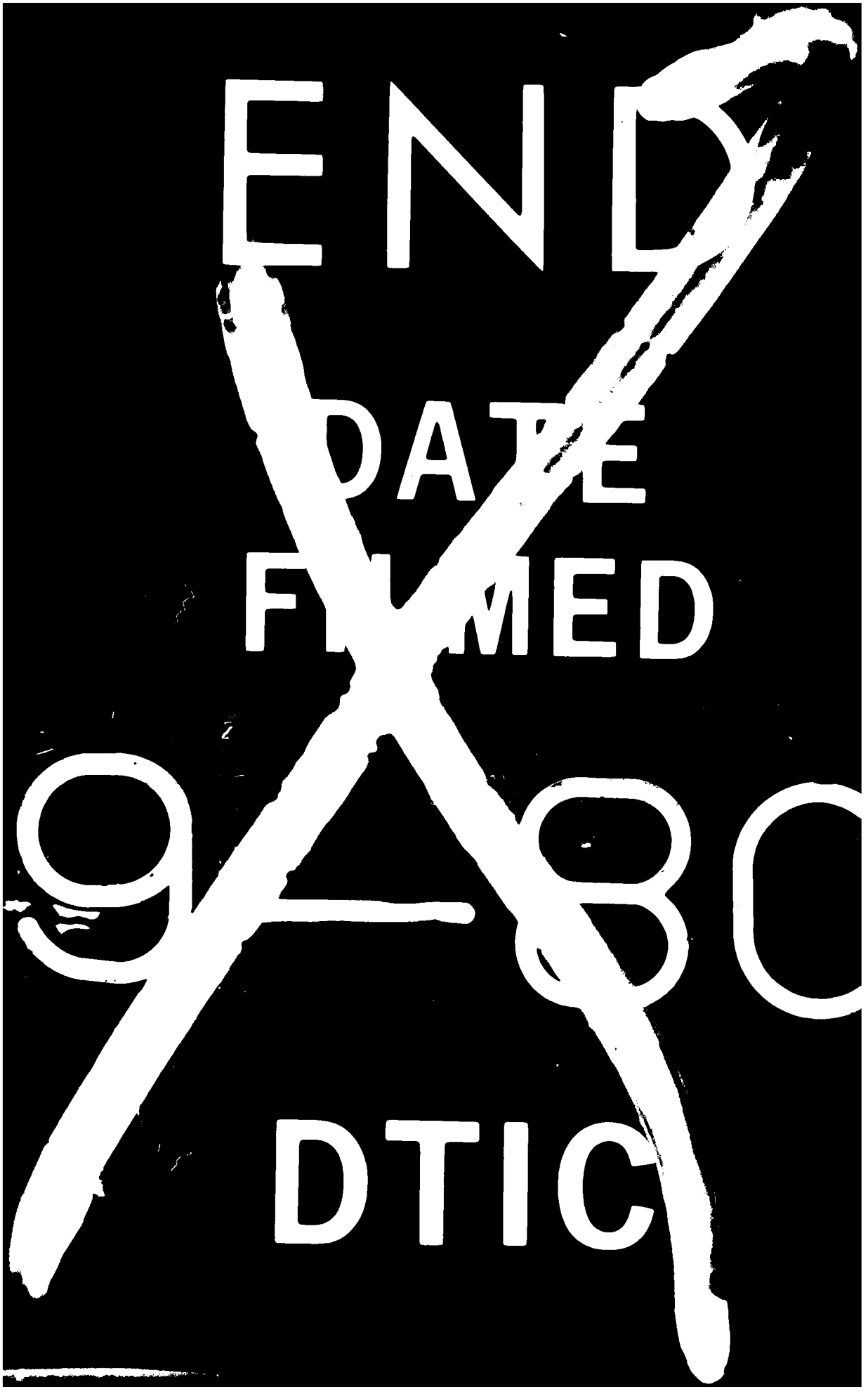
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PFU	plaque forming unit(s)
PMN	polymorphonuclear leukocytes
PR_{50} or PR_{80}	50% or 80% plaque reduction
RBC	red blood cells
RES	reticuloendothelial system
RIA	radioimmunoassay
RMSF	Rocky Mountain spotted fever
rRNA	ribosomal RNA
SC	subcutaneous(ly)
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B
SEC	staphylococcal enterotoxin C
SGPT	serum glutamic pyruvic transaminase
UV	ultraviolet
VEE	Venezuelan equine encephalomyelitis (virus)
WBC	white blood count
WEE	Western equine encephalitis (virus)
WRAIR	Walter Reed Army Institute of Research
YF	yellow fever

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SUPPLEMENTARY

INFORMATION

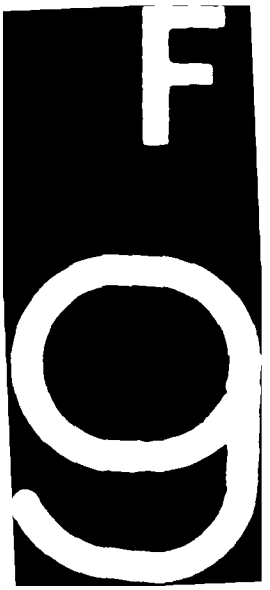
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