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COMMISSION ON EPIDEMIOLOGICAL SURVEY
ANNUAL REPORT
TO THE
ARMED FORCES EPIDEMIOLOGICAL BOARD

FISCAL YEAR 1971

Washington, D.C.

DECEMBER 1971

COMMISSION ON EPIDEMIOLOGICAL SURVEY

ANNUAL REPORT TO THE ARMED FORCES EPIDEMIOLOGICAL BOARD

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DECEMBER 1971

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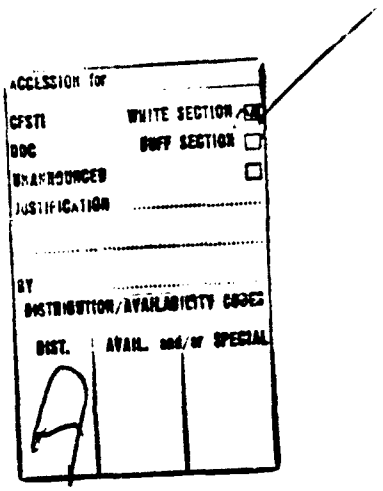

SUMMARY

This is the fourth annual report of the Commission on Epidemiolcgical Survey since consolidation with the Commission on Radiation and Infection. This was a combined meeting with the Commission on Immunization, which is preparing its own report. The annual reports of two contractors, Trudeau Institute, Inc., Saranac Lake, N. Y. (DADA 17-68-C-8124), dated September 1971, and the University of Maryland School of Medicine, Baltimore, Md. (DA-49-193-MD-2867), dated 15 August 1971, are included in the official report to the Armed Forces Epidemiological Board. They are available to other recipients of this report from the Defense Documentation Center, Cameron Station, Alexandria, Va. 22314.

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13. ABSTRACT

Progress is reported in selected areas of research in medical defense aspects of biological agents by the U. S. Army Medical Research Institute of Infectious Diseases and two contractors.

KEYWORDS:
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Hemorrhagic fever
Staphylococcal enterotoxin B
Plague

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FOREWORD

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

Studies employing volunteers in research tests were governed by the principles, policies and rules for medical volunteers as established by Army Regulation 70-25 and the Declaration of Helsinki.

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Wisseman, Charles L., Jr., M.D.

Wood, W. Barry, Jr., M.D.*

* Deceased, 9 March 1971.

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THE DIRECTOR'S SUMMARY REPORT

The combined meetings of the Commissions on Epidemiological Survey (CES) and Immunization was held at the Walter Reed Army Institute of Research (WRAIR) on 23-24 September 1971. The presentations of the Commission on Immunization will be published separately. Dr. Gustave J. Dammin, President, Colonel Bradley W. Prior, Executive Secretary, and Floyd W. Denny of the Armed Forces Epidemiological Board, representatives of the Departments of Army, Navy, and Air Force, personnel of U. S. Army Medical Research Institute of Infectious Diseases (USAMRIID) and guests who attended one or both sessions follow:

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CES

A. Volkman

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MAJ J. R. Warren
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F. E. Cole, Jr.
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Harold N. Glassman, Bio Def Res Lab
Michael B. Gregg, CDC
Kathleen Kos, WRAIR
Carl Lamanna, OCRD/DA
Howard Noyes, WRAIR
John Schachter, Univ Cal Med Sch
Richard H. Zeschke, SUNY/Buffalo

Appreciation was expressed by the Chairmen to Dr. Dammin, COL Pilor and Miss Gilbert for the help and numerous courtesies extended by the AFEB to the Commissions in the conduct of their scientific programs. It was announced that Dr. John Dingle, Advisory Member of the CES, could not attend the meeting but that he served effectively in his advisory capacity. During the year, Dr. W. Barry Wood's distinguished career ended by death on March 9, 1971. The Commission profited throughout its existence from his mature and wise counsel. A Memorial Minute to Dr. Wood was presented by Dr. Woodward and recorded in the Minutes of the Board's spring meeting. Drs. MacLeod, Bennett, Stoner and Bond were unable to attend the meeting because of unavoidable prior commitments.

The agenda for the two-days meeting of the Commission on Immunization and CES was devoted to reviewing the latest work completed or in progress by AFEB contractors of the two Commissions and by investigators of USAMRIID. Emphasis was placed on those scientific areas of mutual interest to members of both Commissions. The scientific program included review of work and discussions of the current status of Venezuelan equine encephalomyelitis (VEE), antibody interaction, antibody formation and methods of assay,

biochemical and humoral aspects of cell mediated immunity and hypersensitivity reactions, development of vaccines for spotted fever, Q fever, gram negative enteric infections, smallpox, mumps, plague, rabies and tetanus; status of epidemic hemorrhagic fever in Latin America; the current knowledge of staphylococcal enterotoxoids and metabolic alterations in infection. This report will summarize that work conducted under the aegis of the Commission on Epidemiological Survey.

Venezuelan Equine Encephalomyelitis

Epizootic in 1971

LTC Spertzel presented a review of the 1971 VEE epizootic. The first case was observed in South Texas on June 23. Vaccination was initiated on June 25 using the USAMRIID developed live vaccine, produced by the National Drug Company. One month later the vaccination area was extended to provide an ocean-to-ocean barrier. Eight states were added in August. USDA had been unwilling to license the production of this vaccine in 1969 or 1970 when the epizootic had occurred in Central America. A provisional license for commercial production and sale was granted in July, 1971.

Host Defenses during VEE Virus Infection in Mice

To determine the relative importance of antibody, interferon and cellular immunity in recovery from primary VEE virus infection, passive transfer experiments were performed by MAJ Rabinowitz in immunosuppressed and healthy mice. Non-immunosuppressed mice which received 30 LD_{50} VEE virus subcutaneously (SC) suffered 100% mortality. Neonatal thymectomy and pretreatment with antithymocyte serum (ATS) resulted in 100% mortality in infected mice which could be completely reversed by passive transfer of hyperimmune serum. Non-immunosuppressed infected mice could be protected against fatal infection with either hyperimmune serum or immune spleen cells. The protective capacity of immune spleen cells in adoptively immunized hosts required intact viable cells and disappeared by 25 days after donor immunization. Administration passively of either Poly I:C or interferon failed to protect against VEE infection. Humoral antibody responses in adoptively immunized mice did not differ significantly from controls. Use of the *in vitro* lymphocyte stimulation test has shown that immune cell preparations contain antigen reactive cells which can be specifically stimulated by VEE antigen.

The results suggest that both antibody and cellular immunity play a major role in protection against VEE infection.

Antibody Formation and Interaction

Sequential Humoral Antibody Responses

Mrs. Wilkie reported the results of a study of the kinetics of antibody responses to inactivated eastern equine encephalitis (EEE) vaccine in

rabbits and in volunteers. Following injection of vaccine, the rates of processing of EEE antigens were apparently complete within 30 days in both man and rabbit; however, 3-4 months later, delayed increases in serological activity were observed, apparently indicating further processing of antigen from an unexplained source. Secondary responses of 3 volunteers appeared to be suppressed, suggesting a tolerance state. Parallelism of human and rabbit responses reinforces the value of the use of the rabbit as a reference model in vaccine evaluation studies.

Microprecipitation and Electrophoresis in Diagnostic Virology

CPT Levitt reported on a microprecipitation test (MPT) for the detection of adenovirus antibody. The new procedure combined precipitation of viral particles with specific antibody, separation of unreacted materials from the resultant electroneutral virus-antibody complexes by electrophoresis, and detection of these complexes with a protein stain. Type-specific antibody was detected in rabbit antisera. Under similar conditions, only antibody to group antigen(s) was detected in convalescent human sera. Paired sera from 57 patients with suspected adenovirus infection were examined by both MPT and the complement fixation (CF) test for significant rises in antibody titer. The MPT was more sensitive, simpler to conduct and required less time and reagents than the routinely employed CF test.

CPT Levitt also described a diagnostic test employing microprecipitation of antigen-antibody complexes. In addition, a simple and rapid method for removal of nonmigrating lipid containing substances from human serum was described. Finally, preliminary studies with VEE virus antigen illustrated the ease of adapting this procedure to other viruses, and its use with sera from various animal species.

Electron Spin Resonance and Antigen-Cell Membrane Interaction

MAJ Kaplan described the mechanisms involved in antigenic activation of the immunocompetent cell. In view of the considerable evidence that lymphocytes possess surface receptors for antigens, it appears likely that activation is triggered by interaction of these receptors with antigen, and possibly by a conformational change in the lymphocyte membrane.

By employing nitroxide spin labels and electron spin resonance (ESR) spectroscopy to detect local membrane changes in viscosity, polarity and anisotropy, responses of cell membranes to various treatments or stimuli were examined. Detection of a response depended on the configuration of the spin label, its site of incorporation into the membrane, and the type of stimulus employed.

For example, treatment with heat or formalin caused irreversible changes in the ESR spectrum of erythrocyte stroma labeled with iodacetamide, but not of stroma labeled with androstane. However, treatment with a lipid-soluble anesthetic, lidocaine, increased mobility of the androstane label.

Procedures were established for in vitro incorporation of spin labels into membranes of intact nucleated cells by culturing suspensions of L-cells in the presence of stearic acid labels or by incubating human lymphocytes (from a long-term tissue culture cell line) with androstane complexed to bovine serum albumin. Spectra of subcellular fractions indicated that these mammalian cells incorporated label in their outer membranes as well as in membranes of subcellular organelles. With nitroxide located in the hydrophobic (12 NS) portion of the stearic acid molecule, the spectrum from 12 NS labeled membranes was isotropic and weakly immobilized, whereas with nitroxide in the hydrophilic (3 NS) portion of the stearic acid molecule, the 3 NS spectrum showed a strong anisotropic component, indicating that mobility of the stearic acid molecule was severely restricted in the hydrophilic region of the membrane.

Inactivation of the spin label in membranes of intact lymphocytes occurred rapidly at room temperature if access to air was restricted, but activity was restored following exposure to air or treatment with potassium ferricyanide. Incorporation of spin label into lymphocyte membranes did not affect biologic function; unlabeled and labeled lymphocytes displayed essentially the same tritiated thymidine uptake following stimulation.

Cell Mediated Immunity

Mechanisms of Cell Mediated Immunity

Dr. Volkman described two models of cell mediated immunity now under study.

(a) Delayed hypersensitivity: The requirements for macrophages in the expression of DTH was shown in a series of intradermal transfer experiments in irradiated sensitized guinea pigs. Cells which carry specific sensivity to skin-test antigen have been shown to be relatively radioresistant.

(b) The local graft versus host (LGVH) reaction: Cell proliferation, as measured by the uptake of tritiated thymidine, is being used to quantitate the intensity of LGVH reactions in the kidneys of F_1 rats after the intrarenal injection of parental lymphocytes. New data show target cell destruction to be nonspecific and not dependent upon macrophages. Results suggest some effector activity by host cells.

Lysosomal Responses during Infection

CPT Canonico reported that enzymatic and physical properties of rat liver subcellular organelles during the infectious process were determined by zonal ultracentrifugation techniques. Livers from rats, inoculated 8-48 hr previously with 10^7 Diplococcus pneumoniae or 10^9 Francisella tularensis organisms, were homogenized and subjected to isopycnic centrifugation in a B XIV zonal rotor. Thirty-three fractions were obtained and analyzed from protein, RNA and 6 enzyme markers of mitochondria, lysosomes and peroxisomes. A computer program was developed to calculate and represent enzymatic activities in the form of standardized equilibrium density distribution patterns.

The distribution patterns of cytochrome oxidase indicated that a progressive degeneration of mitochondrial structural integrity occurs in the course of pneumococcal infection. The activity of the peroxisomal markers, catalase and urate oxidase, was reduced by 82 and 76% respectively during the more advanced stages of the same infection, and were accompanied by a substantial increase in the mean equilibrium density of peroxisomal particles. The activity and distribution of particle-bound cathepsin D, acid phosphatase, and β-glucuronidase were not altered but some increase in their soluble activities was observed. The distribution pattern of β-glucuronidase in addition demonstrated the presence of a minor sedimentable component with a non-lysosomal localization. A loss of this minor component was observed during the most advanced stages of pneumococcal infection. In contrast to these observations, tularemia infection was marked by a sustained rapid loss of the minor sedimentable component of β-glucuronidase and transient changes in the physico-chemical properties of mitochondria and peroxisomes.

The data obtained demonstrate the use of tissue fractionation and zonal ultracentrifugation techniques in evaluating cellular responses and obtaining a greater understanding of the pathogenesis of infection. In addition, this study focuses attention on the potential promise of tissue fractionation techniques in the diagnosis of infectious disease.

Immunity and Hypercholesterolemia

Dr. Beisel discussed the role of hypercholesterolemia in altered immunity to infectious disease. Continuing investigation of the metabolic response has shown that lipids play a fundamental role during infection. Three months of a high lipid diet induced in monkeys abnormally high levels of cholesterol and phospholipids and decreased triglyceride levels. Infection of these experimental animals resulted in decreases in cholesterol and phospholipids and increased triglycerides. Both humoral and cellular immune responses were atypical in hypercholesterolemic monkeys. Colloidal carbon was administered to the infected experimental and control monkeys. Clearance of carbon was then determined.

There was a significant increase in clearance rates in all infected monkeys when compared to uninfected controls. Hypercholesterolemic monkeys had significantly slower clearance rates than the infected normal-diet monkeys.

Since a sizable portion of the U. S. population has overt or unrecognized derangement of lipid metabolism, there may be differences in the susceptibility of individuals to infection and responses to vaccine administered. An effort should be made to evaluate this relationship in man.

Endogenous Mediators of Nonfebrile Host Responses

Dr. Pekarek described significant alterations in trace metal and amino acid metabolism just prior to the onset of febrile illness in volunteers with experimentally induced sandfly fever. It was theorized that the observed metabolic changes in both trace metal and amino acid metabolism may be mediated through a humoral mechanism during infection. To support this theory a heat-labile endogenous mediator was demonstrated in the serum of volunteers infected with Salmonella typhi; which, upon injection into rats caused significant decreases in serum Zn and Fe and a flux of amino acids to the liver in these recepient animals. Endogenous mediator isolated from peritoneal leukocytes was shown to cause dose-dependent alterations in both trace metal and amino acid metabolism, as well as increased synthesis of serum proteins. These changes were independent of dietary intake or excretion and represented a redistribution of these metals and amino acids in the body. Therefore, endogenous mediator(s) was shown to trigger a series of early biochemical events which may be important in nonspecific host defense mechanisms.

Vaccine Studies

Rocky Mountain Spotted Fever Vaccine

Dr. Kenyon described an improved Rocky Mountain spotted fever vaccine. Rickettsiae were grown in duck embryo cells and inactivated by irradiation or formaldehyde treatment. These vaccines were compared with the Lederle yolk-sac grown vaccine. The irradiated cell culture vaccine was more than 300 times and the formaldehyde-treated cell culture vaccine, more than 900 times as active as the Lederle yolk-sac grown vaccine.

Live Q Fever Vaccine

MAJ Robinson reported that a series of 5 lots of R-M (RIF-free M) strain of Coxiella burneti vaccine have been produced and tested according to USPHS regulations. These lots had a mean median infectious dose for eggs of $10^{-10.7}$; no fever was produced when 0.5 ml of undiluted material was inoculated SC into guinea pigs. The median protective dose in guinea pigs contained approximately 0.00005 μg N; no difference in

protection was detected between Phase I and II challenges. The R-M strain did not cause an increased incidence of hepatic lesions when compared to the present killed vaccine.

Studies of Staphylococcal Enterotoxin B (SEB)

Mitogenic Effects

Dr. Adler reported the effects of SEB on lymphoid tissue in vitro. The enterotoxin is a potent mitogenic stimulant for mouse spleen, thymus and bone marrow cells, and human peripheral lymphocytes. The cells able to be stimulated by SEB are probably of several types although the lymphoid series are probably the major type affected. The cells that are stimulated by the SEB are able to induce pathological changes in other tissue culture cell lines in vitro, and the stimulated lymphocytes release substances which cause toxicity and growth inhibition in other tissue culture cell lines.

Staphylococcal Enterotoxoids

COL Metzger discussed the results of studies to develop staphylococcal enterotoxoids. The first phase involves repetition of formalin-toxoiding with pH alterations for types A, B and C enterotoxins. After testing they are combined and used to immunize monkeys. The second phase will consist of studying chemically fragmented SEB enzymatically to locate the fraction or fragment which retains immunogenicity but loses its toxicity. It is hoped that there will be a physical or chemical endpoint which would eliminate the trial and error of repeated safety tests. The system should furnish information to improve the understanding of formalin-toxoiding in general.

Hemorrhagic Fever in Cochabamba, Bolivia

LTC Spertzel presented for Dr. Peters the status of epidemiologic and virologic studies on the recent outbreak of 6 cases of hemorrhagic fever in Cochabamba, Bolivia. The disease differs from that caused by Machupo virus. Using special biological safety cabinets, the virus has been studied intensively. Evidence has been found for the presence of a virus of the Tacaribe complex, but characterization is not complete.

Pathogenesis and Control of Enteric Pathogenesis

Dr. Richard B. Hornick described the results of studies conducted in volunteers at the University of Maryland dealing with pathogenesis and control of enteric infections. He reported the following infective doses of enteric pathogenesis for man: Shigellae -- 10^2, S. typhi -- 10^5 and Escherichia coli -- 10^8 to 10^{10}, which are resistant to the gastric acid effect; Vibrio cholerae -- 10^{10}, which is sensitive to the effects

of gastric acidity; i.e., with low concentrations of gastric HCl, susceptibility to cholera increases. Those pathogenic microorganisms which cause abnormalities in the intestinal lumen or on the epithelial surface and which do not invade the intestinal mucosa are: V. cholerae, Staphylococci, Giardia lamblia and the toxigenic strains of E. coli. Pathogenic strains of Shigellae and E. coli are known to penetrate the superficial epithelial layers of the intestine.

The Maryland studies reveal that smears of stool specimens stained with methylene blue show polymorphonuclear (PMN) leukocytes in patients with bacillary dysentery, occasionally in chronic ulcerative colitis and regional enteritis but not in cholera, salmonellosis or enteritis caused by toxigenic strains of E. coli.

Enteritis caused by toxigenic strains of E. coli is self-limiting and the physiologic abnormalities are in the small intestine simulating the effect of cholera. Invasive strains of E. coli cause illness after an incubation interval of about 11 hr with the clinical picture simulating bacillary dysentery.

Two types of immunity have been demonstrated in cholera: antitoxic and antibacterial. Convalescent volunteers showed an antibacterial type of resistance in the intestine.

Viable strains of Shigellae given orally to volunteers provided some protection after a dose of pathogenic organisms. The question of an antitoxic type of immunity is under consideration.

Several attenuated strains of S. typhi are available for use as oral vaccines. There is more experience with a streptomycin-dependent mutant. Equivocal results show an antibacterial type of immunity after vaccination and oral challenge with a virulent strain of typhoid bacilli. Work continues on optimal dosage schedules.

Dr. Greisman continues to study the host response to bacterial endotoxins; he reported the results of histologic reactions in the skin to endotoxins, whole bacterial antigens, and other test materials such as pyrogen-free saline and alcohol. Small doses of gram negative bacterial endotoxin stimulate an early mononuclear infiltrate which is characteristic of the dermal response of delayed hypersensitivity. Large doses of endotoxin caused a PMN reponse as did antigens consisting of nonviable gram positive bacteria. The reaction to PPD in sensitive volunteers resembled the mononuclear reaction to bacterial endotoxin with PMN representing $< 10\%$ of the early inflammatory response. The rose spot of typhoid fever consists of a mononuclear perivascular response with small lymphocytes and macrophages; $< 5\%$ of the cell population consists of PMN.

Several administrative matters were discussed during the brief executive session. Several weeks prior to future meetings, abstracts of presentations will be transmitted to members.

The next meeting of the CES will be held on 21 and 22 September 1972 probably in the new building at USAMRIID, Frederick, Maryland.

COL Crozier presented a resume of scientific work in progress in USAMRIID and commented regarding the status of personnel positions. The Institute is engaged in an expanded mission and it was stressed that CES ought to retain its identity and continue as an advisory group. It was suggested that a copy of the Chairman's position letter on BW submitted to the Presidential Scientific Advisory Council be distributed to commission members.

In view of the governmental attitude on research programs including stress on curtailment of activities, the decision was made not to add new Commission members. There is need for new expertize in the metabolic, biochemical, and immunologic areas.

The joint meeting with the Commission on Immunization was regarded as successful and unusually productive.

Theodore E. Woodward, M.D.
Director
Commission on Epidemiological Survey

OVERVIEW OF THE 1971 VENEZUELAN EQUINE ENCEPHALOMYELITIS EPIZOOTIC

Richard O. Spertzel, LTC, VC*

The etiologic agent of Venezuelan equine encephalomyelitis (VEE) is a member of Casal's group A arboviruses. There are currently 4 major antigenic subtypes, of which one is subdivided into 5 minor antigenic variants.[1] The nonepizootic sylvatic subtypes occur endemically throughout major portions of Central and South America and in parts of North America,[2] while major epizootics with variants 1A, 1B and 1C have occurred in South America.[2] The present epidemic of highly virulent subtype 1B probably had its origin in Ecuador, and was introduced into Guatemala in 1969. From there, the spread was rapid through El Salvador and portions of Honduras and Nicaragua. In 1970, the disease reoccurred in Honduras and spread into Costa Rica and Mexico.[3] Although slowed in 1970, VEE continued its inexorable spread toward the United States; by April, 1971, the disease was seen near Tampico, Mexico. By early June, equine cases were occurring within 35 miles of Brownsville, Texas. The first recognized encephalitic horse in south Texas was sick on June 23.

Vaccination was begun on a voluntary basis on 25 June in a 13-county area of south Texas. After confirmation of VEE by virus isolation on 9 July, vaccination was extended statewide in Texas on 13 July, and to New Mexico, Oklahoma, Arkansas, and Louisiana on the 16th. A fee-basis, federally supported vaccination program was initiated in these 5 states. On 25 July, the vaccination area was extended to California, Arizona, Mississippi, Alabama, Georgia, and Florida, in order to create an ocean-to-ocean barrier. More recently, on the recommendations of a triagency task force, the area was extended to 8 more states: South and North Carolina, Tennessee, Kentucky, Virginia, Maryland, Delaware, New Jersey, and the District of Columbia. Missouri was invited to participate, but declined.

A State and Federal quarantine for Texas was established on 13 July; for Oklahoma, New Mexico, Arkansas, and Louisiana on 19 July; and Mississippi on 2 August. State quarantines were established in Florida, Georgia, and Alabama. Interestingly, embargoes were placed by Canada on United States horses on 14 July. Several European countries also banned import of U. S. horses.

In addition to vaccination and quarantine, a mosquito-control program, consisting of low-volume aerial spraying of Malathion or Dibrom was maintained along the coastal counties of Texas until 90% of the equine population was vaccinated.

* U. S. Army Medical Research Institute of Infectious Diseases.

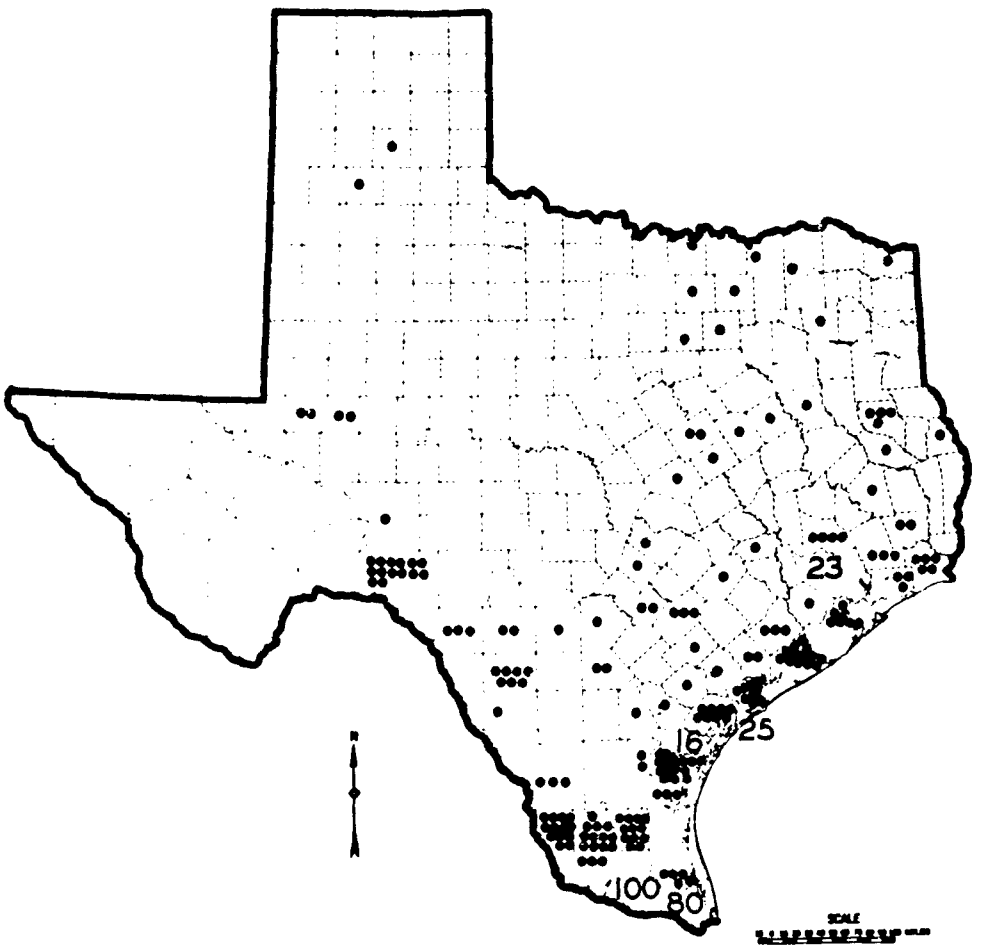

FIGURE 1. ENCEPHALITIS CASES REPORTED ON OR BEFORE 24 JULY 1971.

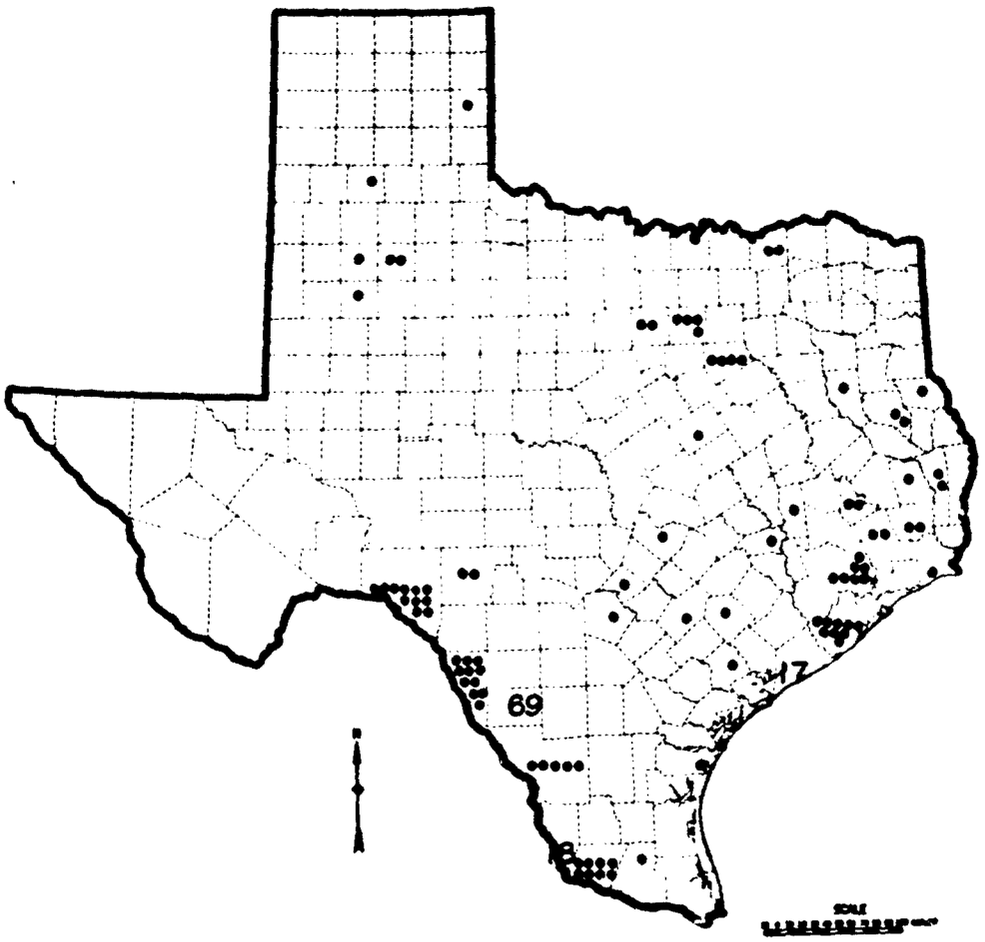

FIGURE 2. ENCEPHALITIS CASES REPORTED AFTER 24 JULY 1971.

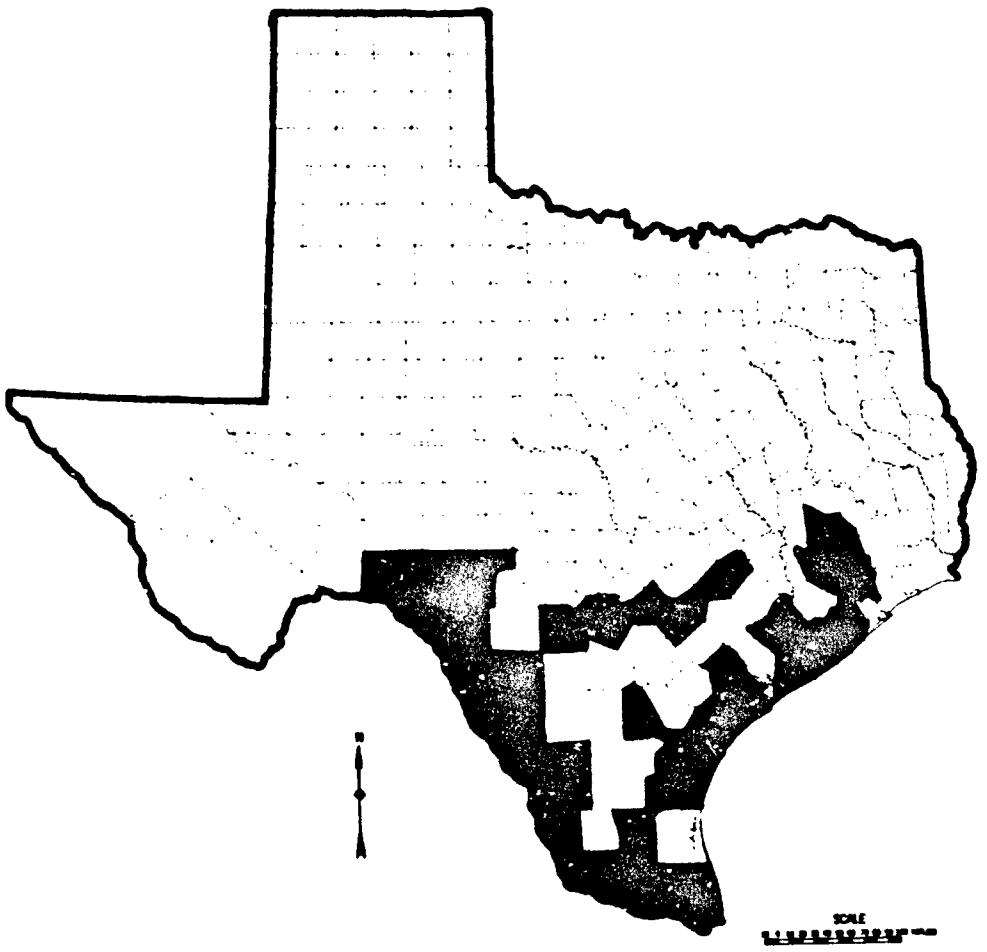

FIGURE 3. CONFIRMED VEE CASES REPORTED, 1971.

Although statewide vaccination was not allowed until 13 July, suspect encephalitis cases in horses were reported in counties outside the allowable vaccine area by 10 July.

The epizootic apparently reached its maximum extent by 24 July, Figures 1 and 2. Within the epizootic area, however, encephalitis in nonvaccinated horses is still occurring. Counties in which VEE virus was isolated, or specific antibody was detected in sera from nonvaccinated horses, are shown in Figure 3. (Ed.: 4 more counties have been added 19 Oct).

Two border parishes of Louisiana and 2 border counties of Arkansas also reported an unusually high incidence of suspected cases of equine encephalitis. Cessation of such reports within 10 days after VEE vaccination leads us to suspect infection with VEE there.

The lack of laboratory confirmation in many counties is not surprising. The relationship of viremia, fever, illness, and onset of detectable antibody, relative to symptomatic signs of encephalitis and death, is depicted in Figure 4. Obviously delay in reporting and investigating suspect cases in the field would minimize the opportunity for diagnosis by serology or virus isolation.

After the 1969 Central American epizootic and the large demand for live attenuated VEE vaccine, numerous attempts were made by personnel of the U. S. Army Medical Research and Development Command to induce the U. S. Department of Agriculture (USDA) to license the vaccine for export and for contingency use within the United States. Unfortunately, USDA officials were reluctant to use the vaccine before VEE was diagnosed in the United States. In spite of extensive use of vaccine in Central America, where it appeared to be both safe and effective, USDA officials continued to express serious reservations about the dangers of reversion to virulence, should horse-to-horse transmission occur.

Vaccine administration to Equidae is characterized by a low, irregular viremia with a transient fever in approximately 50% of animals. Unlike man, where 35-40% of vaccinated individuals may show some reaction to the vaccine, only 1% of horses show even a transient reaction consisting of anorexia and depression for 12-24 hr. Although no evidence of reversion to virulence was observed during serial passage of the virus by subcutaneous or intraperitoneal (IP) routes in small laboratory animals, several laboratories attempted horse-to-horse passage of the virus. As mentioned earlier, viremias with the vaccine strain are low-level, irregular, and unrelated to fever. Thus, Johnson, who used fever as a guide, failed twice to recover virus beyond the 2nd passage; and McConnell, who selected the 72-hr sample for transmission, was unable to infect recipients on the 4th passage.[4] USDA personnel in Mexico City collected 100 cc of serum from each of 5 animals daily for 5 days postinoculation. These samples were pooled, and an aliquot was given to each of 5 additional horses.

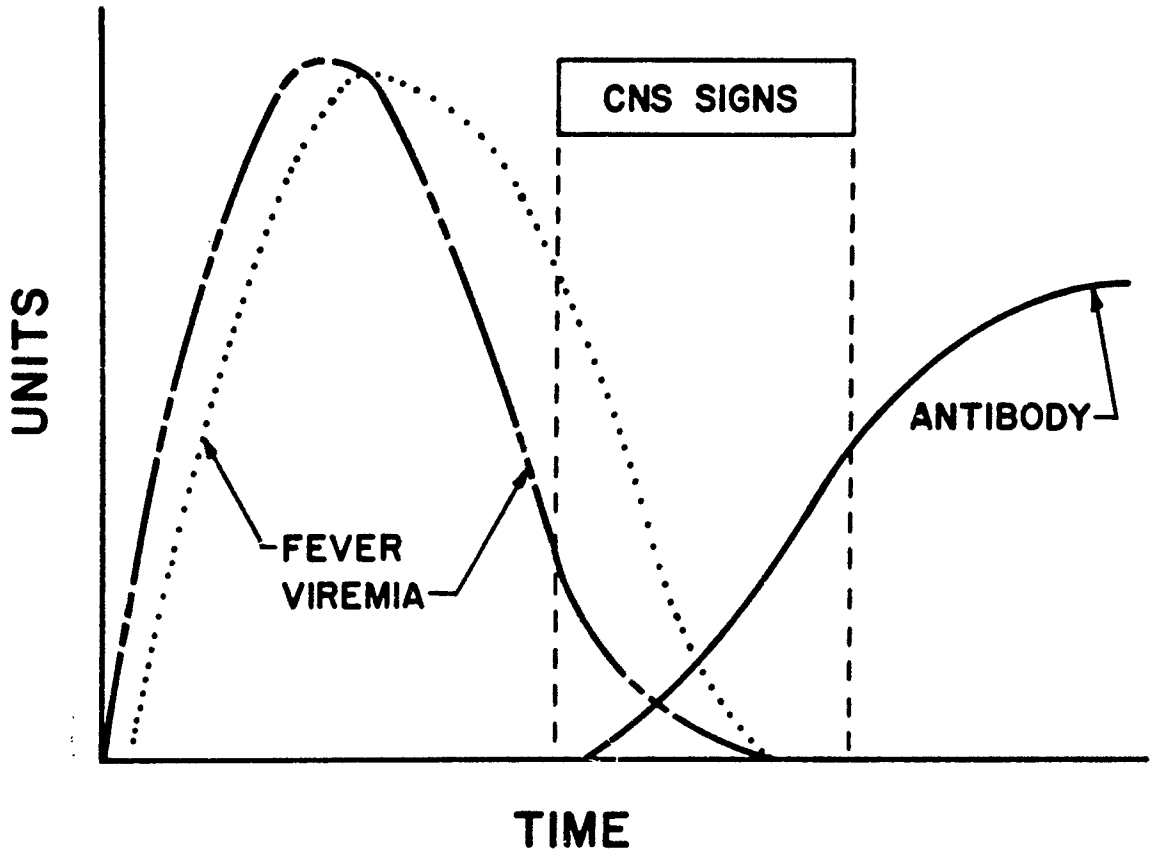

FIGURE 4. SCHEMATIC VEE INFECTION.

By this method, they attained 5 passages, with no indication of reversion to virulence. At the U. S. Army Medical Research Institute of Infectious Diseases, a slightly different approach was used, Figure 5. Burro No. 1 was given 3.84 suckling mouse intracerebral median lethal doses $SMICLD_{50}$ of TC-83. Serum was collected at 12-hr intervals; a portion of each sample was immediately inoculated IP into weanling mice and IC into 1-2-day old mice. Serum from each bleeding was stored at -20 C in 1- and 10-ml volumes.

If tests in mice indicated the presence of virus, shown by the solid diamonds, the 1-ml serum sample was titrated for its viremia level. On the basis of these titrations, the 10-ml serum sample corresponding to the highest level of virus, represented by the large open diamond and shown on the right, was selected for inoculation into the next burro. Note the irregular viremia pattern of Burro No. 1 and the absence of fever in Burros 1, 2 and 5; consequently, transmission studies based on these reponses could readily be unsuccessful. The prolonged viremia and the high viremia levels observed in Burros 3 and 4 are consistent with data on primary vaccination with TC-83 reported previously by our laboratory, and do not indicate an increase in virulence as seen with Burro No. 5.

With this method, 4 passages have been attained. No evidence of reversion to virulence, as indicated by lethality for weanling mice or clinical signs in burros, has been observed.

In addition to these back-passage studies, additional safety studies were conducted in the field.

Observation of approximately 22,000 Equidae by USDA and/or U. S. Public Health Service personnel in 5 separate states indicated a reaction rate of $< 1\%$.

These results were consistent with those reported by our laboratory,[5] and with empirical observations in Central America; they are in conflict with results of the limited study at the Center for Disease Control,[6] in which 3 of 6 animals showed severe depression and anorexia.

In 1969 and 1970, numerous field observations attested to the efficacy of the vaccine; deaths of nonvaccinated animals were documented in herds where all vaccinees survived. These same observations have been made in Texas. A not-uncommon herd report, from an area with active encephalitis cases, follows.

On 20 July, 35 "working" horses and 3 stallions were vaccinated but the owner considered it too much trouble to round up the remaining horses. In mid-August, encephalitis and death began to occur in nonvaccinated animals. A field investigation was made on 31 August. Horses were pastured in noncontiguous areas on the ranch.

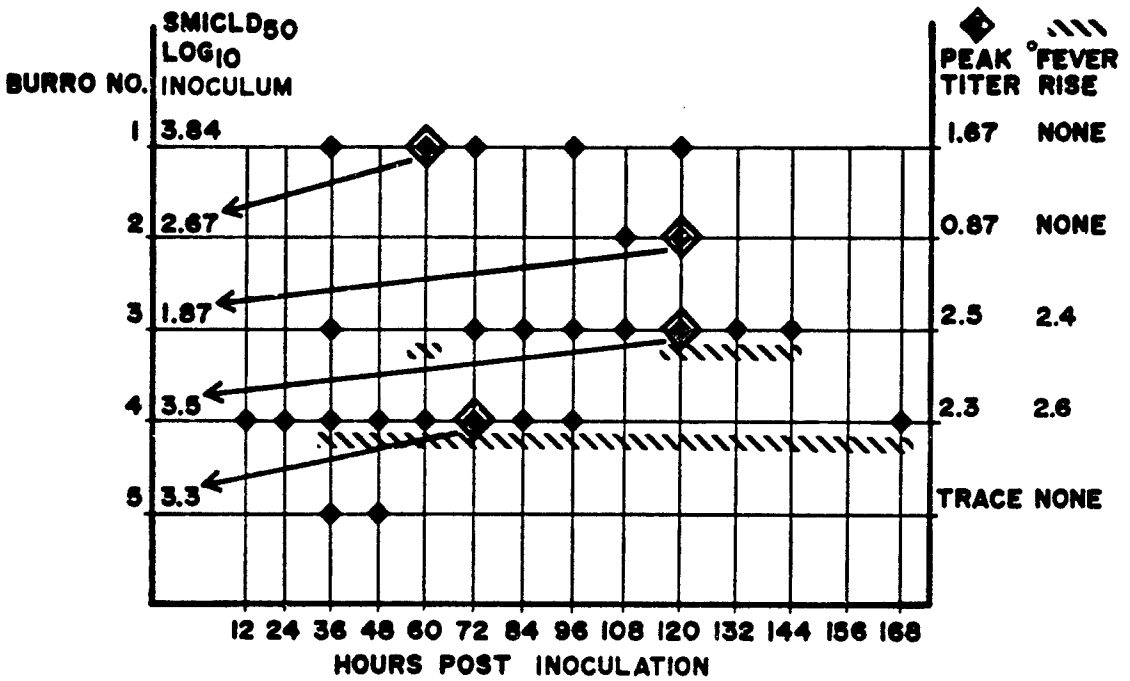

FIGURE 5. SERIAL PASSAGE OF 10 ml OF BLOOD FROM TC-83 INOCULATED BURROS.

The vaccinated horses remained healthy, while 3 of 5 unbroken geldings on the same pasture died and one was sick at the time of investigation. In Pasture A, all 16 colts and 11 of 16 mares died; the other 5 mares were noticeably encephalitic. Similar results were seen for Pasture B and C.

The remaining 15 horses not obviously sick were vaccinated on 31 August. Additional follow-up information on this herd is presented in Table I.

TABLE I. OBSERVATIONS ON ONE RANCH (Case 292)

	NUMBER	NO. SICK	NO. DEAD	VACCINATED
Pasture A	16 mares	5	11	No
	16 colts	0	16	No
Pasture B	11 mares	1	6	No
	10 colts	5	3	No
Pasture C	7 yearlings	6	1	No
	1 gelding	1	0	No
Pasture D	35 "working"	0	0	20 July
	3 stallions	0	0	20 July
	5 geldings	1	3	No

This striking protection with 1-dose immunization is consistent with the high degree of serologic conversion observed in field use of this vaccine. Of 157 paired serum samples collected during the Mexican vaccination campaign in 1970, all pairs with preimmunization titers of $< 1:10$, 150 (96%) had HI titers $\geq 1:20$ within 30-45 days of vaccination. With this abundance of field and laboratory information, and the pressure of necessity, a provisional license for commercial production and sale of live attenuated VEE vaccine was granted to the Jensen-Salisbury Subsidiary of Richardson-Merrill in July, 1971.

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ANALYSIS OF SEQUENTIAL HUMORAL ANTIBODY RESPONSES

Mary H. Wilkie, M.S.*

These studies are part of a program to develop an animal reference model system for evaluating the efficacy of experimental vaccines and to define parameters that, by extrapolation, could be employed to predict immune responses in man.

The rabbit was the most suitable laboratory species in which a model analytical system could be established. Other than in man, the immune response of the rabbit has been the most intensively characterized.[1] While immunoglobulin class relationships are simpler in the rabbit, the kinetics of synthesis and structure of the major classes of both species have been shown to be similar. Man varies immunologically from the rabbit primarily in degree of responsiveness, antigen recognition, and certain metabolic rates.

It is hypothesized that antigen management by man could be predicted by determination of the rates of antigen processing in the rabbit,[2] as reflected in the rates of synthesis of IgM and IgG. Dosages in man could be estimated from the quantities of antigen required to produce sufficient IgG in the rabbit to institute effective memory.

To establish a set of standard curves for the kinetics of responses of rabbits to simple protein antigens, bovine serum albumin (BSA) was chosen as reference antigen. Two doses of 25 mg each of BSA, with or without Freund's adjuvant, were employed to obtain a strong primary response and IgG production. Blood samples were obtained weekly for up to 4 mon and periodically thereafter through 12 mon. Each fresh specimen was chromatographed on Sephadex G-200. Antibody activity in all fractions was determined with the ^{131}I-BSA binding technique of Farr.[3] Total IgM activity of each serum sample was calculated as the sum of BSA bound in the first peak; total IgG was calculated as the sum of BSA bound in the second peak of the chromatogram.

Ten to 12 mon after the primary stimulation, all animals were injected with 10 mg BSA to evaluate memory responses. Bleedings were obtained at 4-day intervals. The sera were chromatographed and IgM and IgG activities were calculated as in the primary series.

IgA activity was not determined, since its activity in the rabbit is too low to affect IgM and IgG values during early responses.

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A summary of the results can be seen in Figure 1. On the left are 2 kinds (A and B curves) of typical primary responses. In the early phase, the curves of antibody activity of the group immunized with adjuvant (B) are the same as those of the group immunized without adjuvant (A). IgM activity reached a maximum value by day 7; IgG activity began simultaneously with IgM and increased rapidly by day 14. In those animals immunized with adjuvant, levels of IgG were sustained for months. In the group without adjuvant, IgG synthesis appeared to cease abruptly about day 14; activity decreased at a rate approximately equivalent to a 7-day half-life.

The figures on the right show secondary responses to the small dose of BSA administered at 10-12 mon. The upper graph, secondary A, represents the response of animals immunized with BSA alone. The booster dose resulted not only in a rapid and sustained synthesis of anamnestic IgG, but also in delayed synthesis of IgM at about day 14. Late appearance of IgM suggested that a new primary cycle was initiated following the typical anamnestic response. The dashed lines represent projections for IgG postulated to follow the primary IgM response. The response of animals immunized with BSA and adjuvant in the primary series, B, are shown below. Apparently, the booster dose exceeded memory capability; excess antigen initiated a new primary response, indicated by the simultaneous appearance of IgM with anamnestic IgG by day 7.

A unique opportunity to evaluate applicability of our hypotheses arose when a killed commercial vaccine to Eastern equine encephalitis (EEE) was administered to volunteers. Since this arbovirus produces complement-fixing, precipitating antibodies at a rate comparable to that of well characterized proteins, it appeared to belong to the same class of antigens as the model BSA. A feasibility study was initiated in which procedures described for the BSA system were employed for parallel studies in humans and rabbits. Antibody activity was determined by hemagglutination inhibition (HI), complement fixation, and neutralization tests.

Although the study is not yet complete and <u>in vitro</u> testing of samples has been delayed because of a lack of diagnostic antigen, and in spite of its fragmentary nature, the data are being presented. The problems that have arisen emphasize the value of analysis in reference models and have already resulted in a revised design of a rabbit analytical program for killed EEE vaccine.

One group of 8 volunteers was given two 0.5-ml doses of the EEE vaccine 28 days apart, a second group, two 0.25-ml doses at the same interval. Preimmunization and postimmunization samples of sera were collected weekly through day 56 and on day 90.

Dosage titrations or schedule variation in the rabbits were not attempted in the feasibility experiment. Instead, we desired to elicit a strongly positive response and study its characteristics. Four rabbits were given two 0.5-ml doses of EEE vaccine, 7 days apart, a schedule optimal for the rabbit and comparable to the BSA program.

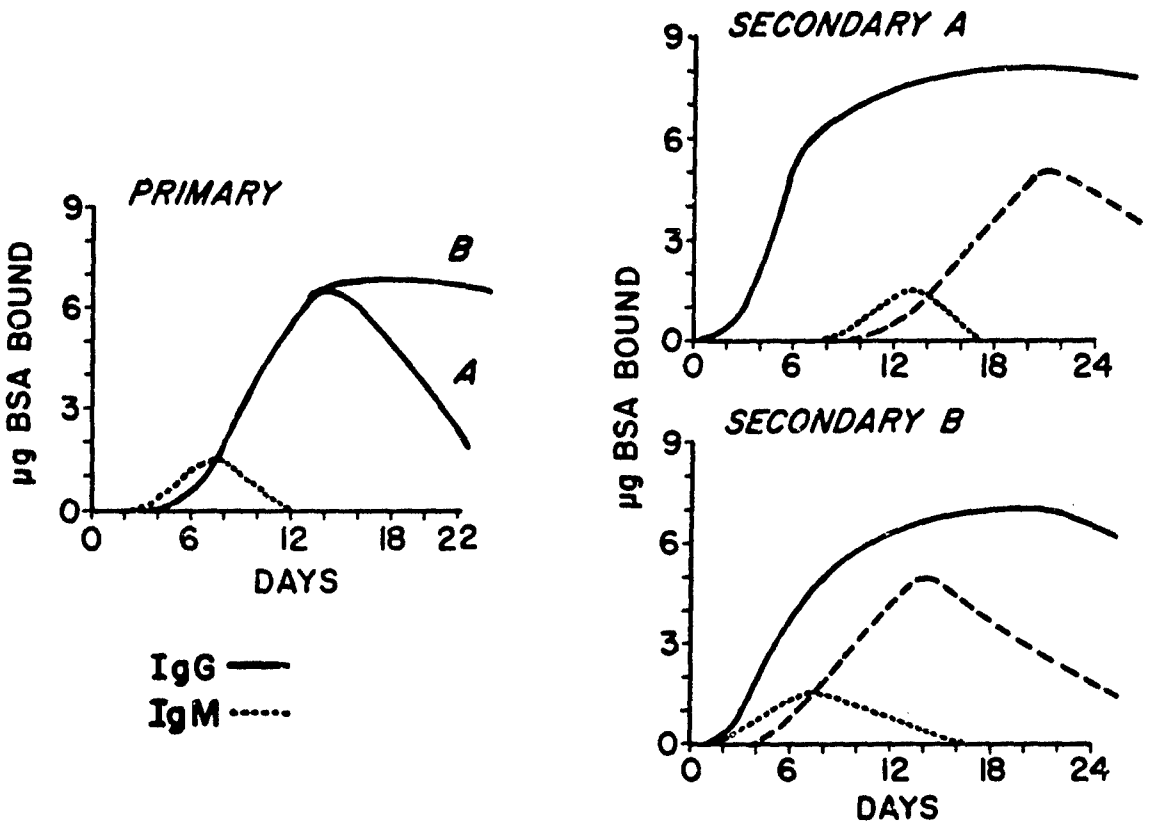

FIGURE 1. RABBIT ANTIBODY RESPONSES TO BSA IMMUNIZATION.

Each fresh, untreated serum was chromatographed on Sephadex G-200. Figure 2 shows a chromatogram of a representative preimmunization serum of a rabbit (OD). The EEE HI titrations for fractions of this control serum are shown. The activity in the 1st peak is that of β-lipoprotein, a nonspecific inhibitor of viral hemagglutination. Herein lies a major technical problem: standard kaolin absorption of inhibitor from the serum prior to antibody titration presented difficulties. It may or may not remove all the inhibitor, partially removes antibodies of both classes from whole serum, and removes all activity from the dilute chromatographic fractions. We cannot determine IgM values until the problem is resolved; some polyanionic methods of lipoprotein precipitation appear promising.[4]

Fortunately, nonspecific activity has not been detected in the 2nd peak from any normal rabbit or normal human sera. The EEE HI activity of the 7-day postimmunization serum from the same rabbit is shown as AFTER EEE. The activity in the 1st peak probably contains IgM masked by lipoprotein inhibitor. However, activity in the 2nd peak is typical of that in all 7-day rabbit sera. Activity in the 2nd peak was considered to represent IgM antibody because it varied quantitatively with the stage of immunization in the same manner as specific IgG against other antigens.

Standard HI titrations, employing whole serum absorbed with kaolin, were performed with all rabbit sera. Since the titers varied little, mean values are shown in Figure 3. Activity of whole serum increased rapidly for 14 days and then decreased. Through 42 days, the pattern of response to EEE vaccine was the same as that seen with BSA; consequently, it was assumed that the vaccine had been degraded and processed at approximately the same rate as BSA. However, with no known stimulation, a late, unexpected rise in titer occurred in all rabbits by day 140.

All titrations in the IgG area of each chromatographed serum were combined into a value for the total number of HI units/ml of serum; these more comprehensive values are shown as a dashed line. IgG activity calculated from chromatograms confirmed the pattern observed for titrations of whole sera, including the late, unstimulated rise.

Kaolin-treated samples of whole human sera were titrated for HI activity in the same manner. Figure 4 presents plots of the titrations of selected individuals. On the left are the HI responses of 3 men in the high dose group, Group I. One man apparently had had prior experience; he is included to show an exaggeration of the trends shown in the others. Following each injection, the rate of rise appears typically anamnestic; however, the rate of decline parallels the rate in the rabbit rather than that expected from the 21-day half-life of human γ-globulin. Also, as observed in rabbits, an unstimulated increase in activity occurred at 90 days. The 2 inexperienced individuals yielded lower values but showed the same effect. By titrations of chromatographed fractions of 56- and 90-day sera from 2 individuals, we have confirmed that these delayed responses involved increases in IgG. Three individuals, Group II, who received the lower dose responded similarly, as shown in the top right graph. Mouse neutralization indices of $\geq$ 1.7 logs were obtained with all 6 sera.

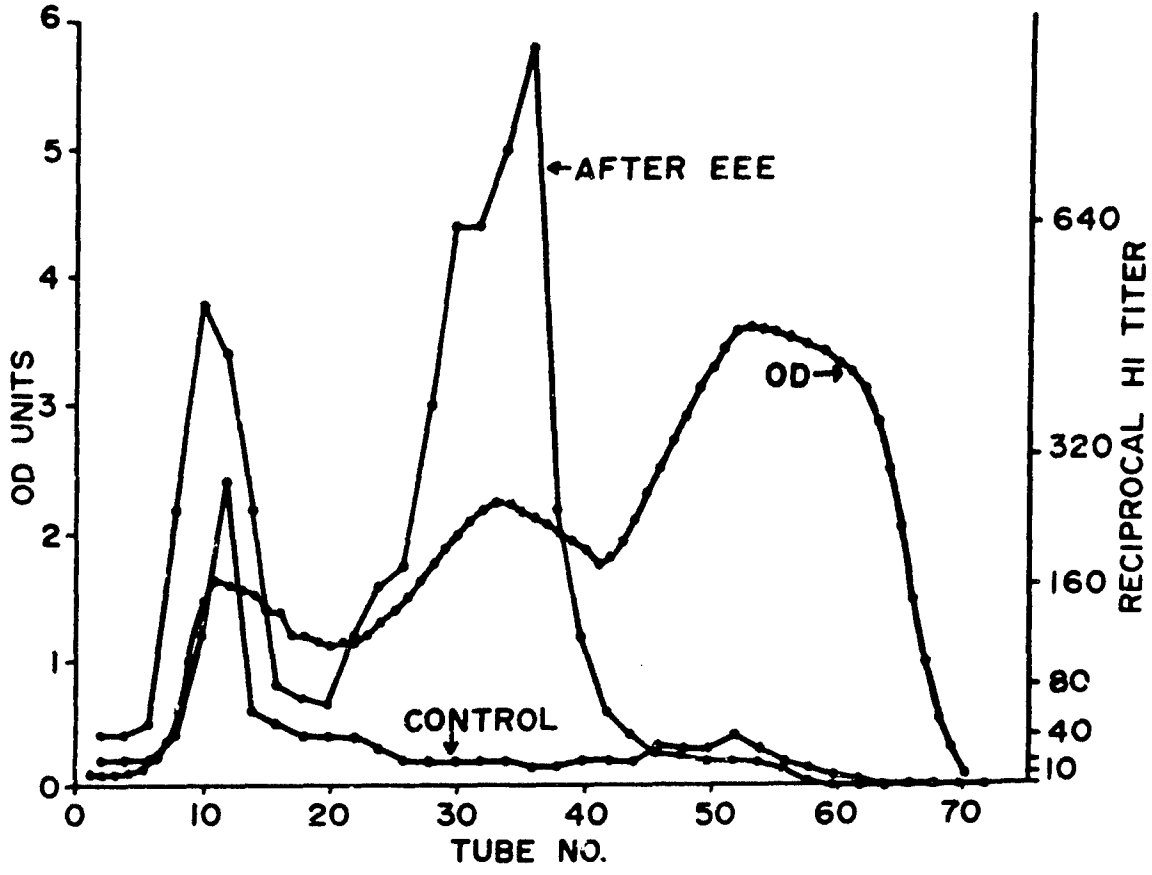

FIGURE 2. CHROMATOGRAPHIC SEPARATION OF NORMAL RABBIT SERUM AND HI TITERS BEFORE AND AFTER EEE VACCINE ADMINISTRATION.

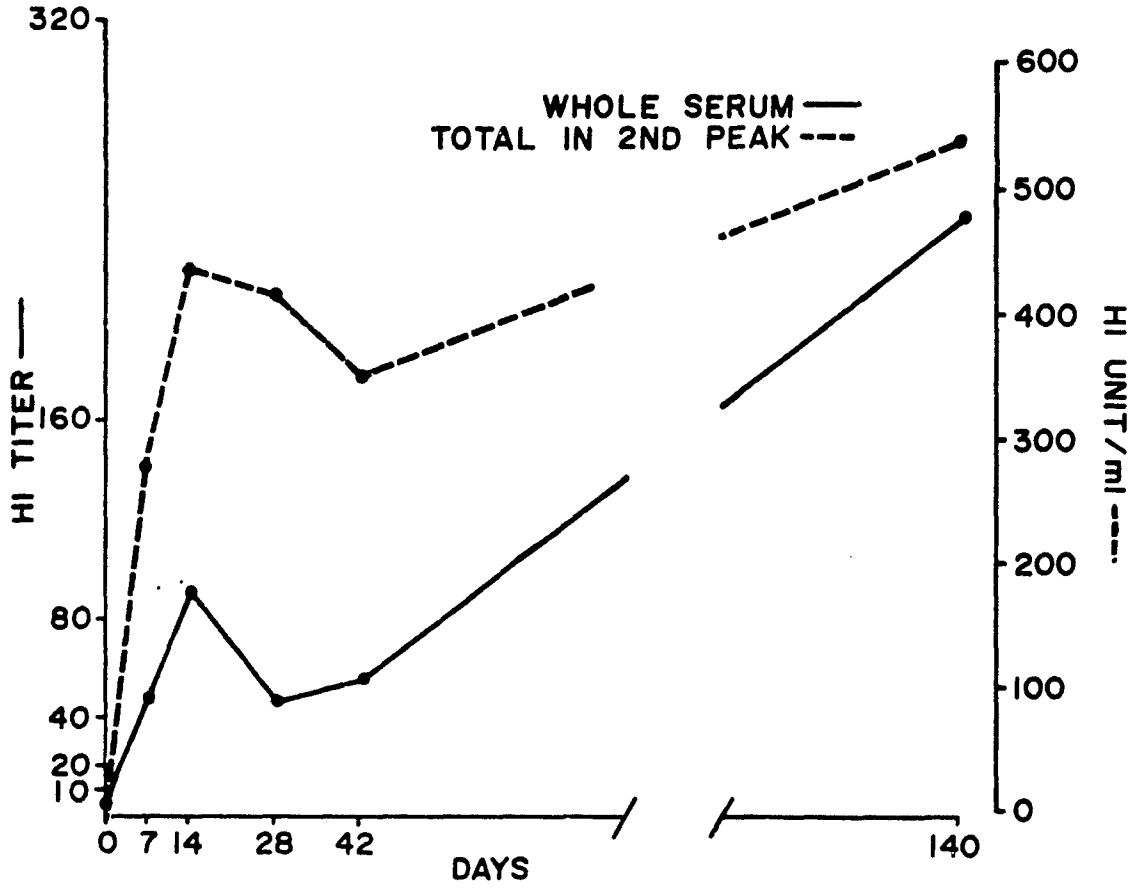

FIGURE 3. EEE HI TITER OF WHOLE RABBIT ANTISERUM AND HI UNITS IN THE SECOND CHROMATOGRAPHIC PEAK.

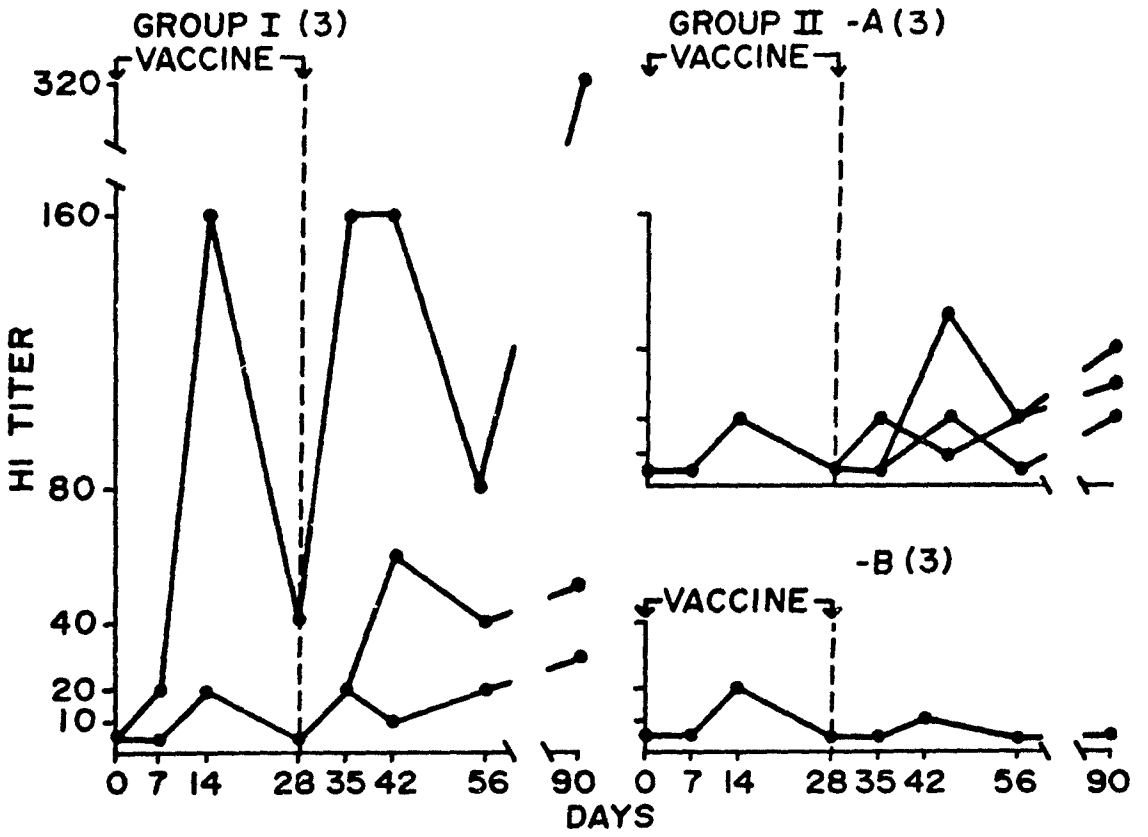

FIGURE 4. EFFECT OF 2 DOSES OF EEE VACCINE ON HUMAN HI TITER. (GROUP I: 0.05ml; GROUP II 0.025ml).

We cannot explain this late increase in IgG in either human or rabbit sera. Among possibilities to be considered are: (1) technical error, (2) the presence of synthesizing but not replicating viral RNA, (3) other poorly degraded viral hemagglutinins, and (4) a change in the affinity of IgG.

On the lower right, are titrations of sera from 3 individuals in the low dose group who showed no evidence of secondary response. Chromatographic analyses have confirmed that 2 of these 3 never produced detectable IgG activity; also, they had low neutralization indices which disappeared during the secondary period. Responses of the 3 men were identical to responses that we observed in rabbits administered low doses of BSA intravenously. The anamnestic response was suppressed by a small primary dose, demonstrating a form of low-dose tolerance[5] in rabbits.

In these studies, responses in humans to the commercially prepared vaccine were very low, compared to previous experience with lots prepared in the laboratory. While we realize fully that the program is incomplete, the data indicate that detailed observation of the immune response beyond 28 days should be included in all vaccine evaluation programs.

Of great practical importance in vaccine evaluation in man is the observation that suppression or tolerance may be induced by primary stimulation with low doses of antigens. It will be necessary to determine whether tolerance or genetic unresponsiveness is involved. If, in fact, low-dose tolerance can be induced in man, it is clear that safe low-dose limits as well as high-dose limits must be established for any vaccine. In addition, risks of tolerance may indicate that studies of means of safely terminating tolerance in man should be initiated. For such studies, our animal reference model system could be invaluable.

In summary, standard curves of kinetics of the early immune responses of rabbits to a standard protein antigen have been established. The importance of the IgM response as an indicator of processing of free antigen during any stage of the immunization sequence has been highlighted. In addition, preliminary results of the evaluation of EEE vaccine in volunteers have been reported; they appear to validate the hypotheses regarding use of a rabbit reference model system.

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ROLE OF ANTIBODY AND CELL-MEDIATED IMMUNITY IN PROTECTION AGAINST VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS INFECTION IN MICE

Stanley G. Rabinowitz, Major, MC*

Although both neutralizing antibody and interferon have been shown to possess definite antiviral activity, only recently has attention shifted to studies concerned with delineating a role for cell-mediated immunity (CMI) in protection against viral infection.[1,2]

The present study was designed to provide information on the relative contribution of humoral antibody and CMI in protection against experimental infection of mice with Venezuelan equine encephalomyelitis (VEE) virus.

White, CD-1 or C57 BL/6 strains of mice utilized to investigate humoral immunity had undergone neonatal thymectomy or been treated with antithymocyte serum (ATS) to eliminate or reduce their capacity to mount a CMI response. Subcutaneous injection with Trinidad strain of VEE indicated that the median lethal dose (LD_{50}) was the same for both mouse strains. The only difference in response was that time from onset of symptoms to death was shorter in the white mice.

To investigate the protective efficacy of immune serum, neonatally thymectomized CD-1 mice were employed. One group was inoculated subcutaneously with 30 LD_{50} of Trinidad strain VEE. A second group was similarly inoculated, but was also given simultaneously normal guinea pig sera intraperitoneally (IP). A third group was inoculated in the same manner, but treated with immune guinea pig serum. Subgroups were reserved for antibody determinations using the hemagglutination inhibition (HI) test. A comparison of mortality rates is presented in Table I. The protection afforded by immune sera in neonatally thymectomized mice is evident.

To ensure that these mice were thymectomized, serial neck sections of all animals were prepared for histologic examination at the time of death or upon completion of the study. No macroscopic or microscopic evidence of thymus tissue could be found.

As further confirmation of the efficacy of immune sera, C57 mice were pretreated with a commercial rabbit anti-mouse thymocyte serum of certified potency (Microbiological Associates, Bethesda, Md., Lot 13020). Evaluation indicated that the experimental dosage employed effected a more than 50% reduction in total white blood cells, predominantly lymphocytes, within 24 hr. No other adverse effects were observed.

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TABLE I. COMPARISON OF MORTALITY RATES FOR NEONATALLY THYMECTOMIZED (TX) CD-1 MICE FOLLOWING INFECTION WITH 30 LD_{50} VIRULENT VEE VIRUS AND SIMULTANEOUS ADMINISTRATION OF NORMAL OR IMMUNE SERUM

NEONATAL TX + VEE	DEAD/10	MORTALITY RATE %
Control	10	100
Normal sera, 0.25 ml IP	10	100
Immune sera, 0.25 ml IP, (HI = 1:640)	0	0

Table II shows survival of mice pretreated with 2 injections of ATS, 48 hr apart, and inoculated subcutaneously with 30 LD_{50} virulent VEE virus with or without simultaneous administration of immune sera. Again, presumably in the presence of a depressed ability to mount a CMI response, immune sera afforded complete protection. Also of note is the fact that mice pretreated with ATS and then infected with VEE virus developed signs of illness and died at a time entirely analogous to mice infected but without pretreatment. HI antibody titers following infection of pretreated mice did not differ significantly from those of untreated animals. Hence, in this study ATS had no appreciable effect on endogenous antibody production in VEE infection.

TABLE II. COMPARISON OF MORTALITY RATES FOR MICE PRETREATED WITH 2 DOSES OF ATS[a] AND INFECTED WITH 30 LD_{50} VIRULENT VEE VIRUS WITH OR WITHOUT SIMULTANEOUS ADMINISTRATION OF IMMUNE SERUM

ATS + VEE	DEAD/TOTAL	MORTALITY RATE %
Control	9/9	100
Normal serum, 0.25 ml IP	10/10	100
Immune serum, 0.25 ml IP (HI = 1:640)	0/8	0

a. 2 injections of 0.25 ml ATS IP given 51 hr and 3 hr before injection.

We next investigated protection afforded by passive transfer of spleen cells from immunized animals.

In these experiments, 6-10 week old male C57 BL/6 mice were used exclusively. Infection was produced by subcutaneous inoculation of 30 LD_{50} VEE virus. Spleens were harvested from mice immunized 7 days previously with

3×10^3 PFU of live, attenuated, TC-83 strain, VEE virus. Washed spleen cell suspensions contained $1\text{-}2 \times 10^8$ cells/ml, 95% of which were mononuclear. Cell viability was between 70-85% as determined by trypan blue exclusion. Each recipient was injected IP with $5.5 - 9.0 \times 10^7$ cells.

Table III summarizes results from 3 experiments in which immune sera or immune spleen cells were used in passive transfer experiments. Both immune cells and immune sera afforded protection against death, but also suppressed clinical signs of illness.

TABLE III. EFFECT OF INTACT IMMUNE AND NORMAL SPLEEN CELLS AND NORMAL AND IMMUNE SERA ON MORTALITY IN MICE INFECTED WITH 30 LD_{50} VIRULENT VEE VIRUS AT TIME OF ADOPTIVE IMMUNIZATION

TREATMENT	DEAD/TOTAL	MORTALITY %
Immune spleen cells[a/]	2/15	13
Normal spleen cells[b/]	15/15	100
Normal sera, 0.25 ml IP	14/15	93
Immune sera, 0.25 ml IP (HI= 1:640)	1/16	6
Control	13/13	100

a. $5\text{-}9 \times 10^7$ spleen cells/recipient IP in volume of 0.5 ml.
b. $4\text{-}7 \times 10^7$ spleen cells/recipient IP in volume of 0.5 ml.

To ensure that cell transfer was not serving as a vehicle for passive transfer of antibody, the supernate from the last washing was used as another modality of therapy. No protection was conferred by the supernate, 100% succumbing to subcutaneous inoculation with 30 LD_{50} Trinidad strain (Table IV). In addition, when an aliquot of this material was titrated for HI activity, no antibody was detected.

Another group of mice were injected with nonviable, immune spleen cells that had been disrupted by exposure to ultrasound for 30 sec. The response of this group of mice to infection with VEE was identical to that of the controls. It appeared then that intact, viable cells were necessary for protection.

We next investigated the possibility that immune spleen cells were mediating protection via elaboration of interferon. Twelve mice were injected IP with 100 µg Poly I:C a potent inducer of interferon,[3] at the time of virus administration. As can be seen in Table V, 11 of 12 mice died when treated

TABLE IV. EFFECT OF DISRUPTED IMMUNE SPLEEN CELLS AND SUPERNATANT OF WASHED IMMUNE SPLEEN CELLS ON MORTALITY IN MICE INJECTED WITH 30 LD_{50} VIRULENT VEE VIRUS AT TIME OF ADOPTIVE IMMUNIZATION

TREATMENT	DEAD/10	MORTALITY %
Supernatant-immune cells[a/]	10	100
Disrupted immune cells[b/]	10	100

a. $4.5\text{-}7.5 \times 10^7$ spleen cells IP in 0.5 ml.
b. 0.5 ml IP.

TABLE V. EFFECT OF POLY I:C AND INTERFERON INJECTED IP ON MORTALITY OF MICE INFECTED WITH 30 LD_{50} VIRULENT VEE VIRUS

TREATMENT	DOSE	DEAD/TOTAL	MORTALITY %
Poly I:C	100 µg/0.2 ml	11/12	91
Interferon	2.5×10^3 units/0.25 ml	6/6	100

in this fashion. In addition, sera obtained 6 hr after treatment of mice with 100 µg Poly I:C had no protective activity although it contained 10^4 units interferon/ml. These experiments suggest that interferon is not involved in protection afforded by cell transfer.

Consideration was now given to the possibility that the recipients of transferred immune spleen cells were capable of reacting to the challenge virus by production of antibody. It might be possible that the recipients could be protected because the transferred cells mounted an anamnestic immune response. To test this hypothesis, we performed our cell transfer experiments in the usual fashion, but in addition to observing mice for signs of illness and death, we divided a recipient pool of 24 mice into subgroups for determination of HI antibody responses at 2, 4, 6 and 8 days after infection with VEE virus (Table VI). No differences in HI antibody response or the complement fixation (CF) reaction were noted in recipients of either immune or normal cells.

These experiments suggested that immune spleen cell recipients were not afforded protection by the early elaboration of antibody, at least as judged by HI and CF reponses.

TABLE VI. ANTIBODY RESPONSES OF MICE (4/GROUP) GIVEN IMMUNE OR NORMAL CELLS AND SIMULTANEOUSLY INFECTED WITH 30 LD_{50} VIRULENT VEE VIRUS SQ

TREATMENT	RECIPROCAL HI TITER BY DAY				RECIPROCAL CF TITER BY DAY			
	2	4	6	8	2	4	6	8
Control	ND[a]	<10	ND	80	neg	neg	64	128
Normal cells[b]	20	20	2560	5120	neg	neg	32	128
Immune cells[c]	20	20	1280	5120	4	4	32	128

a. Not done.
b. 5-6 x 10^7 cells per recipient in volume of 0.5 ml IP.
c. 6-8 x 10^7 cells per recipient in volume of 0.5 ml IP.

Finally, in conjunction with Dr. William Adler (Pathology Division), the in vitro lymphocyte stimulation test was employed to investigate whether immune spleen cell preparations were sensitized to VEE.

Lymphocyte cultures prepared from spleens harvested as described above, were incubated with a preparation of the Trinidad strain that had been inactivated by exposure to γ-radiation. Cultures were incubated for 48 hr, with tritiated-thymidine being added for the last 16 hr. Radioactivity of trichloroacetic acid precipitable material was measured in a liquid scintillation counter.

Figure 1 depicts results with spleen cells harvested 4, 7 and 13 days after immunization with TC-83 strain virus. Maximum lymphocyte stimulation was observed with 7-day spleen cells; 4-day preparations showed approximately one-half as much stimulation and day 13 cells, no stimulation. In Figure 2, the ratio of peak CPM of thymidine incorporation by immune cell preparations to the peak CMP of control preparations is plotted. The very obvious stimulation with 7-day spleen cells is apparent.

When immune spleen cells, harvested at 4 and 13 days, were transferred to recipients, the 4-day preparations provided some protection against VEE infection, 4 of 10 mice survived; 13-day preparations conferred no protection, 0 of 10 survived. Hence, the responsiveness of immune spleen cells to in vitro stimulation with specific antigen served as a good index of their ability to confer protection in vivo.

This series of studies has demonstrated that humoral antibody, in the absence of the cellular components of the immune response, can play a major role against VEE infection. Likewise, immune spleen cells can confer protection. It has been demonstrated that the protective activity of immune

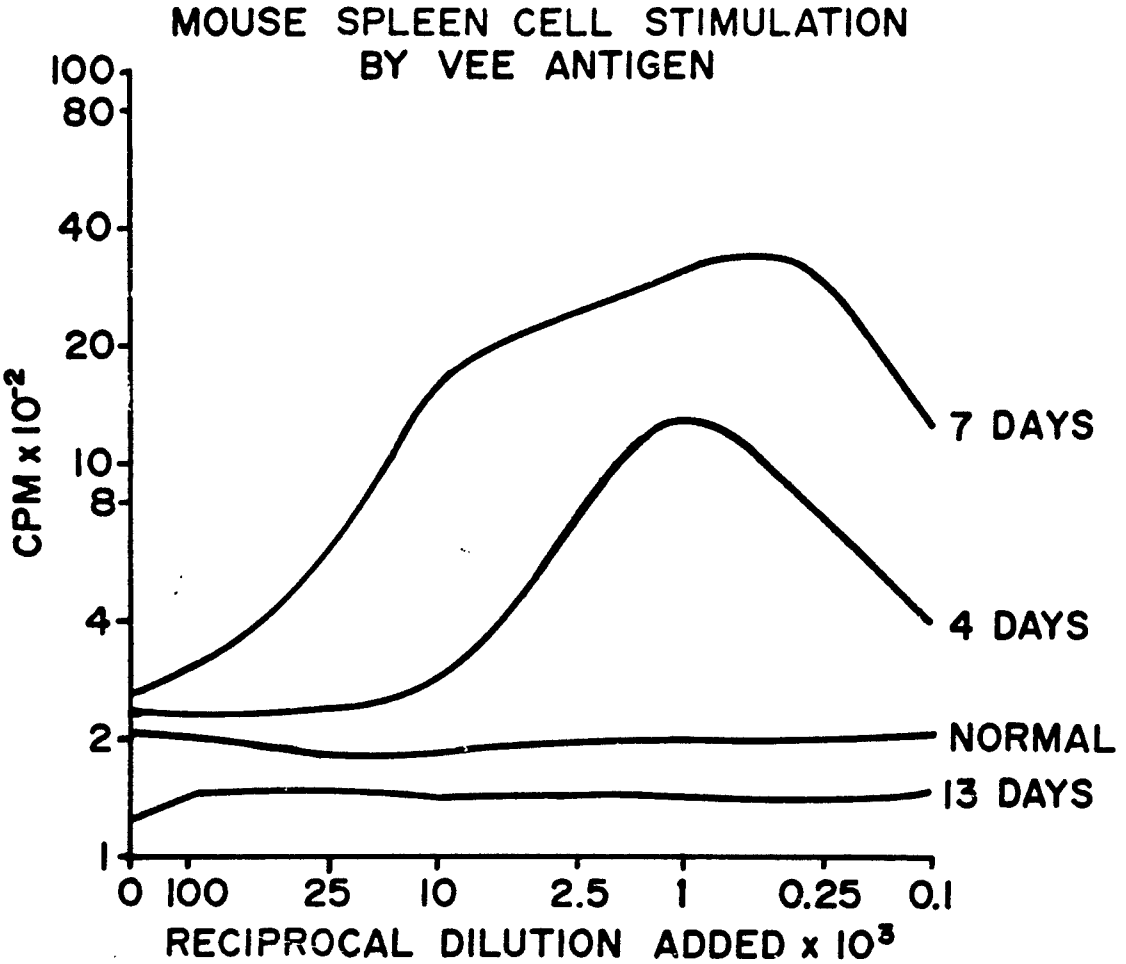

FIGURE I. MOUSE SPLEEN STIMULATION BY VEE ANTIGEN AT VARIOUS DILUTIONS RELATIVE TO DILUTION AND TIME.

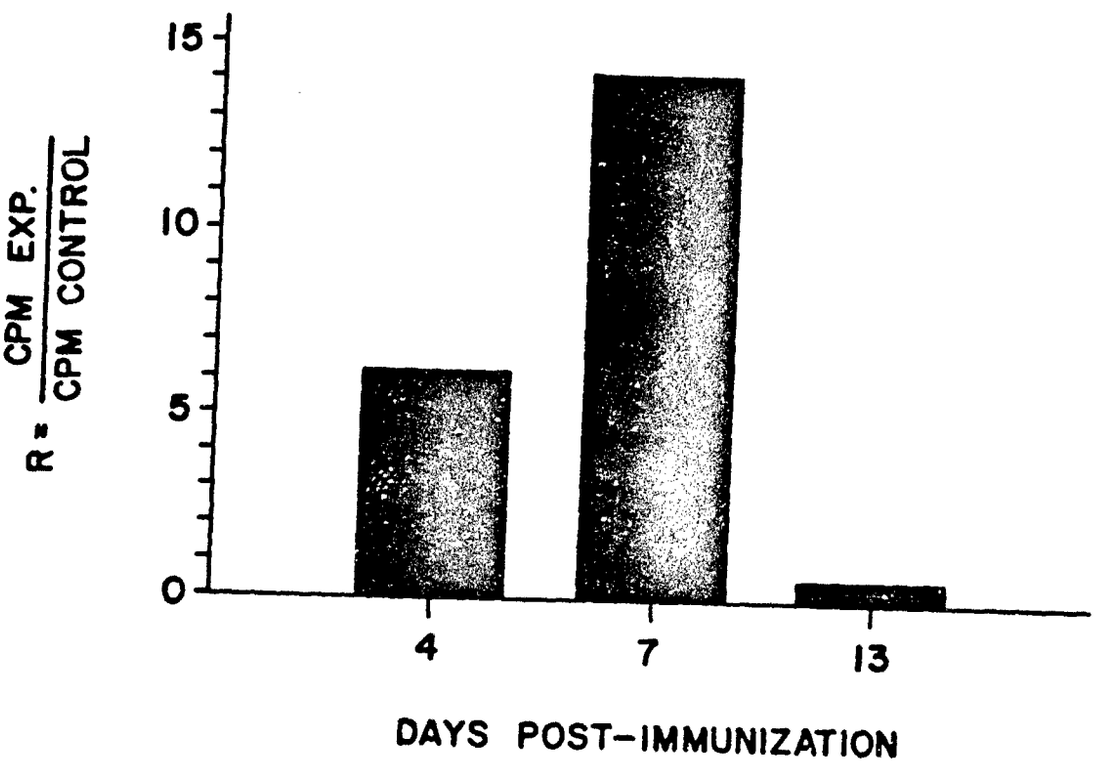

FIGURE 2. PEAK MOUSE SPLEEN CELL STIMULATION BY VEE ANTIGEN AS MEASURED BY THYMIDINE INCORPORATION.

cell preparations cannot be ascribed to transfer of either preformed antibody or to induction of an anamnestic antibody response of donor cells in the recipient. There is no evidence that interferon contributes to passive transfer of immunity. The data suggest the possibility that in vivo stimulation of immune donor cells by infecting virus may initiate a chain of events leading to activation of recipient macrophages. The time after donor immunization is a critical factor in determining responsiveness to in vivo stimulation to transferred cells.

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ELECTRON SPIN RESONANCE STUDIES OF ANTIGEN-CELL MEMBRANE INTERACTION

Joseph Kaplan, MAJ, MC, and William J. Caspary, CPT, MSC *

Sensitized lymphocytes respond to antigen by undergoing a series of changes leading to cell division.[1] Normal lymphocytes undergo similar changes when cultured with such nonspecific stimulants as antilymphocyte sera, Concanavalin A and staphylococcal enterotoxin B.[2] In view of the considerable evidence that lymphocytes possess surface receptors for antigens, as well as for some nonspecific stimulants, it is likely that lymphocyte activation is triggered by the interaction of surface receptors with stimulant. Other nonspecific stimulants may trigger cells by inducing changes inside the cell further along in the hypothetical chain of events leading to nuclear activation. In the case of agents which interact with surface receptors, the actual triggering event could be a conformational change in the lymphocyte membrane. The hypothesis we intend to test is the following:

The trigger for lymphocyte activation is a conformational change in the surface membrane caused by interaction between stimulant and membrane-bound receptors. The method chosen, electron spin resonance spectroscopy (ESR), is uniquely suitable for studying membrane structure and conformational change.

ESR is a type of spectroscopy that measures changes in energy of an unpaired electron (as in a free radical) when the molecule containing the electron is placed in a magnetic field and is excited by microwave radiation. For technical reasons the first derivative of the absorption curve is recorded (Figure 1). This accounts for the strange appearance of the spectra shown.

Most molecules having unpaired electrons are unstable. However, McConnell and co-workers were able to synthesize a stable nitroxide molecule containing an unpaired electron.[3,4]

Since biological systems have relatively few free radicals, such nitroxides could be used as spin labels on molecules of biological interest without danger of interference. Most importantly, the spectra of these nitroxides reflect changes in their molecular environment and in their rate of tumbling in space. Thus, measurements can be made of local changes in polarity, viscosity and anisotropy (i.e., the preferred orientation of a molecule in space). It should be mentioned that the spectrometer has a sensitivity of 10^{-7} to 10^{-8} M in an aqueous environment.

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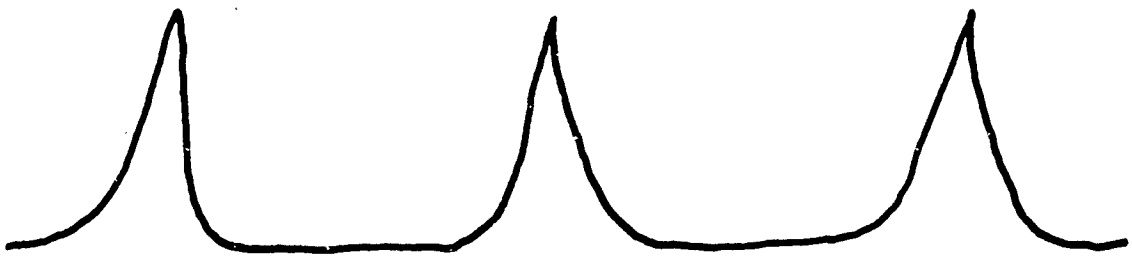
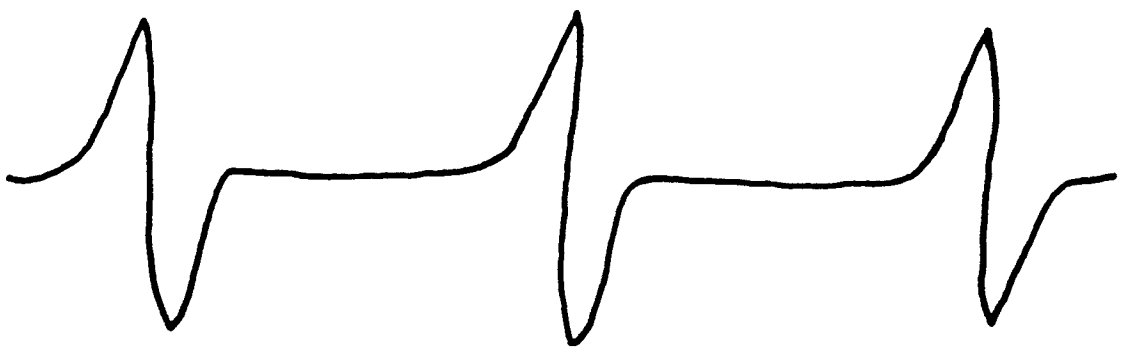


ABSORPTION PEAKS



RESULTING ESR SPECTRUM

FIGURE 1. ESR SPECTRUM DERIVED FROM AN ABSORPTION CURVE.

By placing nitroxides in a glycerol-water medium (which is both isotropic and polar) at room temperature, a 3-line spectrum is obtained (Figure 2). The 3 lines are the result of magnetic interaction between the unpaired electron and the nitrogen nucleus.

The diagram also shows the effect of cooling the solution. Nitroxide labels in the top spectrum at 25 C are tumbling at a rate of 2.5×10^9 times/sec, in the next spectrum at 0 C, at 5×10^7 times/sec and at -25 C at $< 10^7$ times/sec. Therefore, as the rate of tumbling of the molecule is varied, the spectra change drastically.

A number of spin labels, shown in Figure 3, have been found useful for studying the structural properties of membranes. The top 2 bind covalently to protein; the lower 2 labels are amphiphilic and lipid soluble and probably intercollate themselves between the hydrocarbon chains of phospholipids with the acid or hydroxy group anchored in a hydrophilic region of the membrane.

If these labels are incorporated into membranes, changes in the structure of the membrane would most likely be reflected by changes in the polarity and viscosity of the medium immediately surrounding the label.

Anisotropy, as measured by the angular deviation from the membrane surface, is another important parameter that can be measured and may reflect membrane conformational changes.

While we ultimately would like to be able to incorporate spin labels into the surface membranes of sensitive lymphocytes to determine if conformational changes occur with antigenic stimulation, a number of technical capabilities had to be acquired: (1) techniques of labeling a membrane had to be perfected; (2) determination of what specific labels would best reflect protein-membrane interactions; (3) development of methods to allow observations of surface membrane changes separate from those occurring intracellularly, and (4) it was important, to insure that membrane incorporation of the label did not interfere with biological function.

Initially, red cells were used to determine feasibility of labeling cell membranes and observing changes in membrane structure. The characteristic, weakly immobilized spectrum for stroma of sheep red blood cells labeled with an iodoacetamide spin label is shown in Figure 4. This label binds covalently to membrane proteins. Labeled stroma were prepared by incubation in a buffered solution of iodoacetamide label at room temperature for 3 hr, followed by extensive washing.

Table I shows the effect of heating on mobility, or rate of tumbling, of spin label in the membrane as measured by the Tau value. The smaller the value the more rapid the rate of tumbling of spin label. It can be seen that an irreversible change occurs in the membrane protein reflected by an irreversible increase in the rate of tumbling.

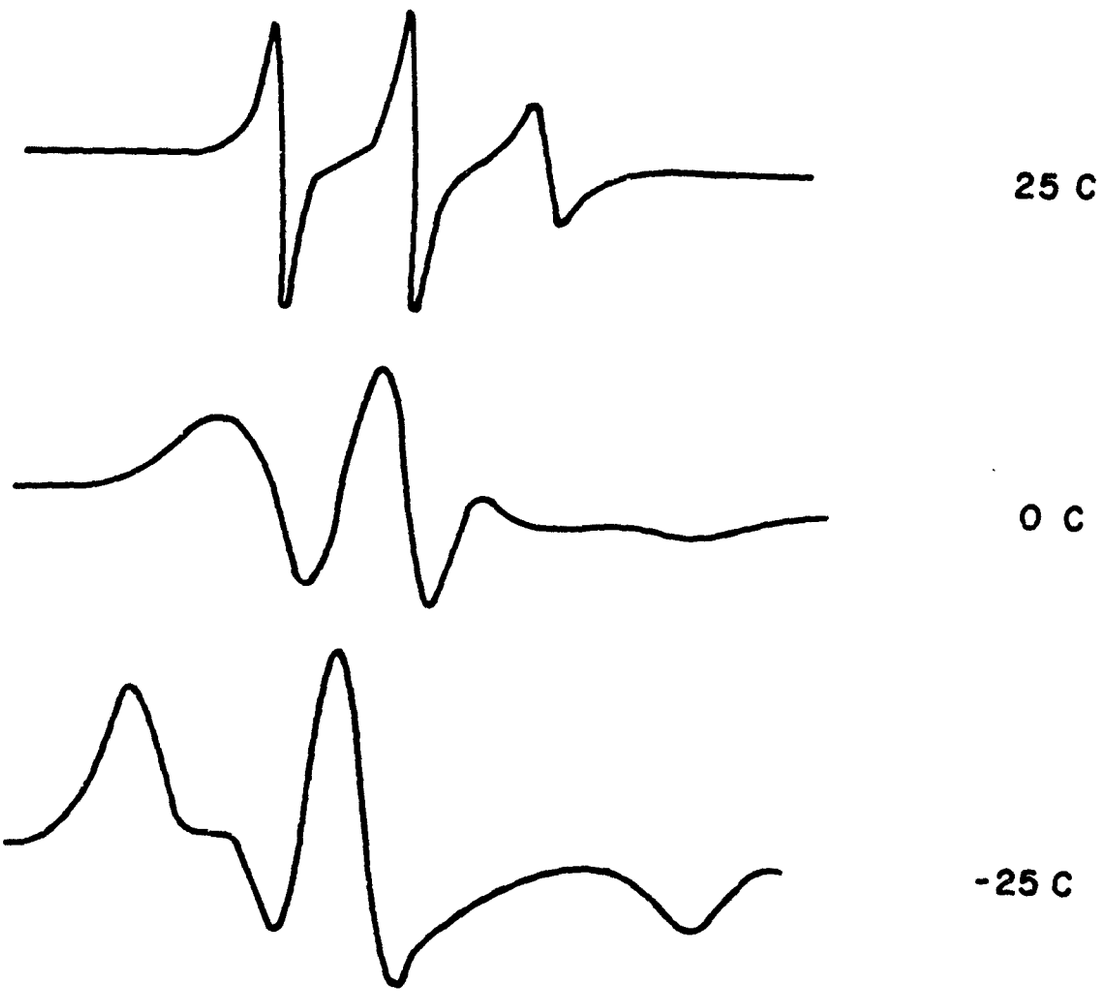
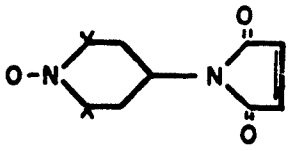
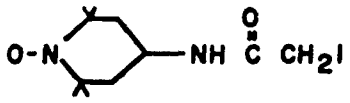


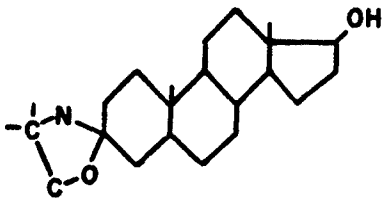
FIGURE 2. EFFECT OF TEMPERATURE ON ESR SPECTRA.



4-MALEINIDO - 2,2,6,6-
TETRAMETHYL PIPERIDINOXYL



IODOACETAMIDE



ANDROSTANE

$CH_3(CH_2)_m-C-(CH_2)_n\ COOH$

STEARIC ACID
m = 5, 12
n = 10, 3

FIGURE 3. EXAMPLES OF SPIN LABELS USEFUL IN MEMBRANE STUDIES.

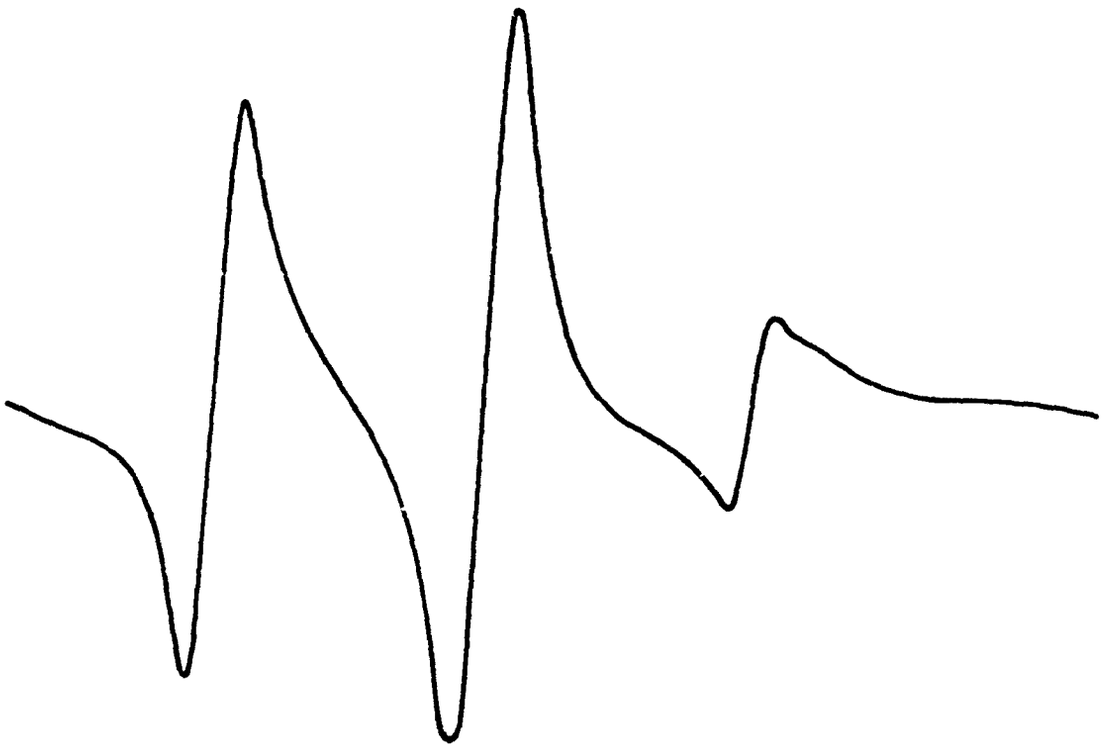


FIGURE 4. ESR SPECTRUM OF IODOACETAMIDE-LABELED SHEEP RBC STROMA.

TABLE I. EFFECT OF HEATING IODOACETAMIDE-SPIN LABELED SHEEP RBC STROMA

LABELED STROMA TREATMENT	TAU VALUE X 10^{-10}
Prior to heating	23.7
Heated at 60 C	2.9
Returned to room temperature	7.9
Reheated to 60 C	2.9
Returned to room temperature	7.4

Figure 5 shows the effect of 38% formalin on the spectrum of sheep red cell stroma labeled with an androstane spin label. Labeling was accomplished by incubating stroma with androstane complexed to bovine serum albumin (BSA). The androstane exchanges from the BSA to the membrane. Mobility of this spin label in the membrane is somewhat less than that of the iodoacetamide label. It can be seen that formaldehyde slowed the tumbling.

Figure 6 is the spectrum of intact sheep red cells labeled with androstane before and after addition of lidocane (a membrane active drug) to a suspension of labeled red cells. The change seen indicates a more rapid rate of tumbling of spin label in the membrane.

In other studies, no consistent changes in spectra were observed when sheep red cells or red cell stroma were labeled with iodoacetamide, stearic acid or androstane and then incubated with varying dilutions of rabbit antibody to sheep red blood cells. One possible explanation for the failure to see changes with antibody attachment is shown in Figure 7. This is a diagrammatic representation of a stearic acid spin label as it may exist in the membrane with the long hydrocarbon axis perpendicular to the cell surface. With the nitroxide label on the 12 carbon, the free electron resides in a fluid region of the membrane. It has been demonstrated that when the nitroxide resides on the 3 carbon at the hydrophilic end of the molecule, the label shows less freedom of motion and being closer to the protein components of the membrane may more readily reflect protein structural changes.[5] We plan, therefore, to use stearic acid labeled on the 3 carbon in future studies.

With these studies, we gained some familiarity with spin labeling techniques as well as information on the kinds of changes and the order of magnitude of changes we might expect to see when we examine lymphocyte membranes under different conditions.

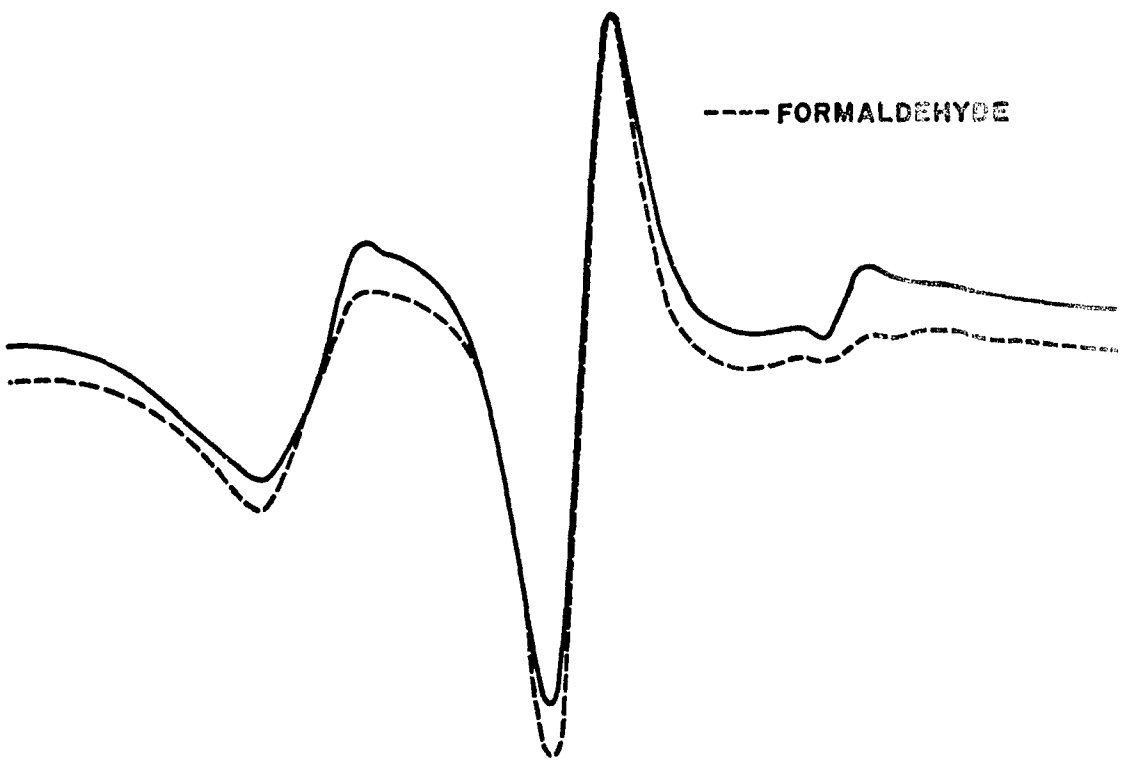


FIGURE 5. EFFECT OF FORMALDEHYDE ON ANDROSTANE-LABELED SHEEP RBC.

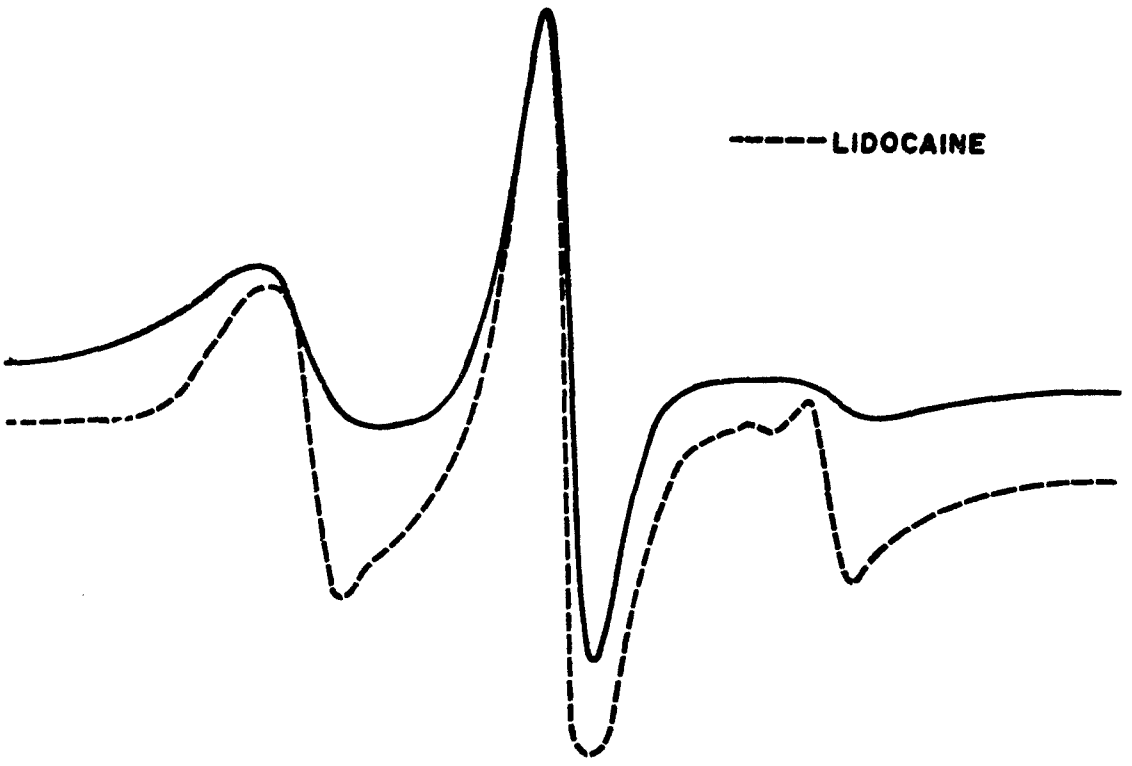


FIGURE 6. EFFECT OF LIDOCAINE ON ANDROSTANE-LABELED SHEEP RBC.

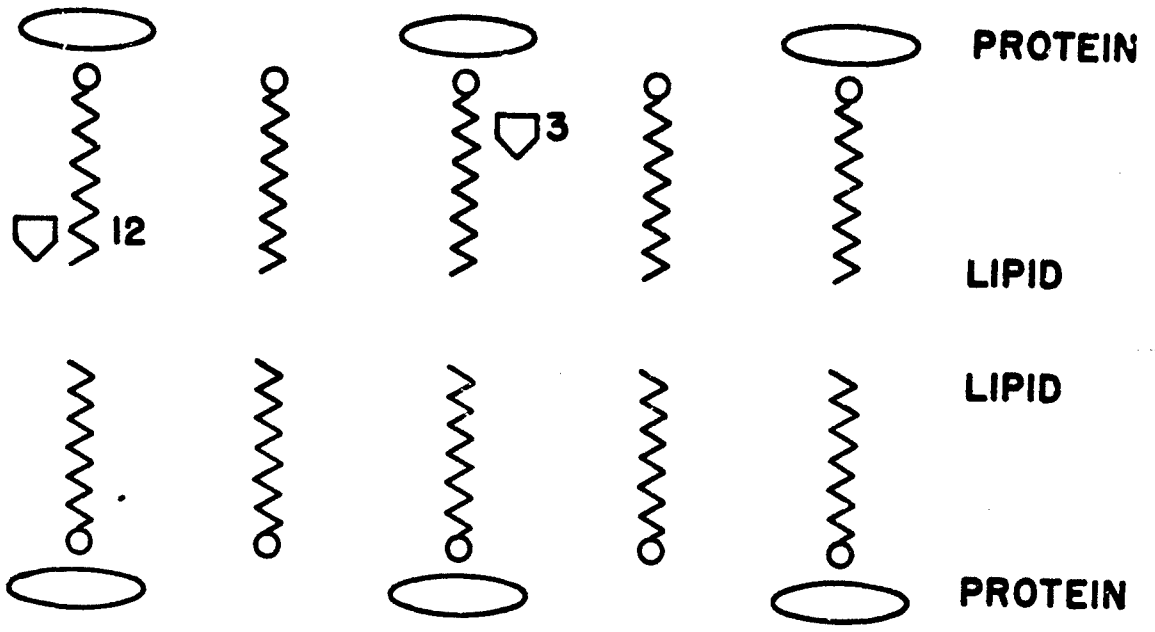


FIGURE 7. DIAGRAM OF STEARIC ACID SPIN LABEL, AS IT MAY EXIST IN A MEMBRANE.

We then attempted to spin label membranes of intact nucleated cells. Figure 8 shows the spectrum of intact L-cells cultured as a suspension for 18 hr in the presence of spin labeled stearic acid. To determine the subcellular distribution of spin label, labeled L-cells were differentially centrifuged; fractions obtained were: nuclei and cell membranes, heavy mitochondria, light mitochondria, microsomes and cell sap. A strong signal was found in the nuclei and membrane fraction as well as in both mitochondrial fractions. A weak signal was detected in the microsomal fraction, and none, in the cell sap.

Figure 9 shows the spectrum of human lymphocytes in long-term tissue culture labeled by incubation for 10 min at a cell concentration of 10^8/ml with BSA-complexed androstane spin label. The spectra of 3 subcellular fractions are also shown. These spectra show that mammalian cells in tissue culture take up the label and that the cell membranes are labeled along with other membrane-containing organelles.

To test the functional competence of spin labeled cells, guinea pig lymph node lymphocytes were spin labeled with BSA-complexed androstane label and then cultured in the presence of the nonspecific mitogen, staphylococcal enterotoxin B. Control, unlabeled lymphocytes were cultured in parallel; tritiated thymidine uptake was measured after 72 hr. It can be seen in Table II that the labeled lymphocytes respond to the same degree as unlabeled lymphocytes in the presence of mitogen.

TABLE II. SEB STIMULATION OF ANDROSTANE-LABELED GUINEA PIG LYMPHOCYTES (25 μg/ml)

CELLS	^{3}H-THYMIDINE cpm
Unlabeled + SEB	4253
Unlabeled + medium	965
Labeled + SEB	4655
Labeled + medium	1275

Technical problems awaiting solution are: (1) determining the conditions required to incorporate spin label into the outer cell membrane exclusively, and (2) obtaining pure preparations of antigen-sensitive cells. In view of the difficulty of obtaining a pure population of antigen-sensitive cells, a technically simpler but informative study would be to examine the changes that occur when labeled lymphocytes are exposed to such nonspecific stimulants as staphylococcal enterotoxin B, Concanavalin A and antilymphocyte sera.

While the technical problems may be great, we feel that the rewards in terms of the possibility of understanding lymphocyte membrane function are well worth the effort.

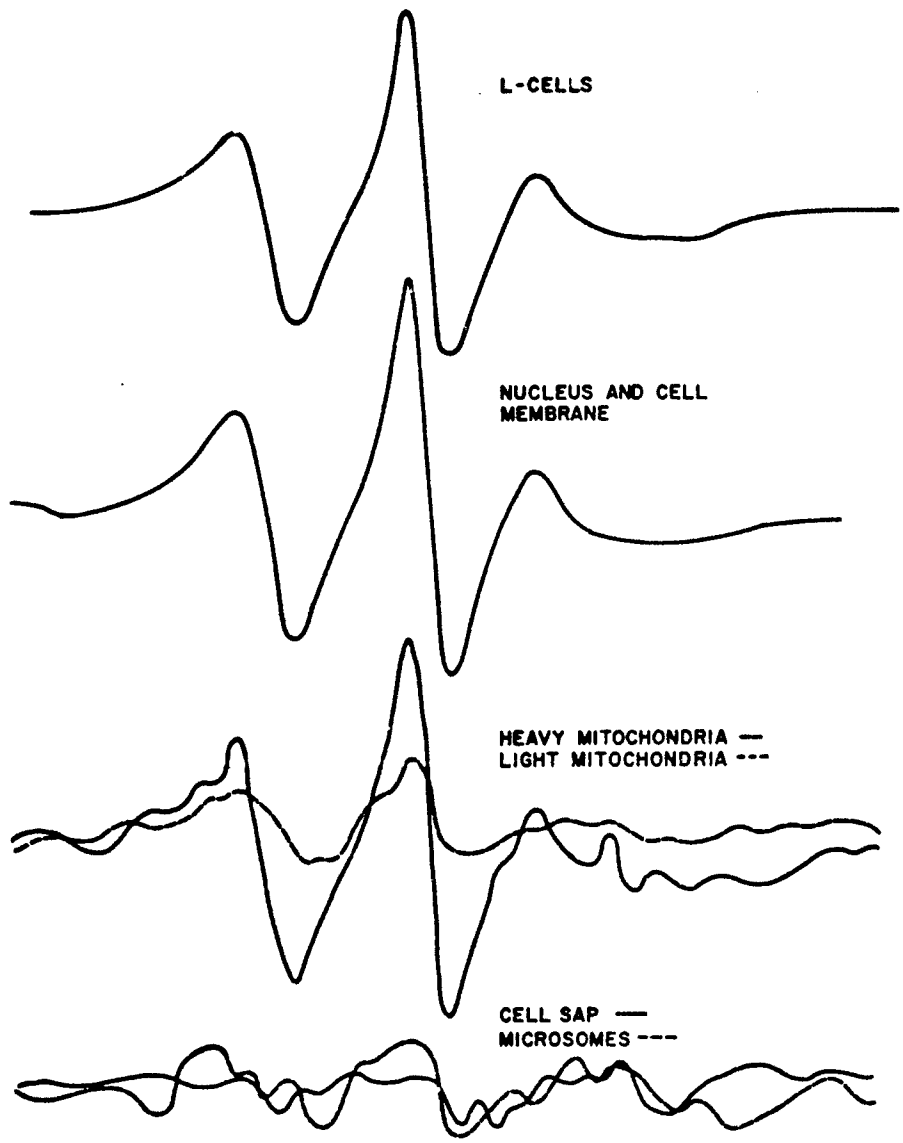


FIGURE 8. ESR SPECTRA OF L CELLS AND THEIR FRACTIONS LABELED WITH STEARIC ACID.

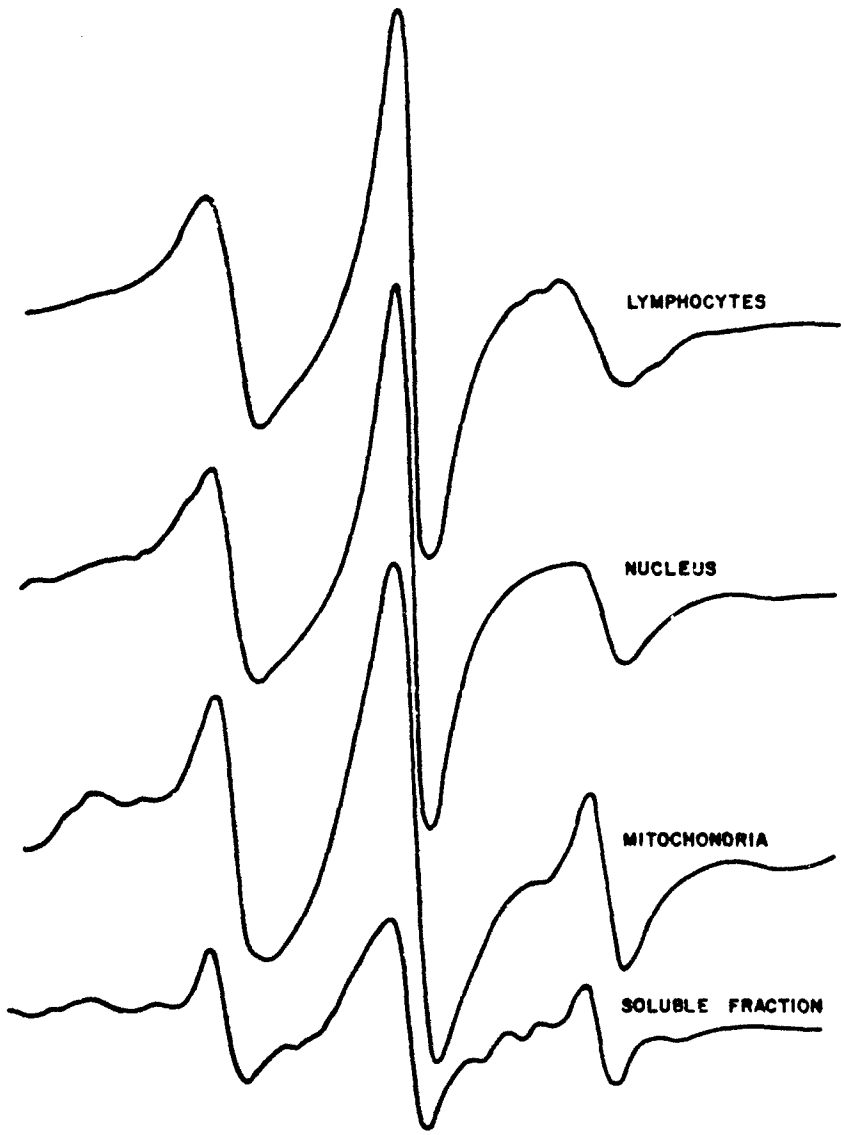


FIGURE 9. ESR SPECTRA OF HUMAN LONG-TERM LYMPHOCYTES AND THEIR FRACTIONS LABELED WITH ANDROSTANE.

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USE OF MICROPRECIPITATION AND ELECTROPHORESIS IN DIAGNOSTIC VIROLOGY

Neil H. Levitt, CPT, MSC*

The definitive laboratory diagnosis of disease of viral etiology is dependent upon virus isolation and/or demonstration of a rise in antibody titer in a serological test against a known viral agent. Although the use of tissue culture techniques has simplified virus isolation and some serological procedures, these methods are still beyond the capability of most clinical laboratories. The trend to more rapid and simplified laboratory methods for diagnosis of virus diseases is exemplified by the increasing number of reports employing modified immunological procedures such as immunodiffusion[1,2] and counter-immunoelectrophoresis[3] for routine use in diagnostic virology.

At last year's session of this meeting we described a procedure[4] which utilized microprecipitation and cellulose acetate electrophoresis for detection of antibody to tobacco mosaic virus. Subsequent studies have demonstrated the applicability of this procedure in the assay of human antibody to selected animal viruses.

This report describes the detection of antibody in sera of patients with an adenovirus infection. Preliminary observations are presented on the usefulness of this procedure for the rapid detection of specific antibody to Venezuelan equine encephalomyelitis (VEE) virus in both human and animal sera.

Figure 1 is a flow chart form of the procedure for detection of specific viral antibody by microprecipitation and cellulose acetate electrophoresis. Equal amounts of concentrated adenovirus antigen and serum dilutions are mixed in a Microtiter plate. The plate is sealed with a plastic cover and floated in a water bath at 37 C for 1 hr followed by overnight incubation at 4 C. Unlike VEE virus antigen, adenovirus required additional overnight incubation to obtain maximum precipitation. After incubation, 10 µl of each antigen-serum mixture is applied to the cathode end of a 1 x 3 inch cellulose acetate plate. The plates are electrophoresed at 220 V for 15 min and subsequently fixed and stained for 15 min in Ponceau S stain. After rinsing to remove excess stain, the plates are dried and examined for elliptical preipitates at the site of sample application.

Figure 2 shows stained cellulose acetate strips. The negative cathode is on the left and the positive anode on the right. On the upper plate, 3 samples were applied to the cathode end. The serum proteins have migrated away from the origin and are seen at the certer of the plate. A precipitate ring is present at the origin where virus + immune serum was applied.

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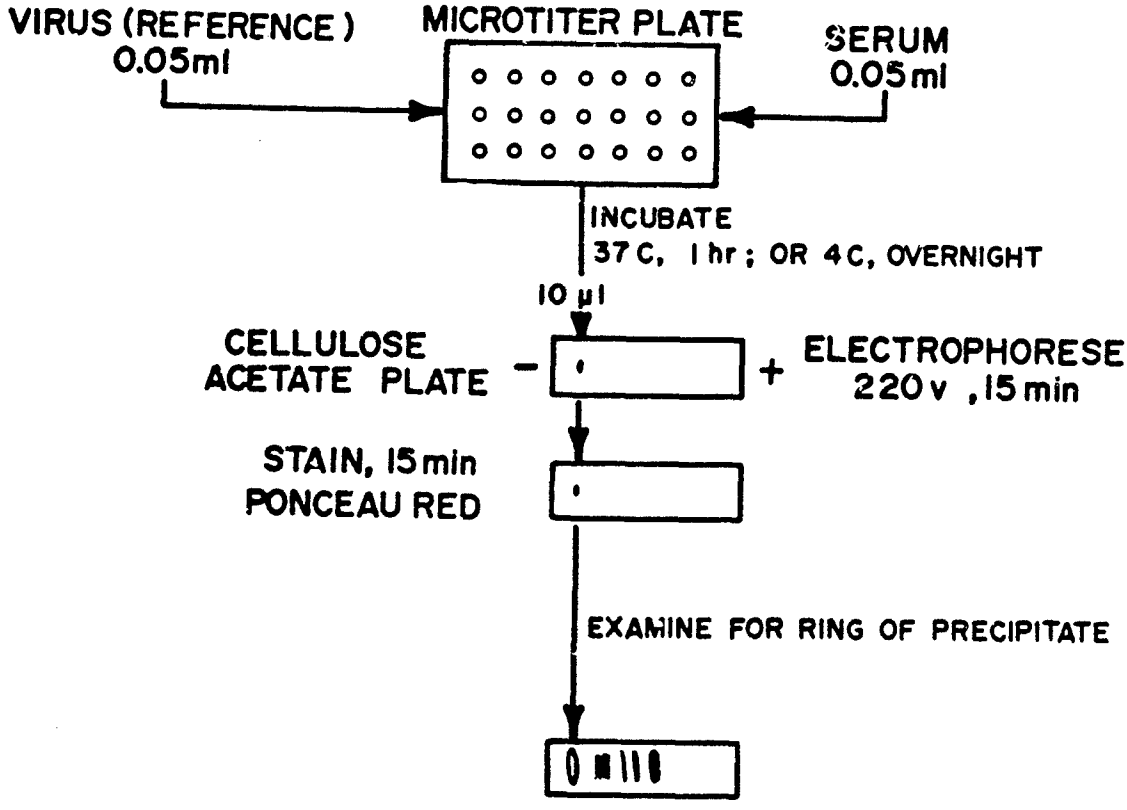

FIGURE 1. SCHEMATIC DIAGRAM OF PROCEDURE FOR DETECTION OF VIRUS ANTIBODY.

VIRUS +

SALINE

NORMAL SERUM

IMMUNE SERUM

1:80

1:160

1:320

FIGURE 2. STAINED CELLULOSE ACETATE STRIPS.

The lower photograph illustrates a graded response. Equal aliquots of virus were present in all 3 samples. The top sample received a 1:80 dilution of immune serum, the middle 1:160, and the bottom 1:320. The endpoint titer would be recorded as 1:160 (the middle sample).

Of prime importance in the precipitation reaction is the requirement of a concentrated virus antigen. The adenovirus antigens used in this study were prepared by using a modification of the method described by Green and Pina.[5] Adenovirus types 4 and 7 were grown in roller bottle cultures of KB cells. The infected cells were frozen and thawed 3 times to release intracellular virus. The cell debris-virus mixture was extracted with Tris buffer for 30 min at 4 C and then centrifuged at low speed to sediment the cell debris. Equal aliquots of supernatant fluid and Genetron-113 (a fluorocarbon) were homogenized in a Waring blendor for 2 min, followed by centrifugation to separate organic and aqueous phases. This procedure removes much of contaminating host cell material. The top aqueous phase, containing the virus, was harvested and centrifuged onto a cesium chloride cushion. The virus antigen was recovered as an opalescent band from the dense cushion. The antigen was dialyzed against Tris buffer and stored at -70 C. Antigen potency was determined by performing a block titration against dilutions of known antisera.

Supernatant fluid from control (uninfected) cells were processed similarly and tested for antigenicity in a precipitation reaction. No precipitation occurred when tissue culture fluid, concentrated antigen from uninfected cells, and infected tissue culture fluid were reacted with adenovirus-7 antiserum. Antigen prepared from infected cells however, reacted strongly with antiserum. At this stage, we felt that the antigen-antibody reaction seen was indeed virus specific, and not cellular in origin.

In 1965, Smith and co-workers[3] reported the precipitation of concentrated adenoviruses and rabbit antisera, to be type-specific. That is, each adenovirus type reacted only with homologous antiserum to form a visible precipitate.

To determine type specificity in our system, we reacted adenovirus 4 and 7 antigens independently with various dilutions of adenovirus -4, -7 and -21 antisera and endpoint titers determined. Table I presents the reactions and antigen-antibody reactions employing rabbit antisera to 3 adenovirus types. Adenovirus-4 antigen reacted with homologous antiserum and showed no cross reactivity with antiserum to types 7 and 21 or with normal rabbit serum. Similarly, adenovirus-7 antigen demonstrated type specificity.

When this experiment was repeated using human sera, cross reactions were observed between virus types. In other words, either antigen (type 4 or 7) could be used to detect adenovirus antibody in human sera. Our procedure appeared incapable, using whole virus antigens, of detecting

TABLE I. SPECIFICITY OF ADENOVIRUS ANTIGEN-ANTIBODY REACTION

ANTIGEN	RECIPROCAL TITERS			
	ADV-4	ADV-7	ADV-21	NRS[a]
ADV-4	320	< 10	< 10	< 10
ADV-7	< 10	160	< 10	< 10

a. NRS-normal rabbit serum control.

only type-specific antibody rises. We were able, however, to detect a rise in titer to adenovirus group antigen, comparable to the routinely used complement fixation (CF) test. A study was then performed which could compare directly the serological results obtained by precipitation and CF tests on sera from patients with suspected adenovirus infection. The isolation, serological results, and paired sera were generously supplied by 5th Army Medical Laboratory and Walter Reed Army Institute of Research.

Before reporting these results, it is important to point out a technical difficulty encountered when human serum is employed in this precipitin test. Human serum, unlike most animal serum, contains lipid and lipoprotein substances which do not migrate in an electric field and will subsequently result in false positive reactions. A simple procedure was developed to remove these substances from serum prior to electrophoresis without affecting antibody concentration. Two milliliters of the lowest dilution of serum being tested (usually 1:10 or 1:20) was homogenized with 5 volumes of Genetron-113 on a vortex mixer for 10-15 sec. The mixture was centrifuged at 600 X g for 5 min; the top aqueous phase (serum) was harvested and filtered for removal of particulate material. The serum was then diluted and used as previously described for rabbit serum. No decrease in immunoglobulin levels or neutralization and hemagglutination-inhibition (HI) titers were detected in serum treated in this manner.

Paired acute and convalescent sera from 57 suspected adenovirus cases were diluted 1:20 with buffered saline and treated with Genetron-113. Serial 2-fold dilutions of each serum were prepared and tested for precipitating antibody against adenovirus-7 antigen. The results of this study are presented in Table II. The number of virus isolations and number of cases showing significant antibody rise as measured by CF, neutralization and precipitin tests can be seen. A neutralization test was performed only when virus was isolated and no significant rise in CF titer was observed. The top line summarizes the data. The CF test showed antibody rises in 34 cases; 8 additional cases showed a rise by neutralization test, and 42 cases demonstrated precipitin rises. These totals were subsequently

TABLE II. COMPARISON OF PRECIPITIN TEST WITH CF AND NEUTRALIZATION TESTS FOR ADENOVIRUS ANTIBODIES

GROUP	NO. CASES	ISOLATIONS	CASES SHOWING ANTIBODY RISE BY:		
			CF	Neutralization[a]	Precipitin
Total	57	39	34	8	42
I	30	30	30	ND[b]	29
II	9	9	0	8	8
III	4	0	4	ND	4
IV	14	0	0	ND	1

a. Neutralization test performed when adenovirus was isolated with no concomitant 4 times rise in CF titer.
b. Not done.

divided into 4 groups: I. isolation, CF rise; II. isolation, no CF rise; III. no isolation, CF rise; and IV. no isolation, no CF rise. Twenty-nine of the 30 in Group I had positive precipitin tests. In Group II, of 9 cases with isolations but no rise in CF titer, 8 demonstrated rises in neutralizing antibodies; the precipitin test detected all 8. The remaining case in this group had only a 2-fold rise in the neutralization test, which was not considered significant. Both the CF and precipitin tests showed rises in titers for each of 4 cases with no virus isolation (Group II). In Group IV, with no virus isolation and no CF titer rise, the precipitin test demonstrated an antibody rise in 1 (repeated 3 times).

These results suggest that the precipitin test is a more sensitive measure of adenovirus antibody response following infection than the routinely used CF test. This greater sensitivity, together with its speed and ease of operation, should make this test a worthwhile adjunct to standard procedures routinely employed for adenovirus diagnosis.

Recent studies have demonstrated the ease of adapting this procedure to the detection of specific antibody to other animal viruses. A concentrated, partially purified VEE virus antigen was prepared and tested in our system against human antiserum to several animal viruses.

Table III shows the specificity of the VEE antigen-antibody reaction. As expected, no cross reactivity was observed when the VEE antigen was reacted with antisera to adenovirus, or even the more closely related Eastern and Western encephalitis viruses. Precipitating antibody to VEE

TABLE III. SPECIFICITY OF VEE ANTIGEN-ANTIBODY REACTION

ANTIGEN	REACTION				
	HUMAN ANTISERA TO:				NHS[a/]
	EEE	VEE	WEE	ADV-7	
VEE	-	+	-	-	-

a. Normal human serum.

was also detected in sera from the rabbit, horse, burro, hamster, and guinea pig, with no cross reactions.

Studies now in progress are attempting to correlate the precipitin test with results obtained by HI and neutralization tests. We are also examining the utilization of this technique in distinguishing between the specific antibodies to the various epidemic and endemic strains of VEE virus.

In summary, we have introduced a diagnostic test employing microprecipitation of antigen-antibody complexes and detection of these complexes by cellulose acetate electrophoresis. In addition, a simple and rapid method for removal of nonmigrating lipid-containing substances from human serum was described. Evidence was presented which demonstrated an enhanced sensitivity of this immunoelectrophoretic technique when compared to the CF test for detection of adenovirus antibodies. Finally, preliminary studies with VEE virus antigen illustrated the ease of adapting this procedure to other viruses, and its use with sera from various animal species.

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MITOGENIC EFFECTS OF ENTEROTOXINS IN LYMPHOCYTES

William H. Adler, III, MAJ, MC*

In 1965, Knight et al first reported the mitogenic effects of staphylococcal culture filtrates on lymphoid cells.[1] In 1967, Ling and Holt extended these findings and proposed that the filtrates of staphylococcal cultures were analogous in their qualitative and quantitative effects to phytohemagglutinin (PHA), a nonspecific lymphocyte mitogen.[2] Since that time several substances such as the staphylococcal hemolysins have been excluded as being the mitogenic substance in the culture filtrates,[3] but no particular substance has been reported to be the active component. In 1970, we first reported on the lymphocyte mitogenic properties of staphylococcal enterotoxin B (SEB) an exoprotein.[4] We will extend that finding and show that purified SEB could be the responsible moiety in a culture filtrate, since its mitogenic properties duplicate in extent and types of cells involved the effects of the crude culture filtrates.

The first observation of the mitogenic nature of SEB was on its effect on mouse spleen cells in culture.[4] Mouse spleen cells, in suspension culture have the morphologic appearance of small lymphocytes; however, when cultured in the presence of SEB, changes in morphology take place. The small mononuclear cells enlarge, the nucleus enlarges, the cytoplasm stains more darkly blue and the cell assumes lymphoblast characteristics which can be quantitated by determining the percent of cells in culture having this morphologic appearance. Using the technique of Allison and Mallucci,[5] lysosomes, stained red by Euchrysine, can be seen in the cytoplasm of the stimulated cells; electron micrographs of the SEB stimulated cells also show the presence of lysosomal bodies in the cytoplasm. The stimulated cells enter a mitotic cycle and at that time can incorporate tritiated thymidine. By determining the degree of radioactivity in trichloroacetic acid precipitates of cell cultures, it is possible to measure the extent of stimulation. Figure 1 shows the dose-response relationship for both SEB and PHA stimulation of mouse spleen cells in vitro. The curves are similar for each of the mitogens; however at low doses it can be seen that the effect of both mitogens together is additive rather than following the curve for doubled amounts of each mitogen individually. This additive effect is not seen at higher combined doses, and the circles demonstrate the combined effect of 3/4 of a peak mitogen dose of one mitogen and 1/4 of a peak dose of the other mitogen which equal the peak effect of each mitogen alone. This peak degree of ^{3}H-thymidine incorporation represents about 60% blast cell transformation of the spleen cells. Figure 2 demonstrates the same type of experiment using human peripheral blood lymphocytes. In these experiments, however, only a slight additive effect is seen at the low doses. Mixing the mitogens results in almost the same degree of stimulation as with higher amounts of each mitogen alone. At peak thymidine incorporation, there are 95% blast cells seen in these cultures.

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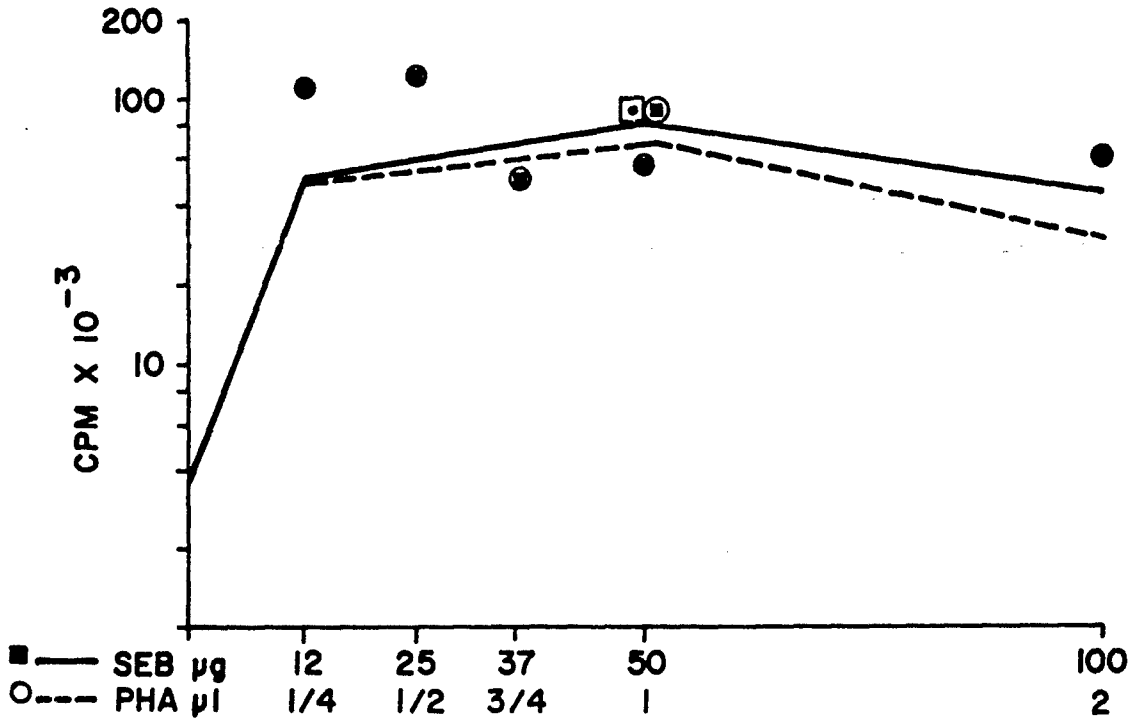

FIGURE 1. DOSE RESPONSE OF MOUSE SPLEEN CELLS TO PHA AND SEB.

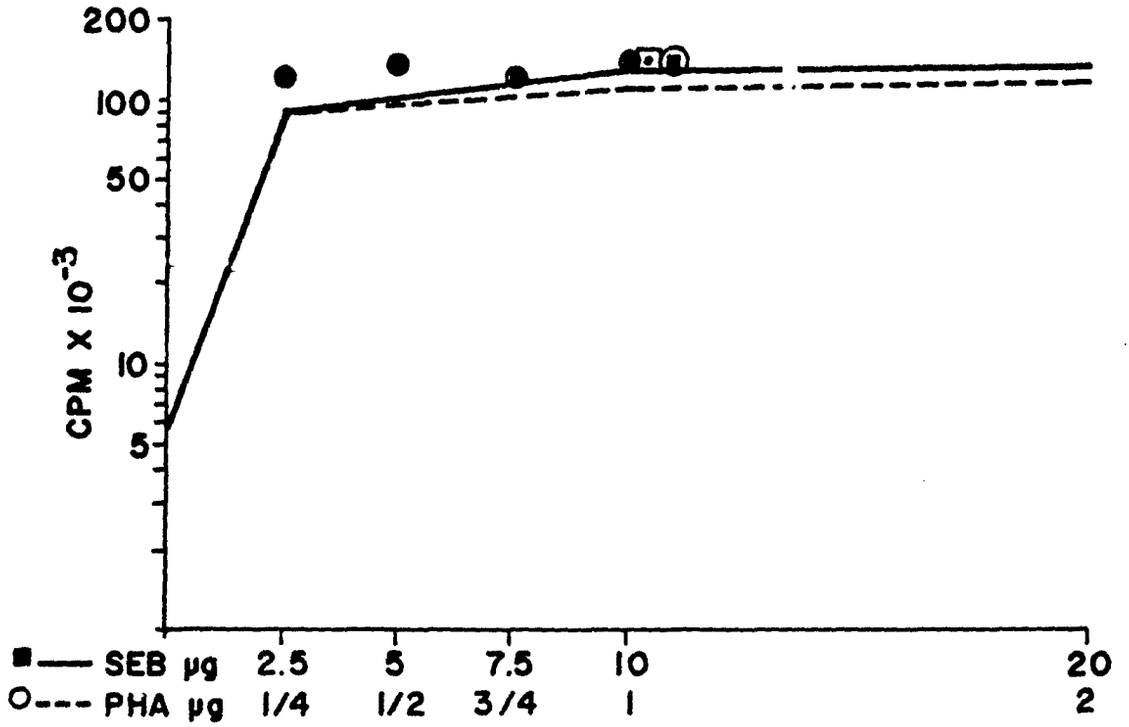

FIGURE 2. DOSE RESPONSE OF HUMAN PERIPHERAL LYMPHOCYTES TO PHA AND SEB.

When mouse thymus cells are cultured with PHA or SEB, as shown in Figure 3, a dichotomy of effects is seen. PHA, which results in only about 5% blast cell transformation, stimulates only a small increment in thymidine incorporation, while SEB-stimulated thymus cells show a high degree of thymidine incorporation and results in 20% blast transformation. If mice are treated with cortisone over a 2-day period the number of mononuclear thymus cells decreases from about 2×10^8 to about 3×10^6 per mouse. However, the cortisone-resistant population, Figure 4, can be stimulated to a high degree by PHA and SEB. Mouse bone marrow cells, Figure 5, are minimally reactive to either PHA or SEB alone, but throughout the total dose range tested, the effects of both PHA and SEB together are additive. Their effect is also evidenced by the assessment of the degree of blast transformation. At a 1.0-µl dose of PHA there are 25% blast cells and at 50 µg dose of SEB there are 20% blast cells; if both mitogens are used together there are 40% blast cells.

The interpretation is, that SEB, like PHA is a nonspecific lymphocyte mitogen; however, SEB is able to affect cells which are not stimulated by PHA. Whether the SEB-stimulated cells are affected directly by the SEB or influenced by lymphocyte mediators is not clear. It is doubtful, however, since in cortisone-treated mouse thymus cells the PHA and SEB effects are the same as on the cortisone-resistant population, and SEB affects 4 times the number of cells in a whole normal thymus cell population. The additive effects seen on bone marrow and on spleen cell cultures probably reflect the different population of cells able to be stimulated by SEB and PHA.

In 1967, Holm and Perlmann[6] demonstrated that staphylococcal culture filtrates, though they did not cause lymphocyte agglutination, could stimulate lymphocytes to destroy a target cell in vitro. Prior to this time PHA had been shown to do this; part of the mechanism of action was thought to be due to its lympho-agglutinating properties. This cytotoxic behavior induced by the staphylococcal culture filtrates can be duplicated by SEB. These effects can be assayed morphologically; it is possible to show that human lymphocytes in the presence of SEB are able to destroy an L cell monolayer, and that mouse spleen cells in the presence of SEB are able to destroy a mouse fibroblast (MFB) monolayer. The cytotoxic behavior can be assayed by release of ^{51}Cr from the monolayer target cell in the presence of lymphocytes and SEB. Figure 6 demonstrates the ^{51}Cr release, or destruction of either L cells or MFB in the presence of lymphocytes and lymphocytes + SEB. In the allogeneic combination (solid lines) the effects are seen earlier, and are greater than the effects seen with the syngeneic combination (dotted lines). However, with an allogeneic combination, after 2 days, there is beginning destruction of the monolayer in the absence of SEB while in the syngeneic combination there is no target cell destruction at 4 days in the absence of SEB.

Supernates of SEB-stimulated lymphocyte cultures were also able to show toxic effects in target cell cultures. Figure 7 illustrates the effects of supernates from unstimulated lymphocyte cultures and the SEB-stimulated

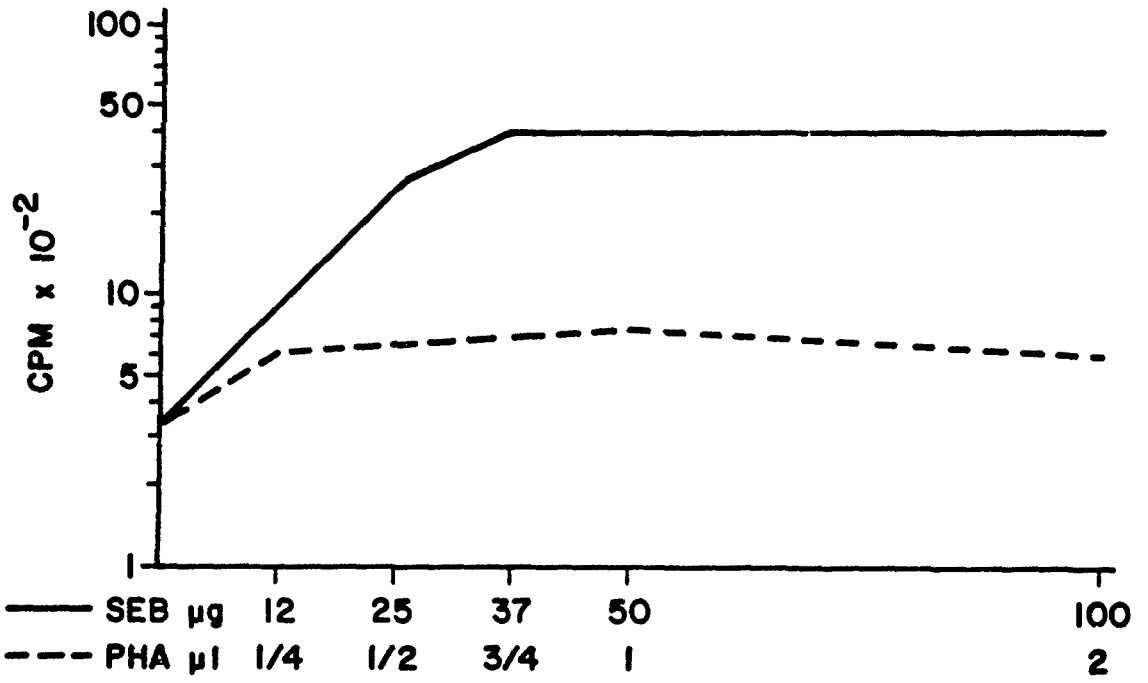

FIGURE 3. DOSE RESPONSE OF MOUSE THYMUS CELLS TO PHA AND SEB.

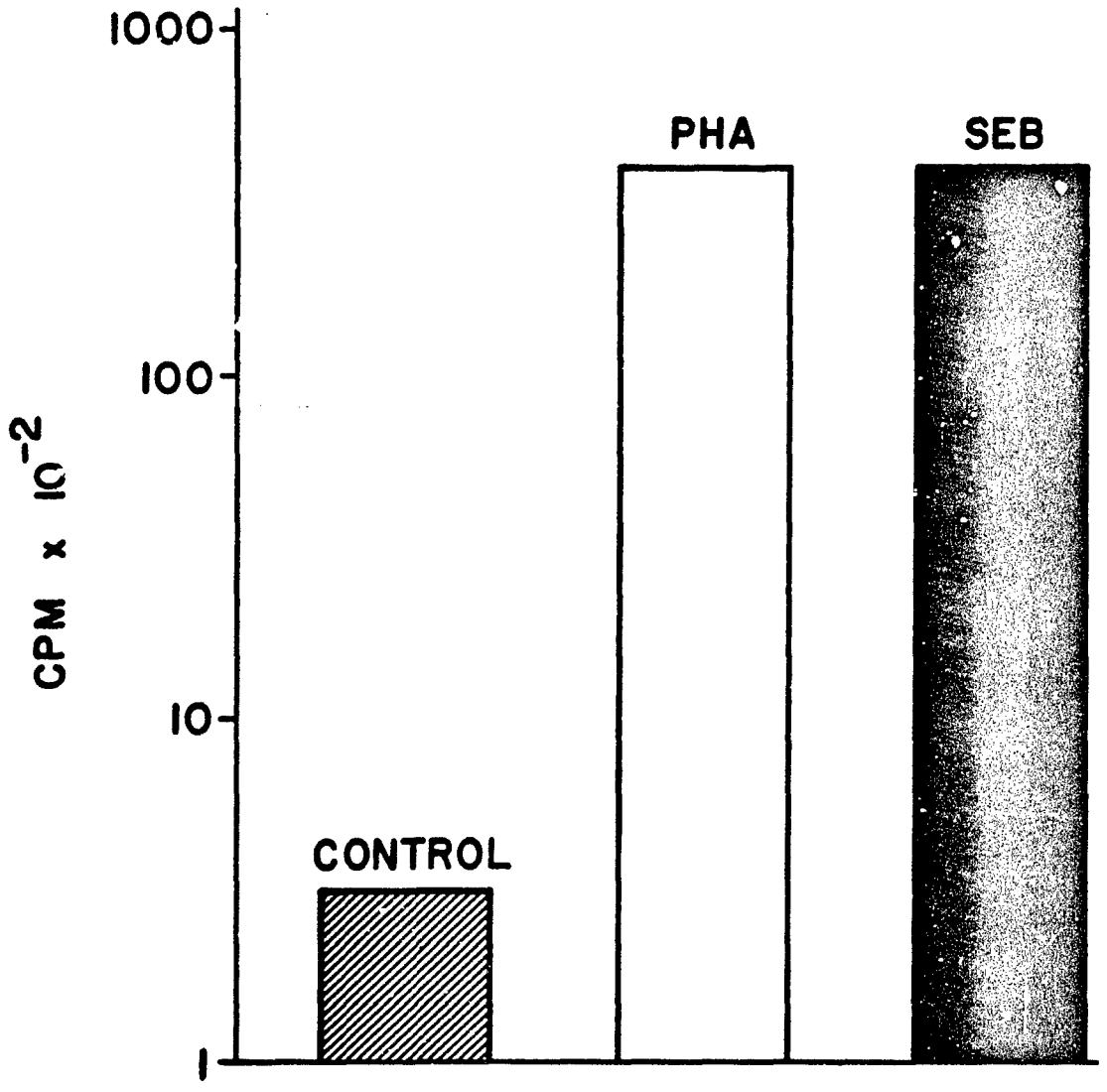

FIGURE 4. EFFECT OF CORTISONE ON MOUSE THYMUS CELLS CULTURED WITH PHA AND SEB.

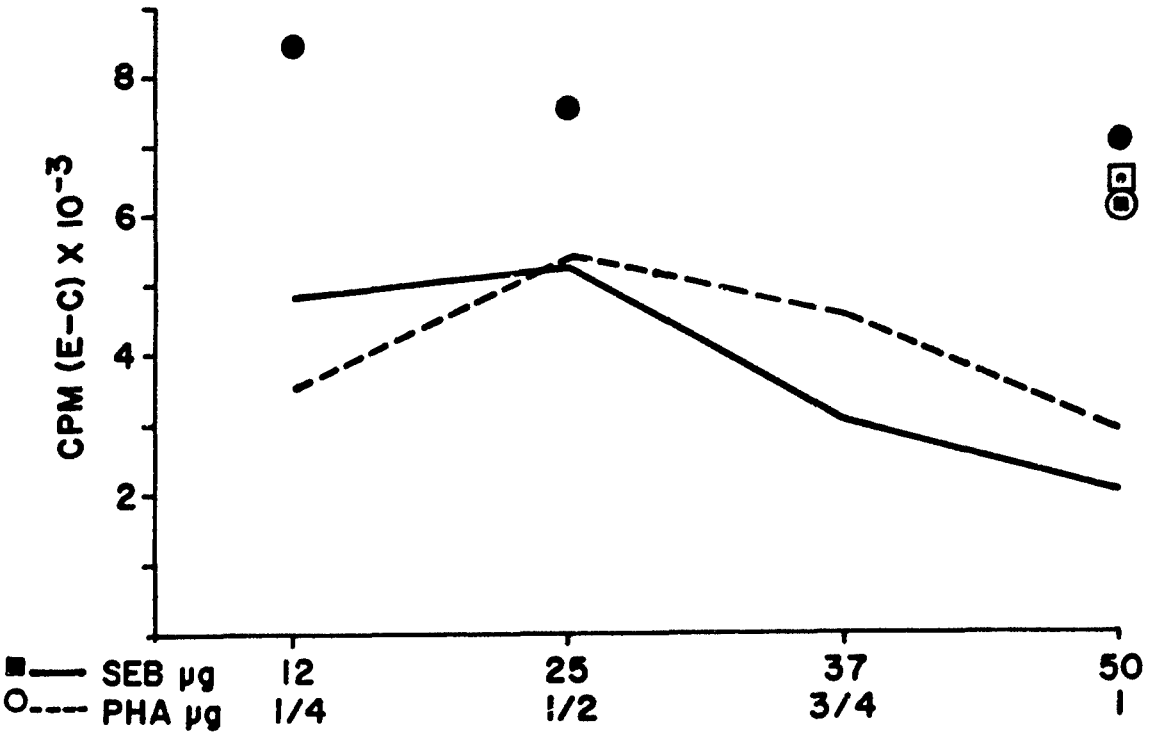

FIGURE 5. DOSE RESPONSE OF MOUSE BONE MARROW CELLS TO PHA AND SEB.

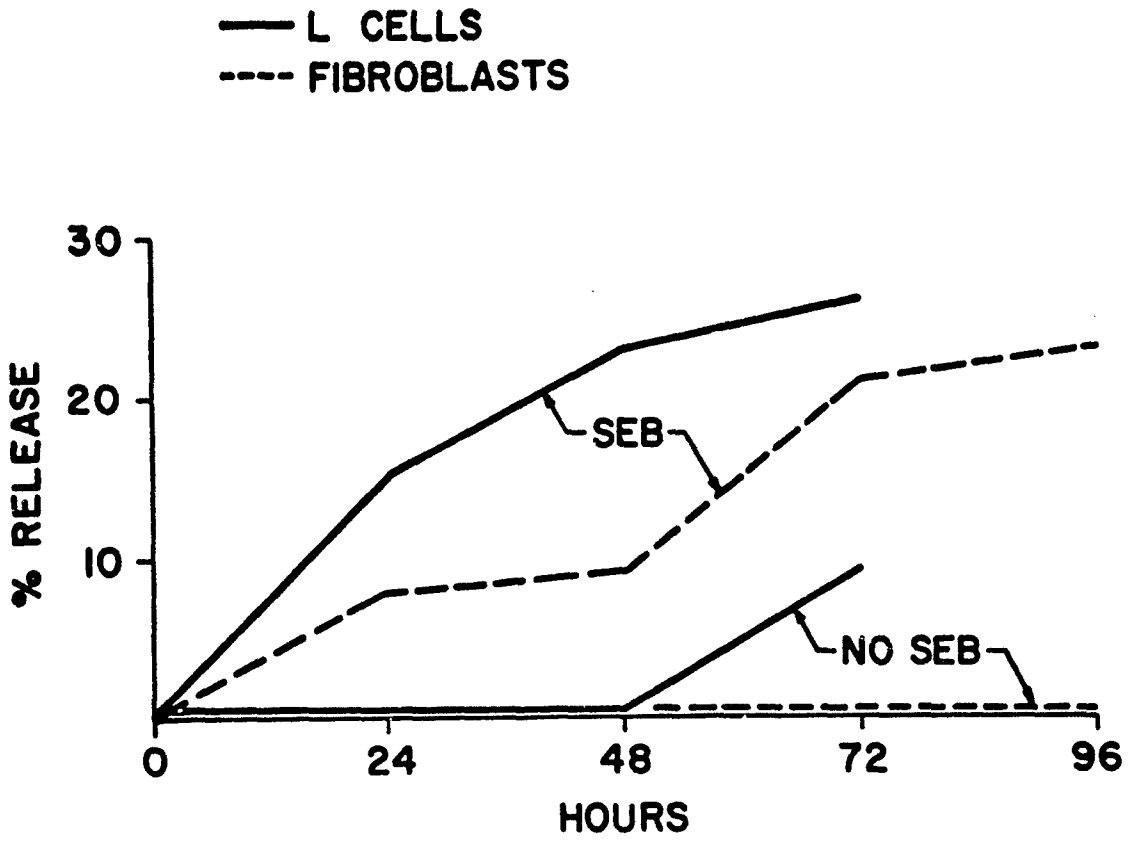

FIGURE 6. ^{51}CR RELEASE FROM L CELLS OR MOUSE FIBROBLASTS BY SEB-STIMULATED MOUSE SPLEEN CELLS.

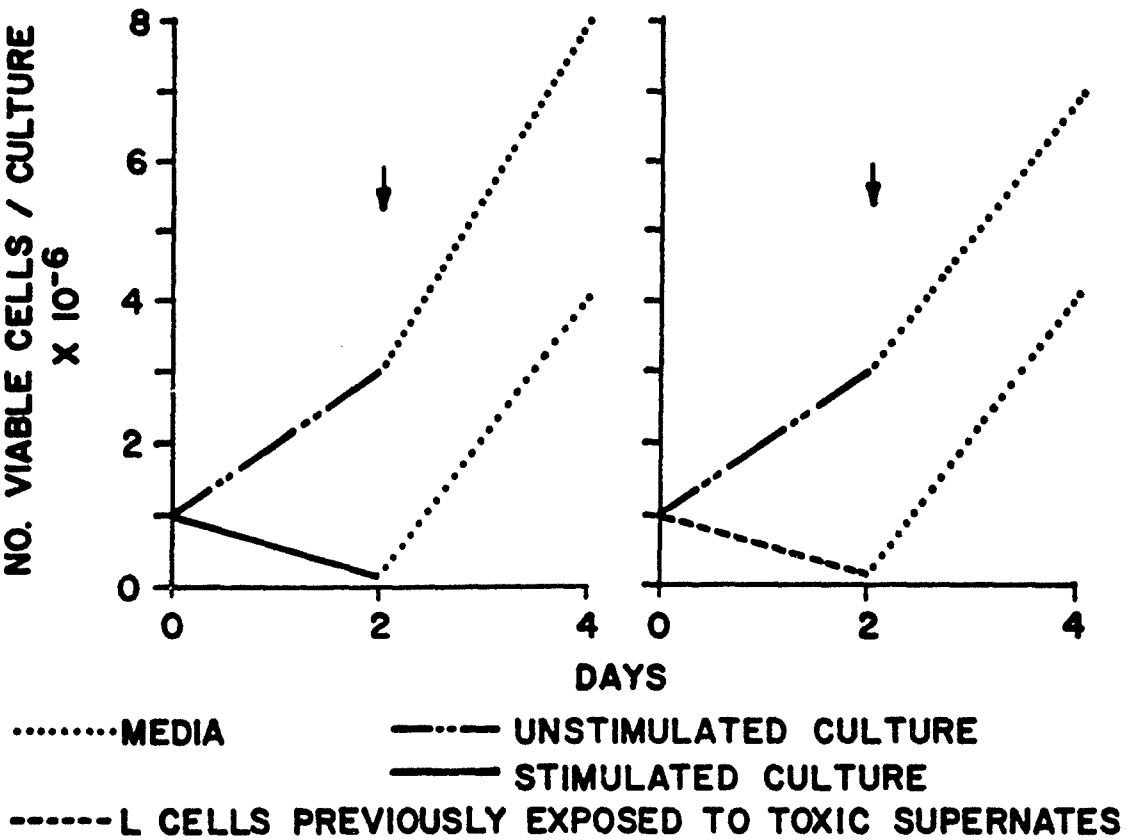

FIGURE 7. EFFECTS OF TOXIC SUPERNATES ON L CELL GROWTH.

cell cultures on L cell growth and on viability in vitro. The stimulated cell supernates cause cell death and growth inhibition which is reversible if the toxic media is replaced by normal media after 2 days (dotted lines). L cells which have survived an initial exposure to the toxic supernates can be adversely affected equally a second time showing that a resistant cell does not emerge (right graph).

The toxic effects of supernates from SEB-stimulated lymphocyte cultures can be measured by the change in morphology seen in the target cell monolayer. The relative toxic activity in the supernates can be judged by testing serial dilutions of the supernates and determining the maximum dilution at which target cell pathology is still seen. Exactly these same toxic effects can also be seen on mouse fibroblast growth.

Figure 8 demonstrates the time of appearance of the toxic factor in the stimulated culture supernates. The dotted line shows the effect of supernates taken from cells stimulated for the first 24 hr with SEB and the supernate removed at each 24-hr period thereafter. The stimulated cells produce the maximum amount of toxic factor on days 2 and 3, the amount decreasing rapidly thereafter. The accumulation of the toxic factor is demonstrated by the solid line in which experiment, lymphocyte cultures were started with SEB; at varied daily intervals the cultures were terminated and the supernates tested for their toxic effects. Since we know that the daily production of toxic factor occurs from day 1-4 it is not surprising to see that the toxic factor accumulates in the media, but what is surprising is that shortly after the cells stop producing the toxic factor, its activity decreases and disappears from the media. Storage of the toxic media at 37 C in the absence of cells, the dashed line, does not result in a decrease of toxic activity; media from 10-day-old cultures do not inhibit the toxic effects of the media from 3-day cultures. If one examines the cells seen in these cultures at this later time period all the cells in the culture resemble macrophages. Using peritoneal macrophages in subsequent experiments, it has been possible to remove the toxic activity from the toxin-stimulated culture supernates. Therefore, we propose that (1) SEB-stimulated lymphocytes produce toxic materials which are released into the culture media and (2) macrophages remove these toxic factors from the media.

In summary, SEB is a nonspecific cell mitogen which affects the lymphocyte population, which is also sensitive to PHA, and at least one other cell population which is not affected by PHA. SEB induces cytotoxic behavior in lymphoid cells which are then able to destroy syngeneic target cells, and the SEB-stimulated lymphocytes release toxic material into the culture supernate which affects cell viability and growth.

The mitogenic mechanism of action of SEB is unknown at present but unlike PHA which is a bean extract containing at least 17 different components,[7] SEB is a single protein of known molecular weight and amino

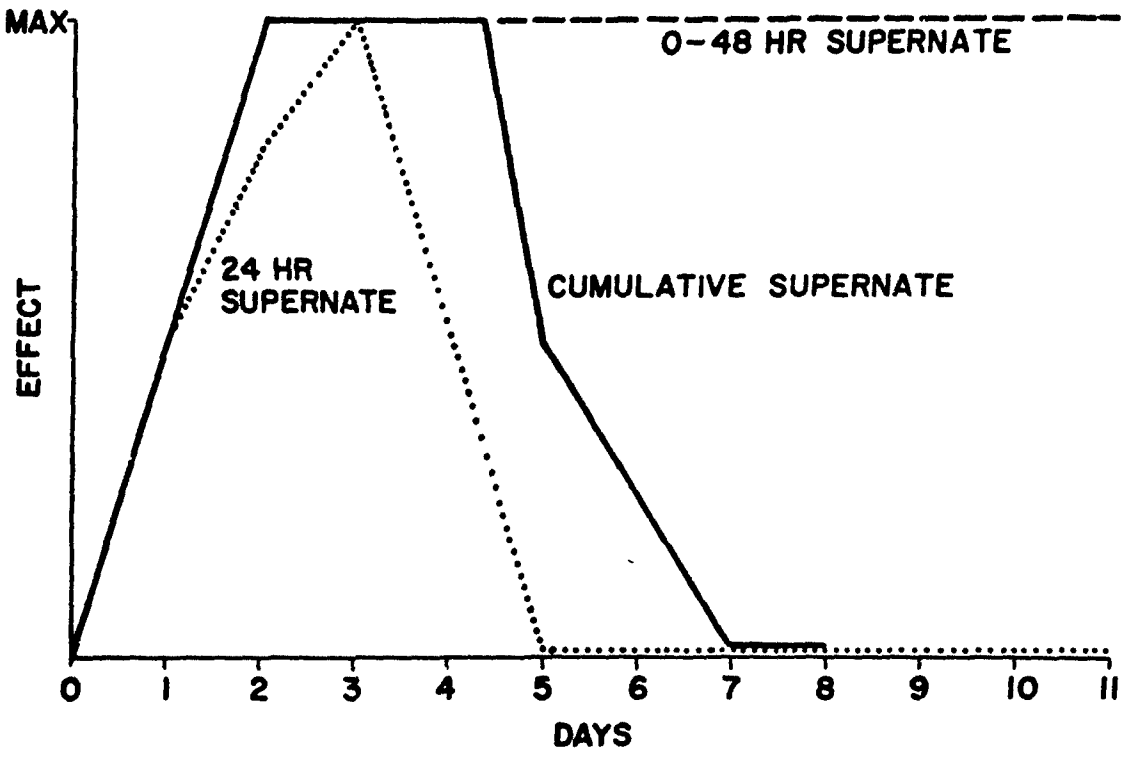

FIGURE 8. EFFECT OF CELL-FREE SEB-STIMULATED MOUSE SPLEEN CELL CULTURE SUPERNATES ON L CELLS.

acid sequence. Working with a pure mitogen may help solve many of the questions on cell activation which were raised by the PHA experiments. Also, using lymphoid cell cultures allows the opportunity to study the physiological and pharmacologic action of a bacterial toxin in an isolated in vitro situation. This may help us to understand the mechanism of action of this toxin and its effect on cellular events.

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MECHANISMS OF DELAYED HYPERSENSITIVITY AND OTHER TYPES OF CELL MEDIATED IMMUNITY

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The data to be reported concern the cell types involved in the expression of cutaneous delayed hypersensitivity and in the host response in the local graft vs. host reaction (LGVHR). Particular attention has been paid to the role of the mononuclear phagocyte in these two models of cell-mediated immunity.

Delayed hypersensitivity

A suppressive effect of sublethal x-irradiation on delayed hypersensitivity in the skin of rats or guinea pigs or the footpad in mice has been described previously.[1] This effect and the spontaneous recovery of skin reactivity were demonstrated in animals with an established state of hypersensitivity following exposure to 400 R whole-body irradiation. Because of the association of renewed production of mononuclear phagocytes with renewed skin activity, it was proposed that the mechanism underlying depression and recovery were dependent upon the fall and later recovery of the output of an effective number of mononuclear phagocytes. This in turn meant that the specifically sensitized population of lymphocytes would have to survive irradiation in numbers adequate to yield a reaction once the monocytes were restored. A second possibility was that the lymphocyte population was also regenerated as the result of a renewed response of surviving cells to persisting antigen. A third consideration was that the damaging effect of ionizing irradiation on sensitized cells resulted in the release of an antibody or other humoral factor into the circulation. If true, one could argue that as increasing levels of such material were attained, reactivity should again be observed. A report by Dupuy et al[2] suggested that such a substance was indeed demonstrable in the plasma of guinea pigs following irradiation. When studied in this laboratory the work of Dupuy and his colleagues was not confirmed.[3] During the past year this problem was pursued further in collaboration with Dr. F. M. Collins. It was found that sublethal whole-body irradiation in the guinea pig had little demonstrable effect on the development of delayed hypersensitivity, but caused a profound, though transient, depression of dermal reactivity in previously sensitized animals (Figure 1). To assess the requirement for mononuclear phagocytes in the observed depression, macrophage-rich peritoneal exudate cell (PE) suspensions (induced with oil) from nonsensitive donors were injected intradermally, together with eliciting antigen, into irradiated, sensitized recipients. Restoration of a significant degree of reactivity resulted; it appeared prior to the time of expected spontaneous recovery (Figure 2). Inocula of lymph node lymphocytes from similar donors, on the other hand, failed

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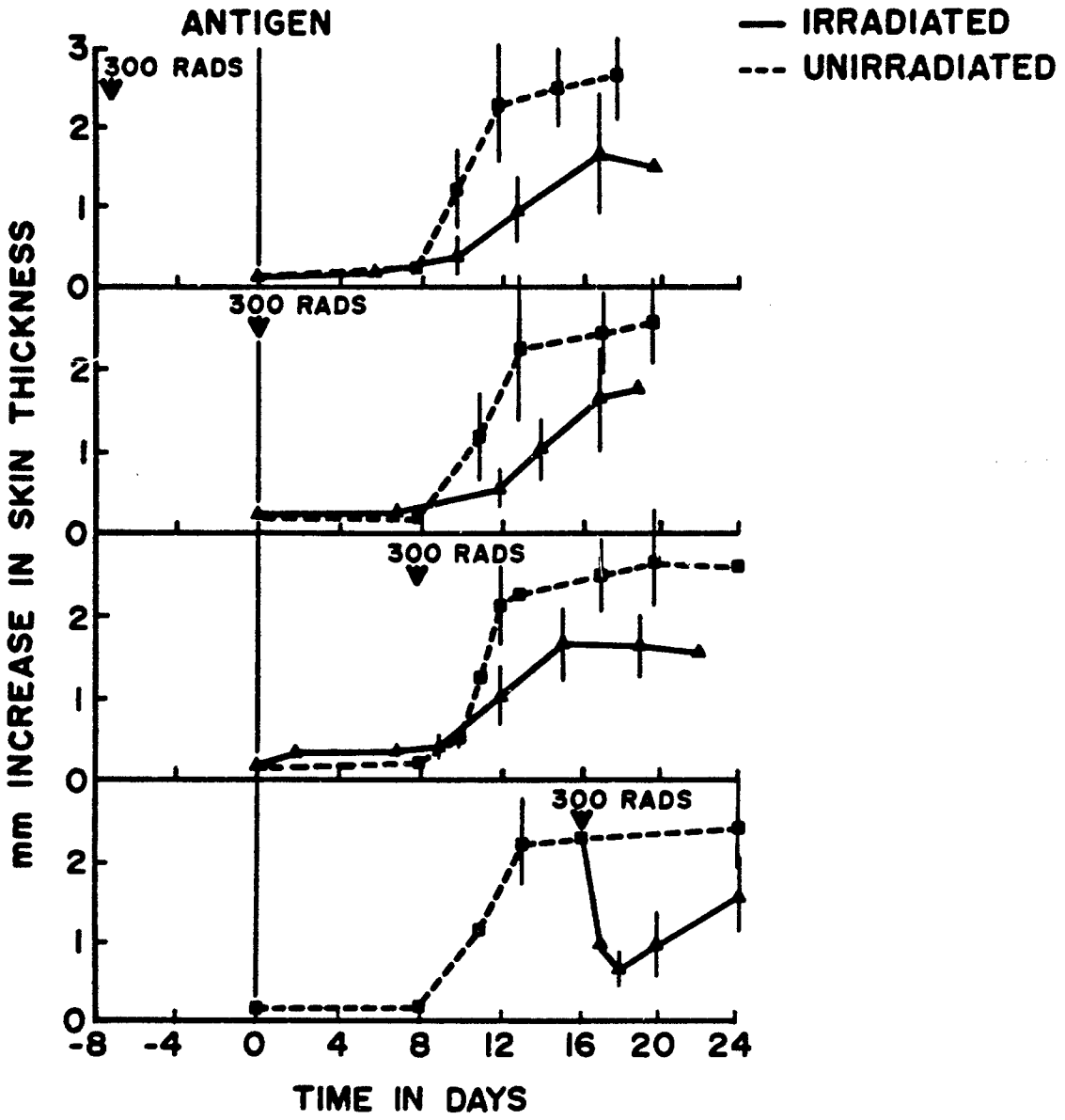

FIGURE 1. EFFECT OF 300 RADS WHOLE BODY IRRADIATION GIVEN AT VARYING TIMES ON DEVELOPMENT OF DELAYED HYPERSENSITIVITY (MEANS OF 5 GUINEA PIGS WITH 95% CL).

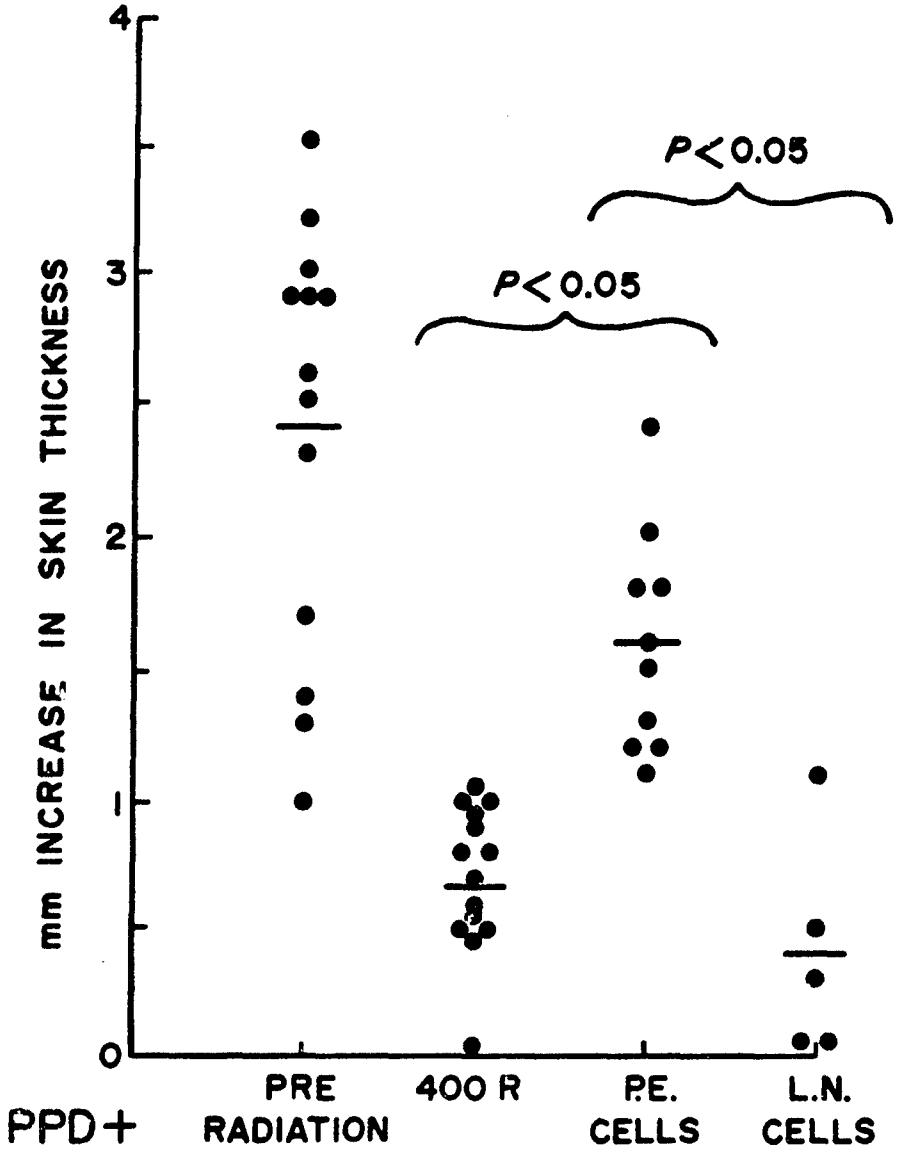

FIGURE 2. EFFECT OF ID INJECTION OF PPD COMBINED WITH PE OR LYMPH NODE CELLS FROM UNSENSITIZED, UNIRRADIATED DONORS ON 24-HR TUBERCULIN REACTIVITY, RELATIVE TO WHOLE BODY IRRADIATION.

to yield positive results. A further demonstration of the dependence upon macrophages was shown in another experiment. Reducing the proportion of macrophages in the cell suspension along with the concomitant doubling of the proportion of lymphocyte-like cells did not increase the ability of the inoculum to restore cutaneous reactivity (Table I). It was also demonstrated that a specifically sensitized cell population does indeed survive

TABLE I. EFFECT OF INCUBATION ON POOLED GUINEA PIG PERITONEAL EXUDATE CELLS IN VITRO

PARAMETER	TREATMENT	
	None	Absorption
No. pools	5	1
No./pool	10	10
DIFFERENTIAL COUNT (% ± SD)		
Macrophages	66 ± 4	38
Lymphoid cells	14 ± 5	39
PMN	20 ± 4	23
Unidentified	< 1	< 1
RESTORATIVE CAPACITY FOR 5 GUINEA PIGS (units ± SD)[a]		
Preirradiation PPD 2.5 μg/0.1 ml	27.5 ± 3.2	24.6 ± 3.5
Postirradiation PPD 2.5 μg/0.1 ml	2.1 ± 2.0	7.4 ± 3.0
Peritoneal exudate cell (10^7/0.1 ml) + PPD (2.5 μg)[b]	17.9 ± 2.0	11.7 ± 1.7

a. Mean 24 hr increase in skin-thickness in Schnelltaster units (10 units = 1 mm).
b. Corrected for thickness due to cells alone, 4.7 for no treatment, and 7.3 for adsorption.

the doses of irradiation used, for specific reactivity was transferable with cells from irradiated donors (Table II). In each animal the magnitude of the combined reaction was greater than that for PE or PPD alone.

The contention that the observed 24-hr increase in skin thickness was due to delayed and not residual immediate hypersensitivity was tested by comparing the effect of PE suspensions in groups of guinea pigs with the respective types of sensitivities (Figures 3 and 4). Clearly PE cells do not enhance immediate hypersensitivity. These data therefore warrant the conclusion that the presence of macrophages is necessary for the expression of cutaneous delayed hypersensitivity. The spontaneously renewed activity which follows irradiation thus becomes a reflection of the recovery of

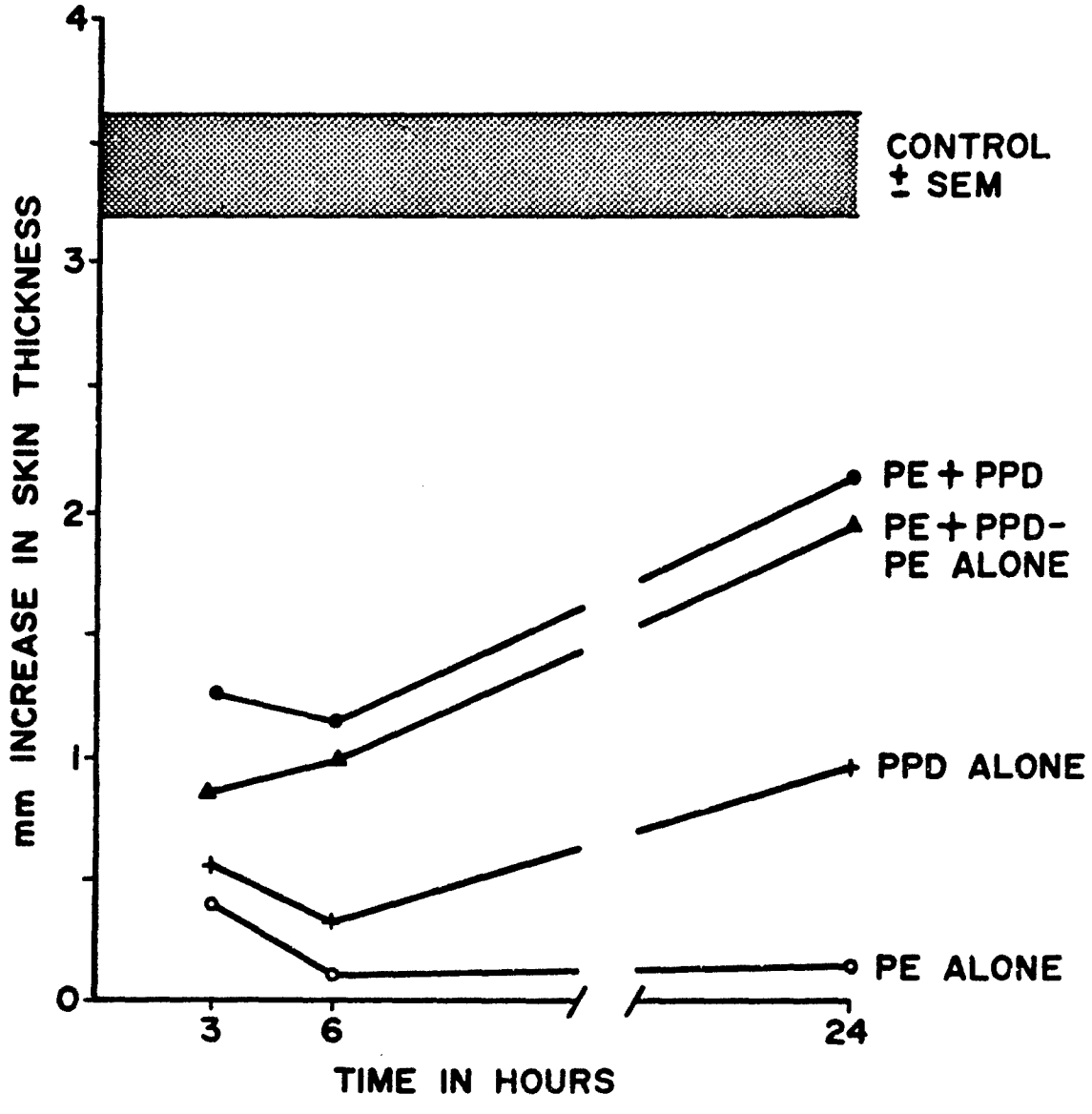

FIGURE 3. EFFECT OF ID INJECTION OF PE CELLS + PPD 48 HR AFTER IRRADIATION INTO TUBERCULIN-SENSITIVE GUINEA PIGS (MEAN OF 10).

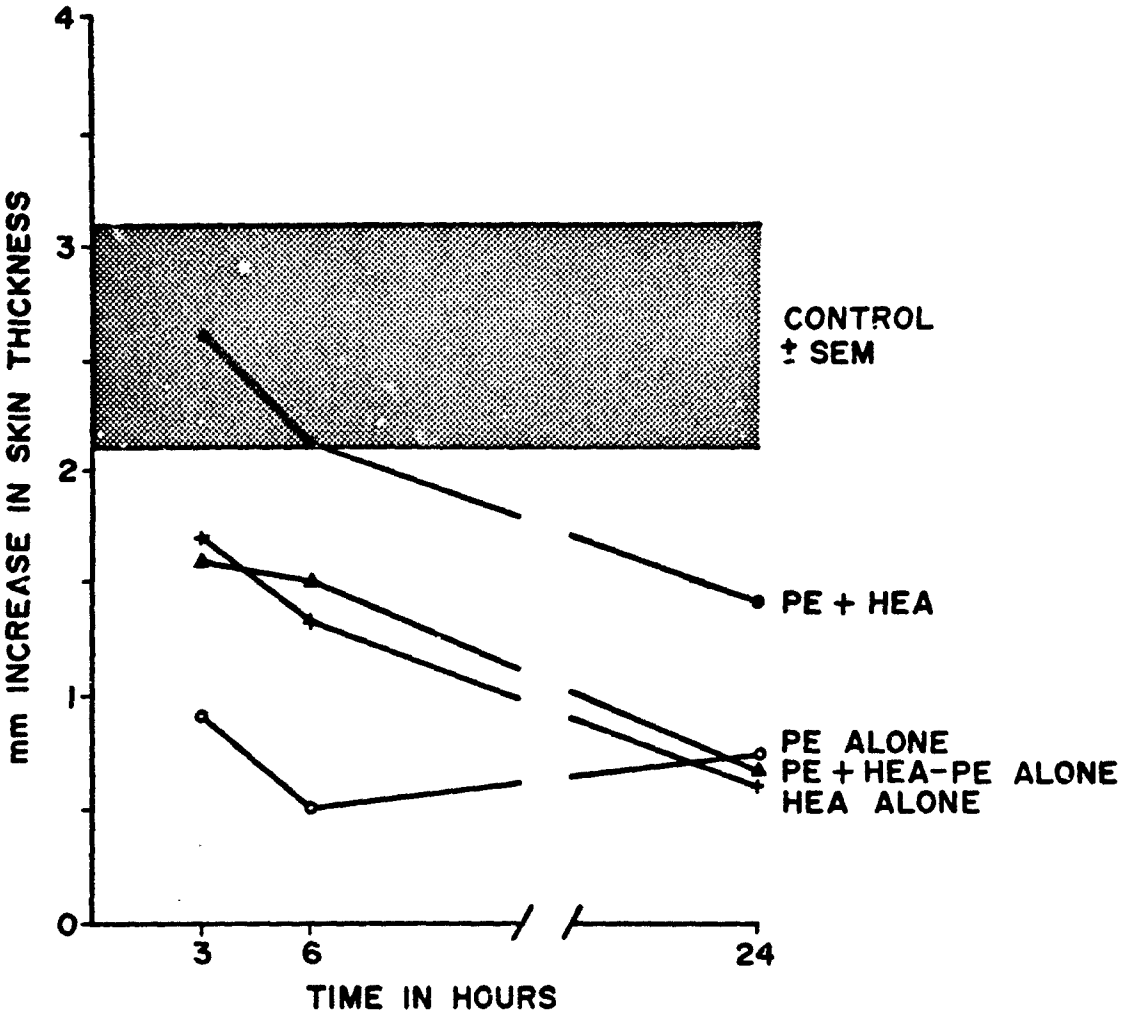

FIGURE 4. EFFECT OF ID INJECTION OF PE CELLS + HEN EGG ALBUMIN (HEA) 48 HR AFTER IRRADIATION INTO GUINEA PIGS WITH PREEXISTING ARTHUS SENSITIVITY TO HEA (MEAN OF 10).

TABLE II. ADOPTIVE TRANSFER OF TUBERCULIN SENSITIVITY FROM GUINEA PIGS, IRRADIATED WITH 400 R ^{137}Cs WHOLE BODY IRRADIATION, TO NORMAL GUINEA PIGS

ANIMAL NUMBER	INCREASE IN SKIN THICKNESS (UNITS) AT 24 HR		
	10^7 PE cells + PPD (25 μg)	PPD (25 μg)	10^7 PE cells
1	23	6	10
2	13	1	9
3	14	1	3
4	21	1	4
5	18	2	6
6	16	1	2
7	23	3	7
8	20	1	5
9	22	1	5
10	20	0	0
Mean ± SEM	19 ± 1.14	2.8 ± 0.05	9.4 ± 0.97

macrophage precursors following radiation injury and not the emergence of a new population of sensitized cells. In addition, the results substantiate the belief that the expression of delayed hypersensitivity in skin requires at least 2 cell populations, only one of which carries the property of specificity. As a corollary, the presence of a sensitized population cannot always be excluded by a weak or absent skin test.

Local graft versus host reaction

When immunologically competent parental cells are injected under the renal capsule of genetically tolerant F_1 recipients, as described by Elkins,[4] a lesion characterized by cellular invasion and proliferation occurs with local destruction of renal parenchyma. This immunologically generated reaction progresses for about a week and then declines, leaving surprisingly little residual damage. It has been shown that a host cell component is necessary to support an ongoing reaction, although the nature and functions of this cell or cells are not completely known.[5] In the present experiments the parental donors were highly inbred Lewis rats and the hosts were (Lewis x BN)F_1 hybrids. In every case the donor cell inoculum was obtained from thoracic duct lymph by conventional cannulation procedures.

Cell proliferation as measured by the incorporation of tritiated thymidine proved to be a suitable parameter for measuring the intensity of the LGVHR. This isotope marker also provided a basis for autoradiographic tracer studies. In a typical assay experiment, host rats are pulse-labeled with a single injection of ^{3}H-thymidine 30 min before sacrifice on varying days after injection of the graft. DNA is extracted from an entire kidney by a conventional trichloroacetic acid procedure and the activity in an aliquot of the extract is measured by liquid scintillation counting. Variations of the basic procedure have been used to estimate the effect of experimental manipulation. In earlier experiments significant differences between experimental and control animals were apparent by the 3rd day following grafting and were more marked by days 5 and 7, at which time peak activity occurred. Corresponding cytological and histological events in these kidneys included increased numbers of mononuclear cells, lymphocytes and macrophages, along with a distinct increase in the proportion of medium and large lymphocytes. The conspicuous presence of the lymphocytes is undoubtedly indicative of lymphoblastic transformation. In tracer studies, in which host cells were labeled *in vivo* with ^{3}H-thymidine, it was found that by the 4th day $\leq$ 30% of the blast-like lymphoid cells were of host origin. This is of interest in view of the fact that the LGVHR is supposed to represent an *in vivo* model of a one-way mixed lymphocyte reaction in which host cells provide only the antigenic stimulus to donor cells. It is reasonable to assume that the transformed host cells seen in LGVHR, on the other hand, have the aggressor capacity expected of any similarly transformed donor cells *in vivo* or *in vitro*. This is of more than speculative interest in view of the fact that Elkins and Guttmann[8] presented evidence to indicate that the destruction of kidney tissue in the LGVHR is nonspecific in the sense that a typical lesion was demonstrable in a Lewis kidney which had been transplanted into an F_1 host following the inoculation of Lewis spleen cells under the renal capsule. This finding is of extreme importance with respect to current concepts of transplantation immunity. It was therefore considered important to examine this problem by means of another experimental system. To do so, young adult Lewis rats were exposed to 800 R whole body ^{137}Cs irradiation. One day later they were hemopoietically reconstituted with 10^{7} (Lewis x BN) F_1 bone marrow cells. Thirty days later white blood cell counts were in the range of 5-6,000/mm^{3} which was considered sufficiently high for testing. It should be stressed that these radiation chimeras were genetically Lewis with respect to tissue but (Lewis x BN) F_1 with respect to white blood cells. Lewis thoracic duct cells (10^{7}) were injected under the renal capsules of experimental and control rats in the usual way. As in other experiments the animals were pulse labeled with ^{3}H-thymidine at 5 and 7 days for evaluation of the intensity of the LGVHR. The incorporation of label in the kidneys of the parental genotype were in every way as good as in the control hosts. The destructive lesions in the radiation chimeras were as severe histologically as those seen in control hosts.

One of the other questions under study in the project was whether the macrophages of the host were necessary for the expression of the LGVHR. It was seen, for example, in earlier experiments that approximately 20-30% of mononuclear cells were macrophages, presumably of host origin. Because of the known origin of these cells from bone marrow, a series of experiments were carried out in which heavily irradiated (1200 R whole body irradiation with ^{137}Cs) F_1 hosts were reconstituted systemically with either thoracic duct lymphocytes or bone marrow. The results were difficult to compare because of differing numbers of white cells in each group. Although the results were erratic, it was quite clear that either combination could give rise to a LGVHR following the injection of parental cells under the renal capsule. Better results were achieved when parental and F_1 thoracic duct cells were mixed in vitro and injected into irradiated hosts (Table III).

TABLE III. LOCAL GRAFT VS. HOST REACTION INDUCED IN KIDNEYS OF HEAVILY IRRADIATED F_1 RATS WITH GRAFT OF PARENTAL + F_1 CELLS

SOURCE OF F_1 CELLS	CPM x 10^{-3} KIDNEY, LESS BACKGROUND BY DAY OF GRAFT[a]	
	5	7
Thoracic duct lymph		
Sample size	4	3
Median	56.0	100.1
Range	15.9-80.3	76.3-104.0
Peritoneal exudate		
Sample size		4
Median	ND	9.2
Range		7.4-17.8
Bone marrow		
Sample size	5	5
Median	42.9	55.5
Range	14.7-53.6	54.1-83.5

a. Graft = 2 x 10^7 F_1 cells + Lewis thoracic duct cells. When Lewis cells are injected alone the mean yield of 10 rats by day 7 = 7.54 ± 1.5 CPM x 10^{-3}.

Histologically, a moderate degree of parenchymal destruction was seen in recipients of thoracic duct lymph (TDL). The bone marrow, which was obtained from F_1 donors previously depleted of lymphocytes by drainage of the thoracic duct for 3 days, also gave LGVH reactions although to lesser degrees than those generated by the combination of parental and host lymphocytes. The results obtained with peritoneal exudate cells, however, did not appear to

be significantly higher than those obtained by the injection of parental TDL cells alone. Monocytes could not be identified in smears of the thoracic duct cell inocula, nor could macrophages be found in the lesions. From these data, it can be concluded that the invasive, destructive and proliferative features of the LGVHR can occur in the absence of mononuclear phagocytes. These experiments thus demonstrate aggressor activity in vivo on the part of lymphocytes acting in the absence of macrophages.

Possibly the sole function of the host cells is to provide a source of histocompatibility antigens which are stimulatory to the immunologically competent cells of the donor. Contrarily, there is the previously stated observation that a relatively high proportion of morphologically transformed cells seen in the LGVHR in the intact F_1 appear to be of host origin. Taken with the demonstrable lack of specificity of aggressor cell activity, it is plausible to argue that cells of host origin express effector activity against cells of their own genotype.

Further experimental evidence obtained in this laboratory in the past year reinforces this view. In an attempt to estimate the number of donor cells engaged in proliferation and transformation, or rather participating in the reaction, Lewis thoracic duct cells were labeled in vitro with ^{3}H-uridine. As early as 3 days following inoculation, few of these labeled cells could be identified in the kidney. It was clearly possible that the rate of division was sufficiently rapid to dilute virtually all the label. An alternative possibility was that the donor cells were rapidly leaving the injection site. To detect the emigration of donor cells via lymphatics, the thoracic ducts of presumptive hosts were cannulated before initiating a LGVHR. Donor cells were then labeled with ^{3}H-uridine in vitro and injected under the kidney capsules of hosts whose lymph was collected over periods of 0-6, 0-24 and 24-48 hr. Additionally, the kidneys and the regional lymph nodes between the kidney and the thoracic duct were removed for autoradiographic examination. Labeled cells could be found both in the kidneys and the regional lymph nodes of 6-hr samples, but no labeled cells were found at this time in controls. In the 24-hr collection of lymph, however, approximately 20% of the label was recovered from this compartment. Again, labeled cells could be found in the kidneys and regional nodes. In the lymph collection representing the interval from 24-48 hr, an additional 20% of the inoculum of radioactive cells could be accounted for, and labeled cells were still present in the lymph nodes, although extremely few were found among the increasing numbers of lymphoid cells in the renal cortex. Although further investigations along these lines were necessary, these results suggest that most of the cellular exudate is of host origin and further support the view that host lymphocytes engage in effector activity.

With respect to the macrophage, it is apparent that the requirements differ in the two models studied. This is not surprising in view of the fact that different parameters and end points of effector activity are being

measured. Thus, in dermal delayed hypersensitivity the maximal development of erythema and edema appears to require the presence of macrophages. In the LGVHR, on the other hand, lymphocytes alone can produce a typical lesion.

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LYSOSOMAL RESPONSES DURING INFECTION

Peter G. Canonico, CPT, MSC*

Basic biological research in the 1960's was characterized by substantial advances in cellular biology and enzyme cytology. These advances have made it possible to evaluate cellular and tissue responses by monitoring changes in enzymatic and physical properties of subcellular organelles. A few months ago, we initiated a study designed to evaluate in rat liver, physicochemical properties of mitochondria, lysosomes and peroxisomes in response to infection. Our specific objective was not only to describe the effects of infection on the enzymatic composition and distribution of subcellular organelles, but to demonstrate the use of tissue fractionation techniques to study cellular responses in pathological and physiological processes.

In the study to be described, male, Lewis strain rats were inoculated subcutaneously with 10^7 Diplococcus pneumoniae organisms. At various times after challenge, liver homogenates were assayed for characteristic marker enzymes of organelles, shown in Table I.

TABLE I. CELLULAR LOCALIZATION OF VARIOUS ENZYMES

ENZYMES	CELLULAR LOCALIZATION				
	Mitochondria	Lysosomes	Peroxisomes	Endoplasmic reticulum	Cytoplasm
Cytochrome oxidase	X				
Cathepsin D		X			
β-glucuronidase		X		X	
Acid phosphatase		X		X	X
Catalase			X		X
Urate oxidase			X		

Cytochrome oxidase was used as a marker enzyme for mitochondria. Cathepsin D, β-glucuronidase and acid phosphatase were used as lysosomal markers. It should be noted that a portion of the β-glucuronidase activity in liver is associated with endoplasmic reticulum, while acid phosphatase activity, as determined by the p-nitrophenyl phosphate method, is found in

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the cytosol as well as the endoplasmic reticulum. Peroxisomes are the most recent of cellular constituents to be described. Originally reported as microbodies in 1960, these particles were renamed peroxisomes in 1965 when it was determined that they contained a variety of peroxidative enzymes such as urate oxidase, L-hydroxy acid oxidase, D-amino acid oxidase and catalase.[1] In general, hepatic peroxisomes are about 0.5 μ in diameter; they are bounded by a single membrane, limiting a granular matrix wherein catalase and other peroxidative enzymes are located. In several species, including the rat, a dense core shown to be the location of urate oxidase is found within the peroxisomal matrix. The physiological role of peroxisomes in liver is still unclear; though it has been suggested that peroxisomes may participate in a variety of liver cell functions including gluconeogenesis, reoxidation of NADH and purine degradation. Catalase activity in the liver is also present in the cytosol.

Figure 1 shows the response of these enzymes following pneumococcal infection. The lysosomal enzymes show moderate increases in specific activity. Cathepsin showing the greatest increase, reaches a value of 132% of control at 48 hr.

The specific, as well as total, activities of peroxisomal enzymes, on the other hand, were markedly decreased. After an initial delay, peroxisomal enzyme activities fell precipitously to 16% for catalase and 28% for urate oxidase (Urox.) of control values by 48 hr. Bacteremia in these animals was observed between 18-24 hr, death usually occurred between 52-56 hr after infection.

To characterize the mutability of the physical and chemical properties of cellular organelles during the course of infection, liver homogenates were subjected to isopycnic zonal ultracentrifugation. Such centrifugation was carried out as shown diagrammatically in Figure 2: 40 ml of an overlay solution followed by 40 ml of a liver post-nuclear supernatant were introduced into the rim of a Titanium B XIV zonal rotor. This was followed by a linear 29-59% sucrose gradient and a 50-ml cushion of 61% sucrose. Centrifugation was for 150 min at 35,000 rpm. Thirty-three 20-ml fractions were collected by displacement of the gradient with 63% sucrose. Fractions were analyzed for enzymatic activities and the corresponding values recorded on cards for computer analysis.

The development of a computer program for analyzing these data represents a significant investment of our research efforts. It has, as a consequence, yielded an important tool for the practical manipulation of the large amounts of data generated by zonal centrifugation experiments.

Our program was adapted from one originally developed and supplied to us by Dr. Pierre Baudhuin of the Physiological-Chemical Laboratory at the University of Louvain, Belgium. The program gives a listing, as well as histographic representations of experimental data in terms of percent of

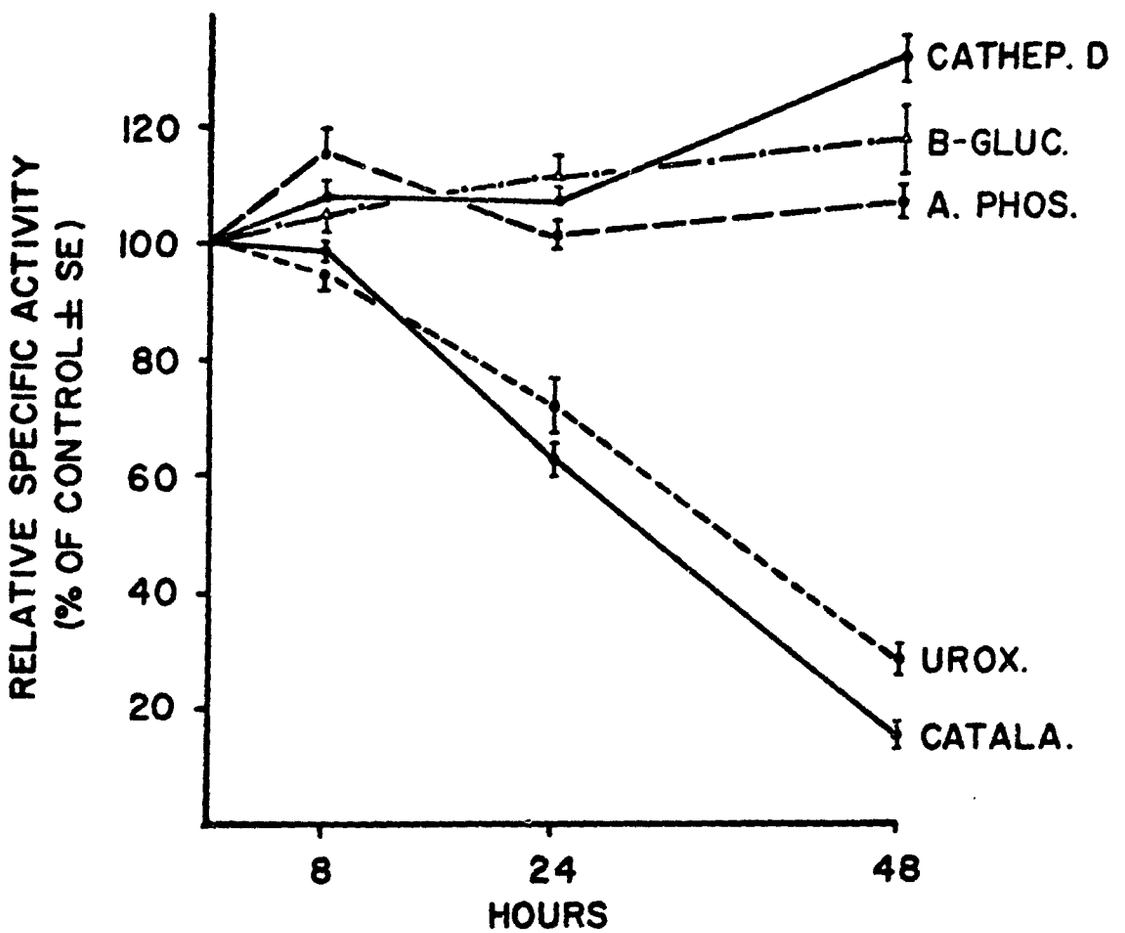

FIGURE 1. SPECIFIC ACTIVITY OF LIVER LYSO- AND PEROXISOMAL ENZYMES FOLLOWING *D. PNEUMONIAE* CHALLENGE WITH 10^7 CELLS (SQ).

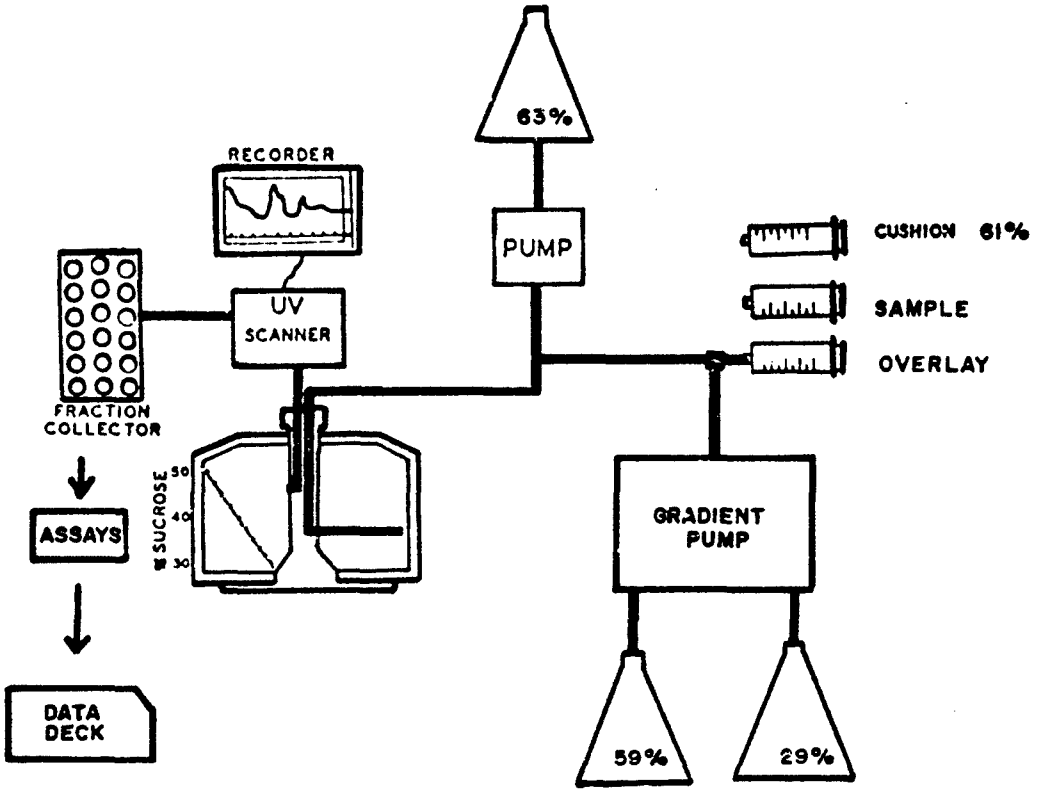

FIGURE 2. LAY-OUT OF APPARATUS FOR ISOPYCNIC CENTRIFUGATION.

total activity/fraction; specific activity/fraction; frequency of activity as a function of density; and finally, as a standardized frequency versus density (Figure 3). The histograms which follow are respresentations of data in the last form, and are commonly referred to as "standardized equilibrium density distribution patterns." These same patterns can be stored by the computer for future statistical analysis of the accumulated data from a number of like experiments. In addition, the program determines the percent recovery as well as the mean distribution for each enzyme. A plot of the sucrose gradient is also superimposed on the first two histograms. While the computer will calculate these results for 7 enzymes in 4.5 min, similar manual calculations might require as much as 18 hr.

Equilibrium density distribution patterns (+ SEM) of enzymes and proteins in control animals are shown in Figure 4. In these graphs soluble nonsedimentable activity is represented by a block having an equilibrium density < 1.12. As judged by the designated standard errors of the mean, the procedures we have employed yield reproducible equilibrium density patterns. Lysosomal enzymes show a characteristically broad density distribution with a mean density of approximately 1.20. The nonsedimentable activity which is observed is probably the result of some lysosomal rupturing during the homogenization procedure β-glucuronidase in addition shows a bimodal distribution. We suspect that the activity in the lesser peak represents that portion of the enzyme associated with endoplasmic reticulum. The distribution of cytochrome oxidase illustrates the homogeneic nature of liver mitochondria having a mean equilibrium density of 1.198. Peroxisomal enzymes show relatively broad equilibrium density patterns with a mean density of 1.225. As previously noted, a substantial portion of the catalase activity is found in the soluble phase of the cell.

The equilibrium distribution pattern 8 hr after inoculation of D. pneumoniae is shown in Figure 5. The areas described by the fine lines represent the experimental histograms and are superimposed over control values. The area delineated by the experimental histograms are adjusted so as to reflect changes in the total activity of the enzyme with respect to controls. At this time period, we find no significant changes in the distribution patterns of the mitochondrial or lysosomal markers except for a slight increase in the soluble activity of the acid hydrolases. On the other hand, catalase shows a moderate decrease in its soluble activity. Peroxisomal markers show an unusual bimodal distribution which is accompanied by an increase in the frequency of protein at the corresponding peroxisomal density. The significance of this bimodal distribution is presently unclear, though it could possibly be due to the synthesis of new peroxisomal particles.

There are essentially no changes in the distribution pattern of lysosomal enzymes 24 hr after challenge (Figure 6). A broadening of the mitochondrial distribution pattern, suggests that the structural integrity of mitochondria is being compromised. Single peaks are again observed in the distribution patterns of particle-bound catalase and urate oxidase, though their total activities are considerably reduced. The non-sedimentable portion of catalase activity has diminished to approximately 50% of control.

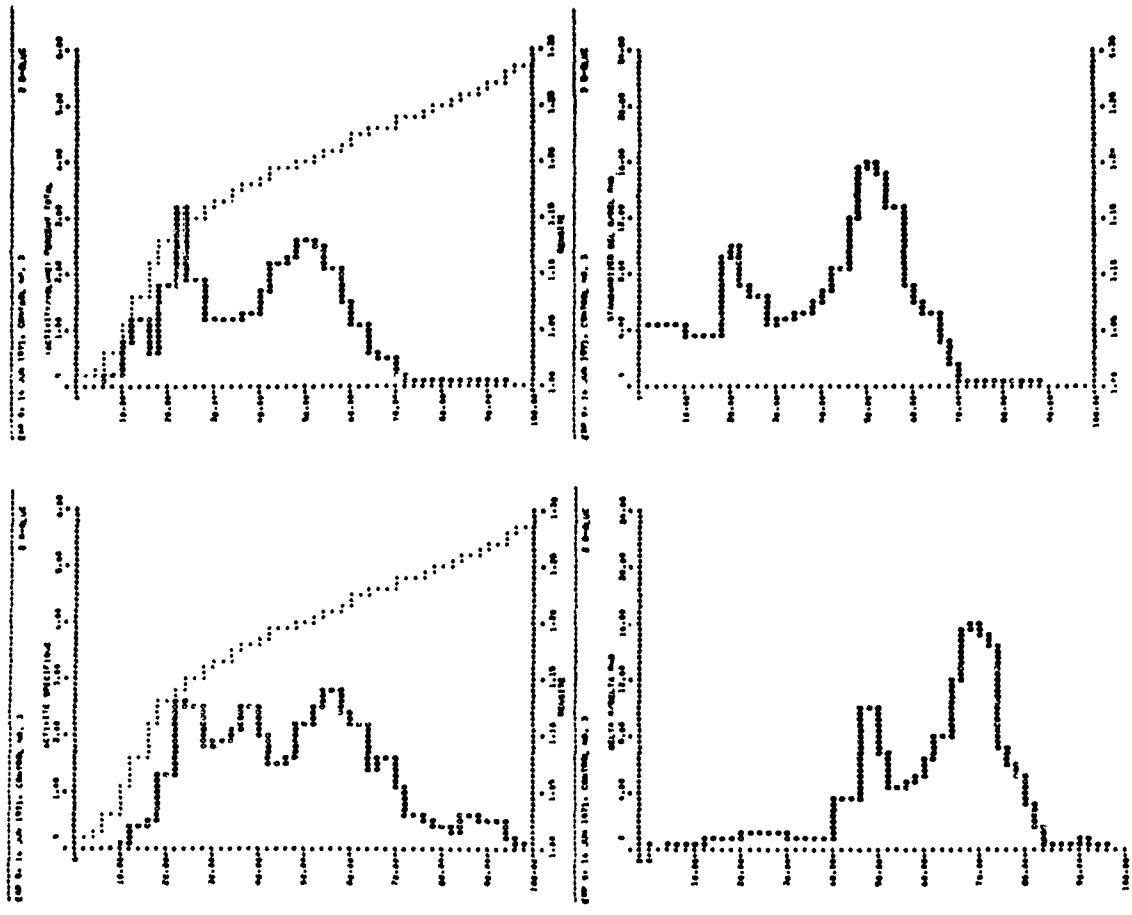

FIGURE 3. COMPUTER-GENERATED HISTOGRAMS. UPPER LEFT: % TOTAL ACTIVITY VS. FRACTION; LOWER LEFT: SPECIFIC ACTIVITY VS. FRACTION; LOWER RIGHT: FREQUENCY OF ACTIVITY VS. DENSITY; UPPER RIGHT: STANDARDIZED FREQUENCY VS. DENSITY.

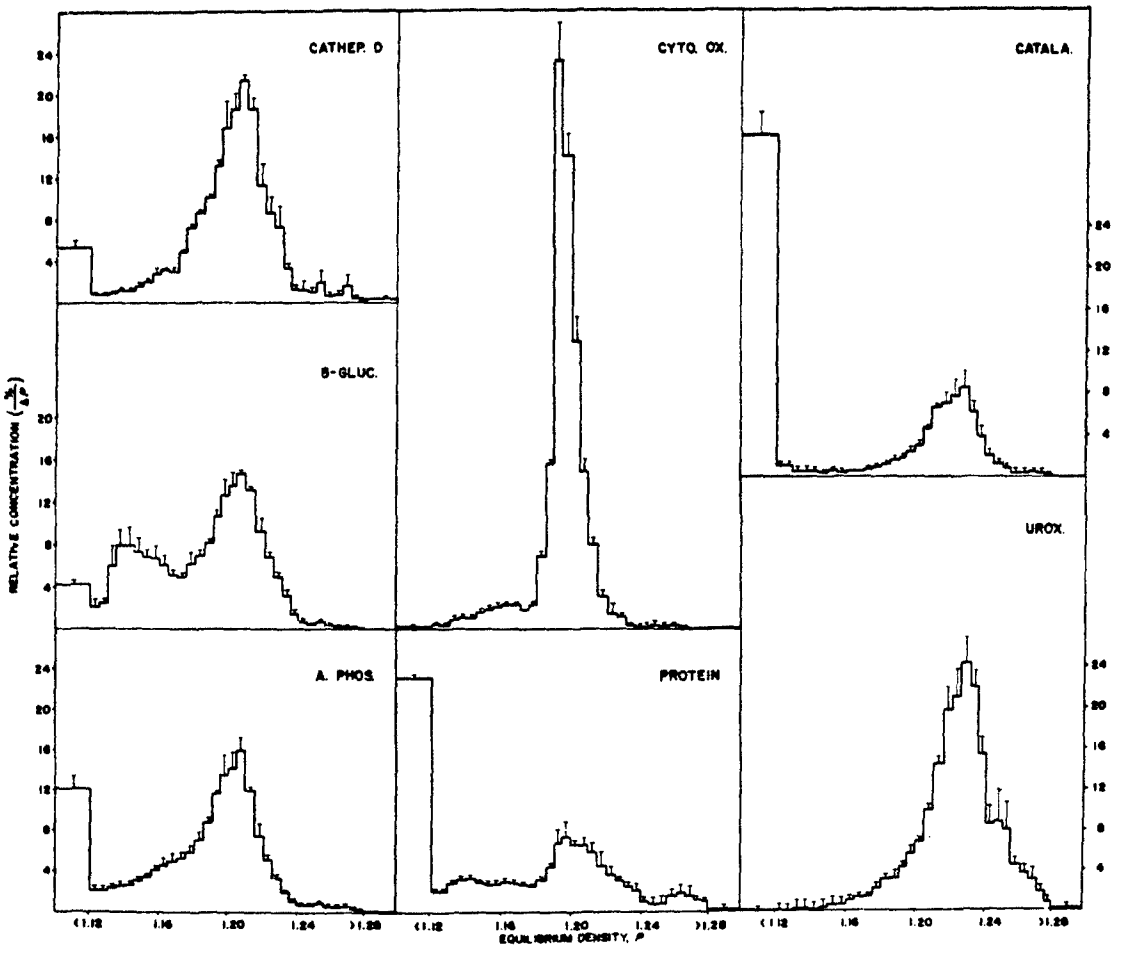

FIGURE 4. EQUILIBRIUM DENSITY DISTRIBUTION OF LIVER ENZYMES OF 3 RATS (± SEM).

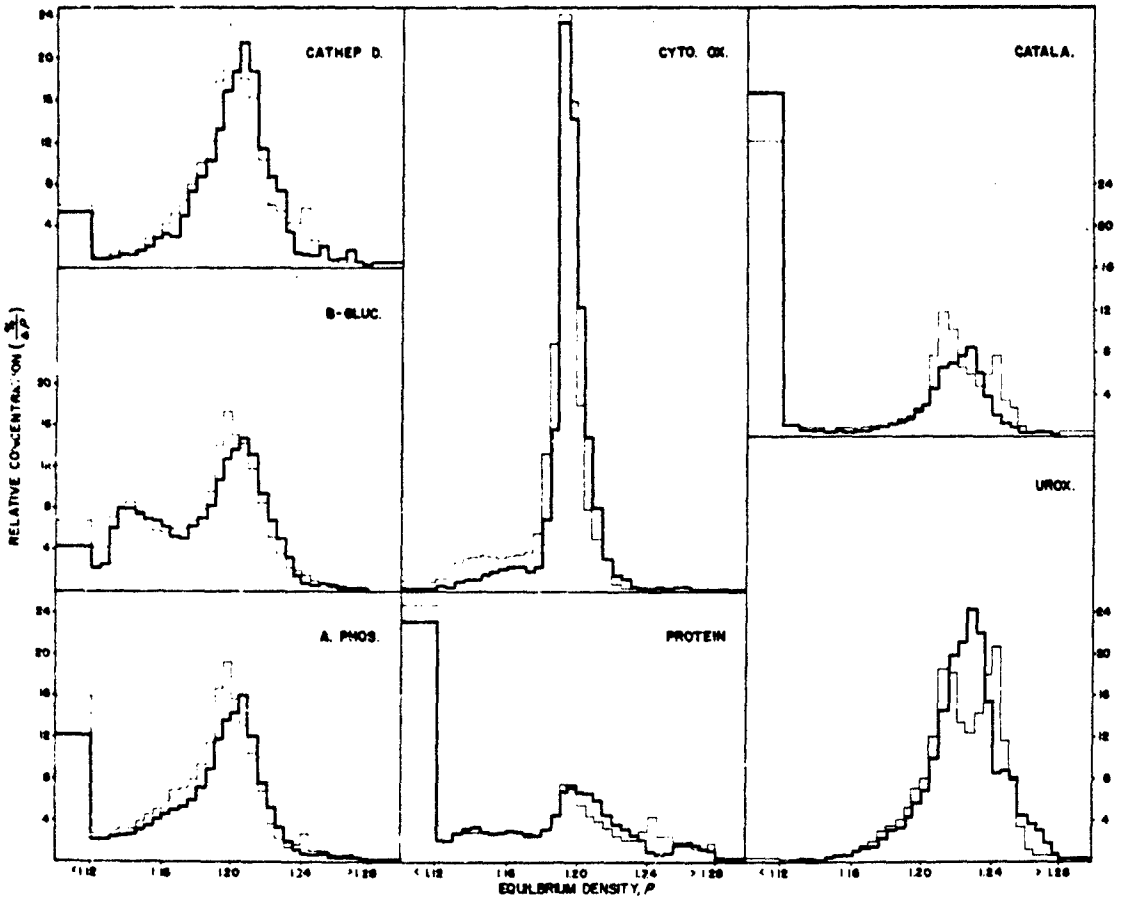

FIGURE 5. EQUILIBRIUM DENSITY DISTRIBUTION OF RAT LIVER ENZYMES 8 HR AFTER *D. PNEUMONIAE* INFECTION.

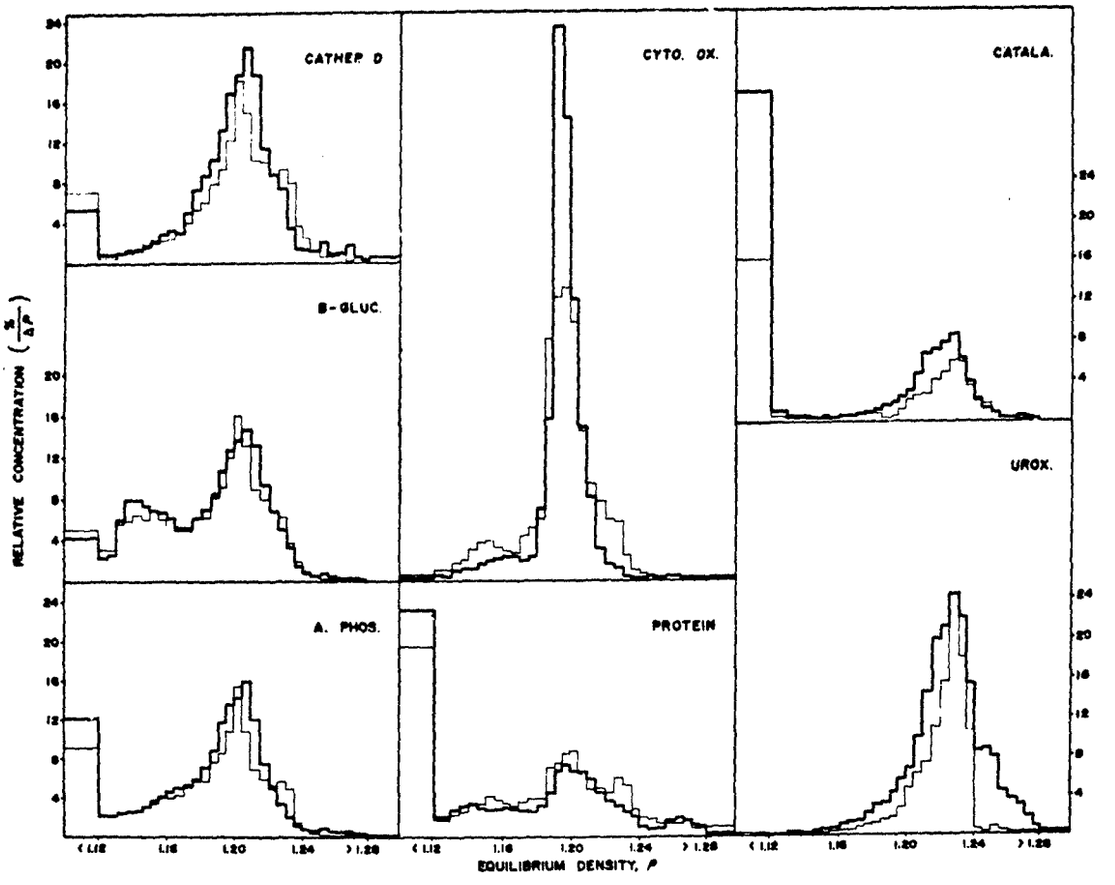

FIGURE 6. EQUILIBRIUM DENSITY DISTRIBUTION OF RAT LIVER ENZYMES 24 HR AFTER *D. PNEUMONIAE* INFECTION.

At 36 hr, there is additional loss of soluble catalase and peroxisomes (Figure 7). The mean density of the distribution pattern of the remaining peroxisomal particles has, in addition, been shifted to a higher density. Further structural degeneration of mitochondria results in a greater heterogeneity of their distribution pattern. Relative to the hydrolases, the most conspicuous observation is the loss of the minor sedimentable component of β-glucuronidase.

Some initial observations from a contrasting study using the living vaccine strain of Franciscella tularensis (LVS) have been made. The striking feature of this experimental model is that there is no precipitating loss of peroxisomal enzymes as observed during pneumococcal infection (Figure 8). Rats are relatively resistant to LVS and survive an intraperitoneal challenge of 10^9 organisms, although we were able to culture LVS organisms from the livers of all animals at 24 hr. Recovery from the infection is indicated not only from the enzymatic activities which tend to return to normal at 48 hr, but also from the fact that less than half of the animals had positive liver cultures at this time.

The equilibrium density distribution patterns 8 hr after inoculation of LVS are shown in Figure 9. There is an increase in soluble hydrolase activity and a skewness of the distribution patterns toward higher densities. There is also a noticeable decrease in the β-glucuronidase activity of the minor sedimentable component. There are no discernible changes in the mitochondrial pattern. Catalase shows a 50% decrease in its soluble activity, while peroxisomes seem to demonstrate a greater homogeneity in their distribution.

At 24 hr (Figure 10) we primarily note the loss of the minor sedimentable component of β-glucuronidase, accompanied by a $2\frac{1}{2}$-fold increase in its soluble activity. An increased heterogeneity in the mitochondrial distribution pattern is also observed.

There is no additional loss of soluble catalase. While a slight reduction in the number of peroxisomal particles is suggested by the distribution patterns of catalase and uroxidase, their mean equilibrium density does not differ from controls. These observations stand in contrast to the effects of pneumococcal infections, where, as noted previously, the loss of activity in the minor sedimentable component of β-glucuronidase coincided with (a) substantial reductions in the enzymatic activities of peroxisomes and (b) increase in their mean equilibrium density.

Shown in Figure 11 are patterns obtained 48 hr after LVS inoculation. A recovery of the mitochondrial structural integrity is suggested by the more homogeneous distribution observed at this time. Peroxisomes show a normal distribution as well, although soluble catalase and the minor sedimentable component of β-glucuronidase remain substantially reduced.

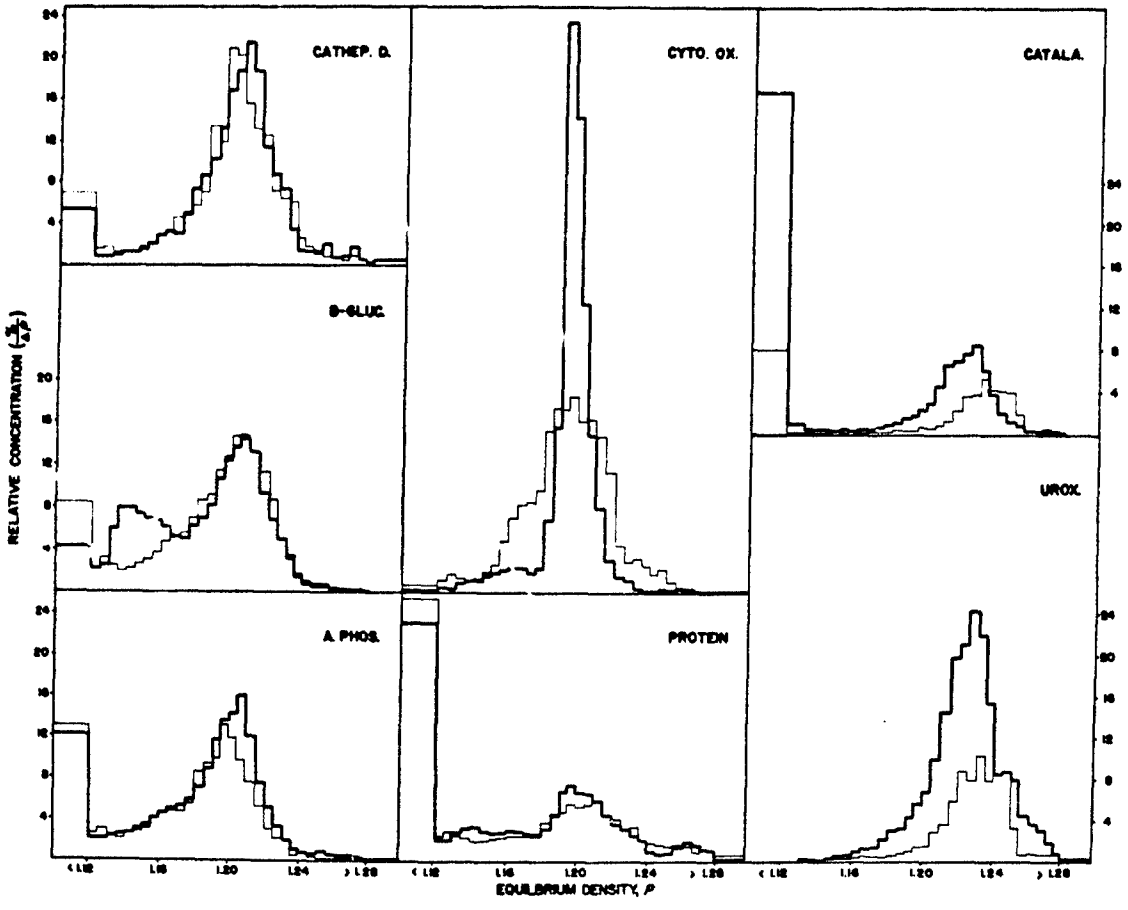

FIGURE 7. EQUILIBRIUM DENSITY DISTRIBUTION OF RAT LIVER ENZYMES 36 HR AFTER *D. PNEUMONIAE* INFECTION.

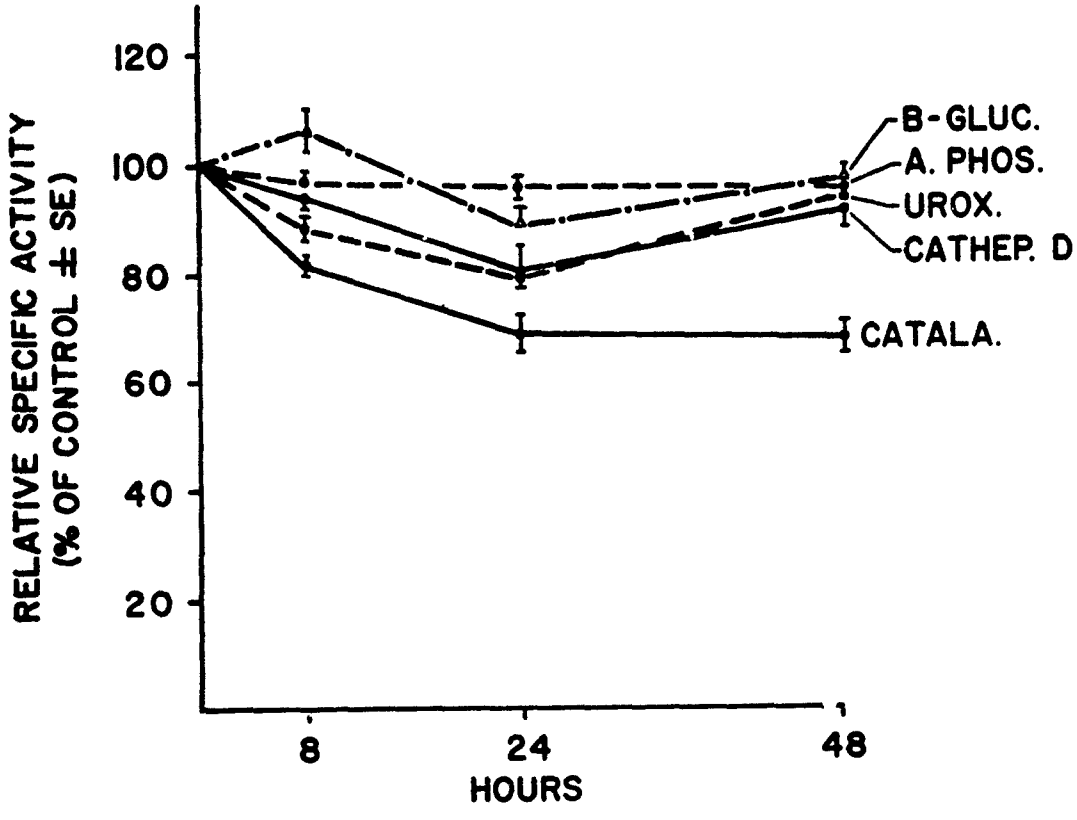

FIGURE 8. SPECIFIC ACTIVITY OF LIVER LYSO- AND PEROXISOMAL ENZYMES FOLLOWING LVS INFECTION (10^9 CELLS IP).

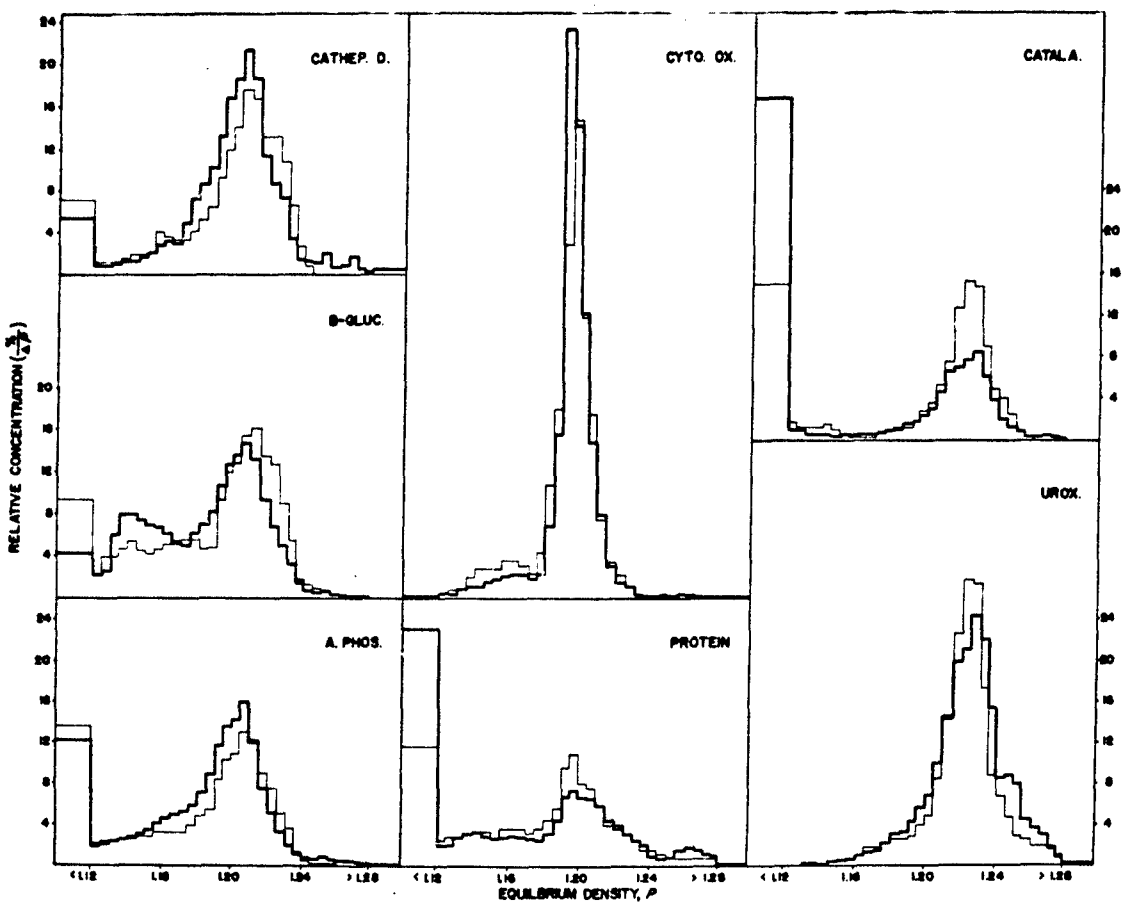

FIGURE 9. EQUILIBRIUM DENSITY DISTRIBUTION OF RAT LIVER ENZYMES 8 HR AFTER LVS.

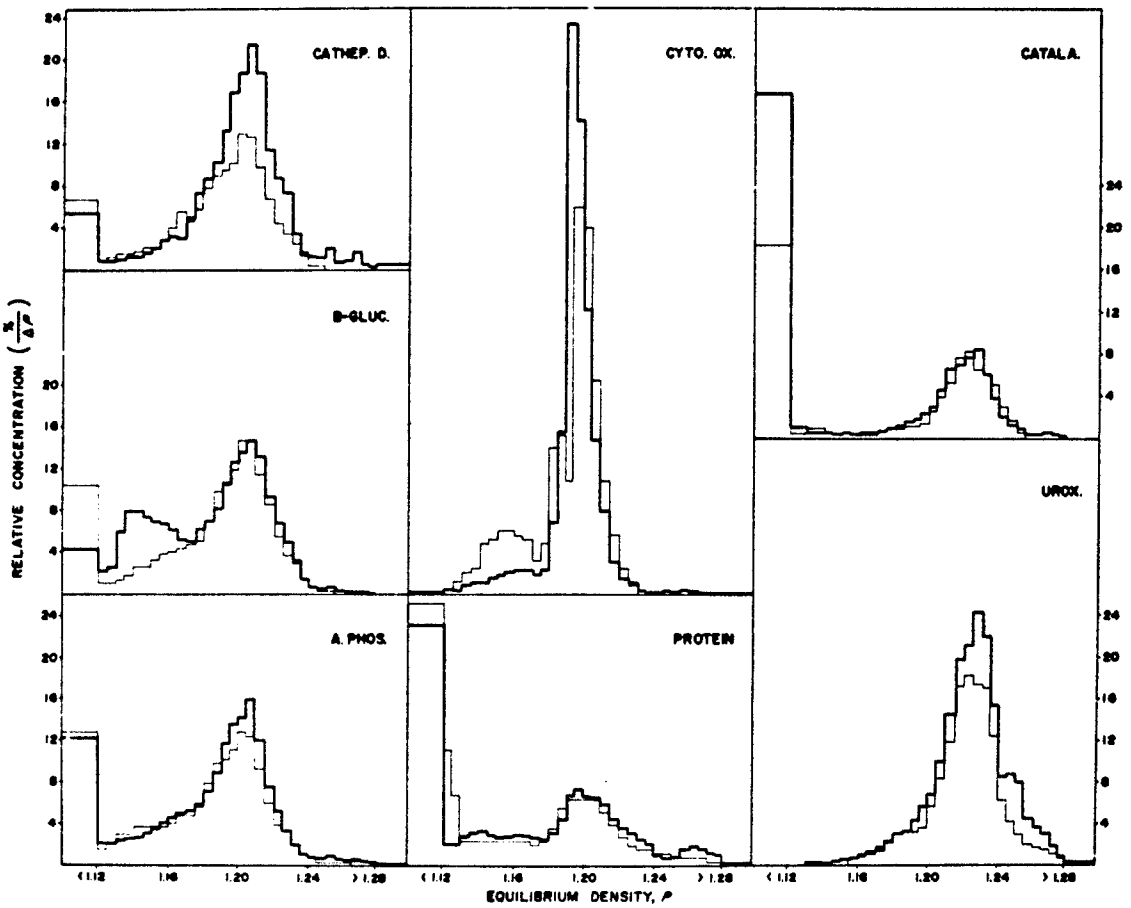

FIGURE 10. EQUILIBRIUM DENSITY DISTRIBUTION OF RAT LIVER ENZYMES 24 HR AFTER LVS.

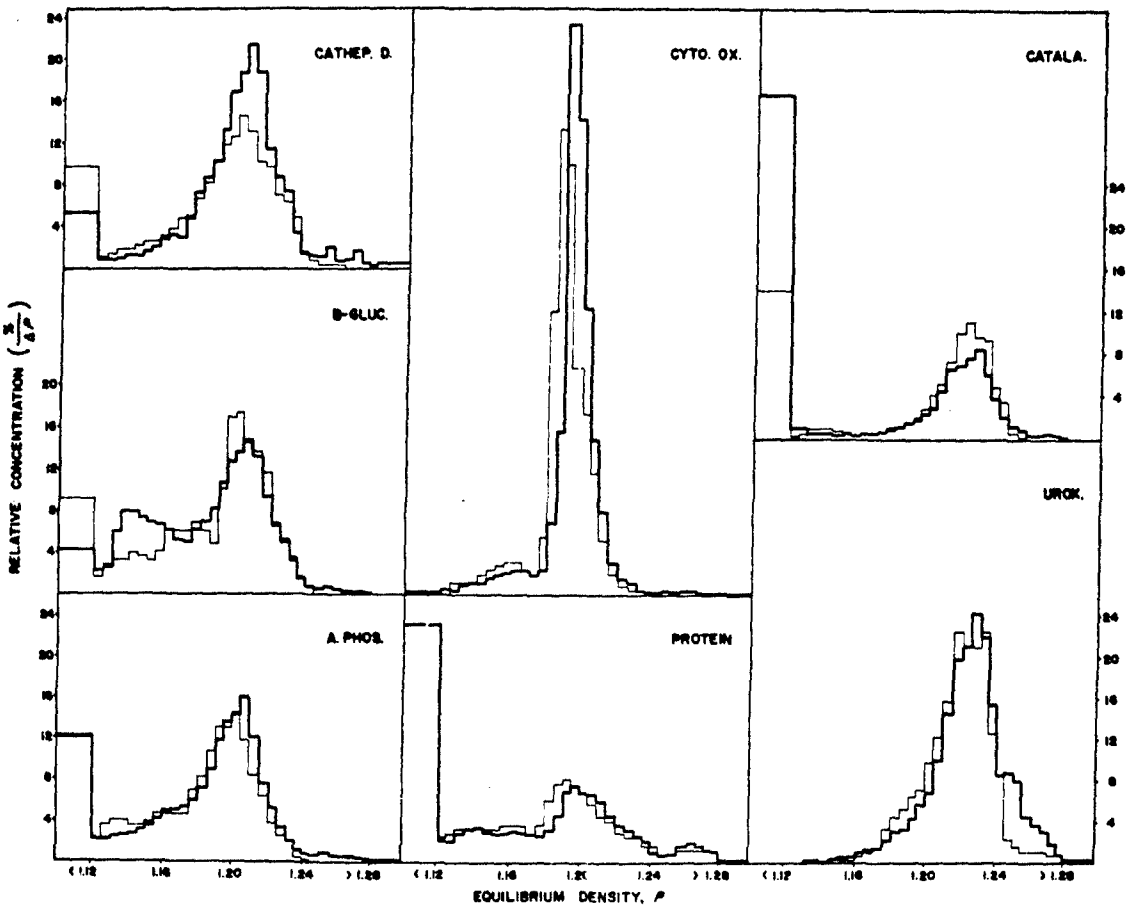

FIGURE II. EQUILIBRIUM DENSITY DISTRIBUTION OF RAT LIVER ENZYMES 48 HR AFTER LVS.

Complete interpretation and discussion of these data are not only beyond the time limits of this presentation, but must await the completion of experiments now in progress. These experiments will include the determination of distribution patterns of liver organelles based on sedimentation coefficients in a manner which will complement the equilibrium density distribution patterns presented here.

The data suggest that following the inoculation of rats with D. pneumoniae or LVS, a number of changes in the physico-chemical properties of liver organelles occur that can be observed by tissue fractionation techniques. The changes that have been observed include alterations in the structural integrity of mitochondria, distribution and turnover of peroxisomes, size and fragility of lysosomes and enzymatic composition of endoplasmic reticulum and cytosol.

It is our intention to extend similar studies to more clinically accessible tissues such as peripheral white blood cells for the purpose of assessing the practicability and usefulness of tissue fractionation techniques in the diagnosis of infectious diseases. It is hoped that this presentation served primarily to demonstrate the promise of tissue fractionation and zonal ultracentrifugation techniques for evaluating cellular responses and obtaining a greater understanding of the pathogenesis of infection.

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HYPERCHOLESTEROLEMIA AND ALTERED IMMUNITY

William R. Beisel, M.D.*

In contrast to the many recent advances in our knowledge of cellular and humoral immunity, the nonspecific mechanisms of host defense against infection remain poorly understood. Included within the category of nonspecific mechanisms are the many metabolic responses which occur during the incubation period of an infection and throughout the days of ensuing illness and convalescence. Our continuing investigations of the metabolic response have shown over the past 3 years that many body lipids play a fundamental role during an infection, a role that supplies the major source of energy for other body functions.

Presented here are a new group of findings which suggest that the metabolism of several important lipids may also influence the adequacy of humoral and cellular immunological defense mechanisms. While preliminary in nature they are considered to be of potential importance.

It should first be pointed out that nutritional deficits are generally believed to reduce the ability of an individual to resist infection. In contrast, it is not widely known that host resistance against experimentally-induced infections may be diminished, as well, by the presence of obesity or by the long-term ingestion of a diet containing abnormally high quantities of fat. Such a conclusion has been reached by investigators who studied either bacterial or viral infections in different species of laboratory animals.[1] However, no indication was given of the mechanism by which a lipid excess was detrimental to the host.

The present study was conducted chiefly by Doctors Fiser and Denniston who have both left the Institute. Other individuals who collaborated in specific aspects of the study included Doctors Kaplan, McGann, Adler and Kastello.

The first studies defined a number of changes in lipid metabolism during experimentally-induced infections in rhesus monkeys. The present study was designed primarily to determine if similar changes in lipid metabolism would occur if a similar infection was initiated in monkeys with long-standing abnormalities of their body lipid composition.

A diet containing excess fat and cholesterol was found by Armstrong's group[2] to produce chronic hypercholesterolemia in monkeys. This diet contains both crystalline and egg yolk cholesterol. Eight monkeys fed the diet and an equal number of control monkeys were fed a normal biscuit diet

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(Table I). Average body weight of monkeys on the cholesterogenic diet remained similar to the average of the control group, and serum protein concentrations remained normal. In addition to special metabolic studies, all monkeys of both groups were tested before and during an acute, experimentally-induced infection for evaluation of their immunological competence.

TABLE I. COMPARISON OF DIETS FOR MONKEYS

COMPONENT	NORMAL %	CHOLESTEROLEMIA-INDUCING %
Protein	20	15
Carbohydrate	76	44
Fat	4	41 (including added cholesterol)

After a period of 3 mon on the high lipid diet, baseline serum lipid values had indeed become abnormal (Table II). In comparison to values of the

TABLE II. PLASMA LIPID CONCENTRATION VALUES (± SEM) OF MONKEYS 24 HR POST-INFECTION

DIETARY GROUP	BASELINE	INFECTION	CHANGE	P
	mg/100 ml			
High Fat Cholesterol (8)				
Cholesterol	412 ± 38	305 ± 37	-107	< 0.001
Phospholipid	582 ± 42	450 ± 54	-132	< 0.01
FFA	25 ± 2	23 ± 3	- 2	NS[a]
Triglyceride	34 ± 5	70 ± 8	± 36	< 0.05
Normal (8)				
Cholesterol	123 ± 4	77 ± 3	- 46	< 0.001
Phospholipid	368 ± 32	278 ± 41	- 90	< 0.05
FFA	20 ± 1	20 ± 3	± 0	NS
Triglyceride	85 ± 13	42 ± 8	- 43	NS

a. Not significant.

monkeys fed a normal diet, serum cholesterol and phospholipid values were significantly elevated and triglyceride concentrations were depressed. These abnormalities persisted during an addition 4-mon maintenance on the diet. Acute infection was then initiated in control and hypercholesterolemic

monkeys by intravenous inoculation of 10^8 virulent Type I Diplococcus pneumoniae. Within 24 hr, all monkeys of both groups developed fever, pneumococcal bacteremia and 2- to 4-fold increases in their neutrophile counts. The serum lipids for the control group changed rapidly during the first day of pneumococcal infection. The hypercholesterolemic monkeys also had decreases in serum cholesterol and phospholipid values as early responses to the infection, but the hypercholesterolemic monkeys developed significant increases (rather than a decrease) in triglyceride values. All monkeys were killed 27 hr postinoculation for special studies of tissue lipids. Atypical patterns of fat mobilization were seen in the hypercholesterolemic group. During the brief period of infection, the clinical responses of both groups were equivalent. There were no differences between groups with respect to gross or microscopic evidence of infection. No monkey had tuberculosis.

The far reaching aspects of the study, however, became evident during evaluation of immunological responsiveness. Once hypercholesterolemia was fully documented in the experimental monkeys, all monkeys of both groups were inoculated subcutaneously with 15 mg of ovalbumin emulsified in complete Freund's adjuvant. Three weeks later, as shown in Figure 1, all were given a subcutaneous booster dose of 15 mg ovalbumin in normal saline. Although the appearance of hemagglutinating antibodies to ovalbumin was similar in both groups, the hypercholesterolemic monkeys (shown as squares) exhibited an altered progression of precipitin antibody responses. During 6 weeks of study, 7 of 8 control monkeys (shown as circles) developed high titer precipitin antibodies as shown in closed circles; the control monkeys generally showed precipitin bands against more than one component of the ovalbumin. In contrast, only 4 hypercholesterolemic monkeys developed precipitin antibodies during this period as shown in the closed squares, and none showed a multiple band response.

Other findings suggested an altered immunological status of the hypercholesterolemic monkeys prior to initiation of acute infection (Table III). Three mon after receiving their ovalbumin booster, all monkeys were skin-tested with 5 μg ovalbumin. A delayed positive reaction, with an area of induration > 10 mm in diameter at 24 and 48 hr, occurred in an equal proportion of monkeys in both groups. Several of the control monkeys, however, displayed combined Arthus reactions during a 4-hr period after skin testing in contrast to only one of the hypercholesterolemic group.

Tuberculin skin tests with 0.1 ml intermediate strength PPD were negative in all monkeys prior to the initiation of the diet, but 5 hypercholesterolemic monkeys converted to a positive tuberculin skin test 2 mon after being immunized with ovalbumin in Freund's adjuvant. Skin tests for tuberculosis in these monkeys remained positive thereafter, whereas all tuberculin skin tests remained negative in the control monkeys on the normal diet (Table III).

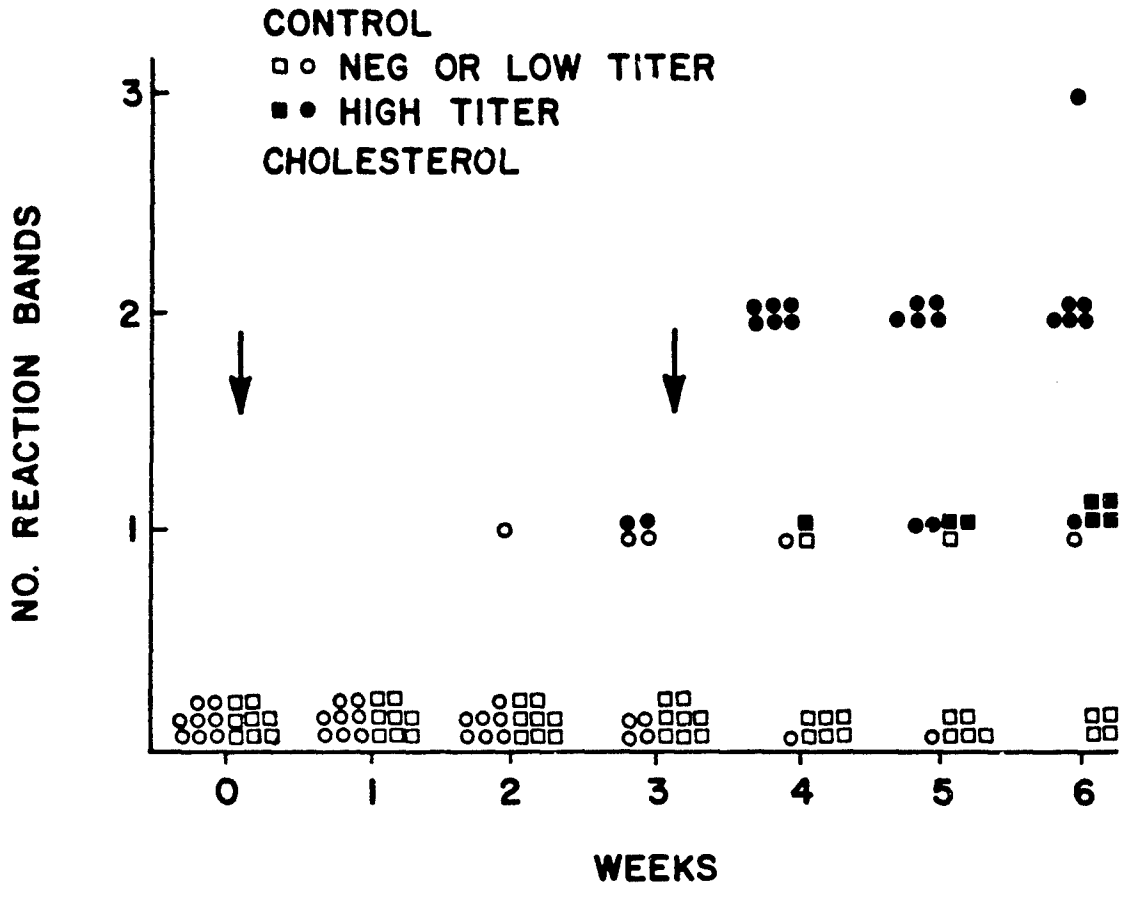

FIGURE 1. PRECIPITATING ANTIBODY RESPONSES IN CONTROL AND HYPERCHOLESTEROLEMIC MONKEYS. 15 mg OA GIVEN WEEKS 0 AND 3 ↓.

TABLE III. RESPONSE OF MONKEYS PRIOR TO INFECTION

IMMUNE MECHANISMS	NO./TOTAL		P
	Normal Diet	Hypercholesterolemia	
Positive TBC skin test	0/8	5/7	< 0.01
HA response to ovalbumin (OA)	7/8	6/8	NS[a/]
Primary precipitin response to OA	4/8	0/8	< 0.05
Multiple precipitin bands to OA	5/8	0/8	< 0.01
Anamnestic response to OA booster	8/8	4/8	< 0.05
Immediate skin test reaction to OA	4/6	0/6	< 0.02

a. Not significant

Specific immunological differences between groups were also noted after the initiation of pneumococcal infection. When tested 24 hr after inoculation with virulent pneumococcal organisms, leukocyte preparations from the control monkeys showed an average 13% incidence of neutrophiles that reduced nitro-blue tetrazolium (NBT) dye. The average value was significantly higher in the hypercholesterolemic monkeys, with 24% of their cells reducing NBT. Greater than 20% dye reduction was noted in 5 of 6 monkeys in the high cholesterol group. Uninfected monkeys on a normal diet averaged only 3 to 4% of cells with a capability for reducing NBT.

Figure 2 depicts the regression lines (with 95% CL) of values for the clearance of colloidal carbon from blood when the test was performed in monkeys 24 hr after their inoculation with pneumococci. During infection, the rates of reticuloendothelial system (RES) clearance in both groups were significantly increased above rates for normal controls; however the infected hypercholesterolemic monkeys had a significantly smaller increase in the clearance time for colloidal carbon than the average increase measured in the group of infected control monkeys.

These findings indicated that experimentally-induced hypercholestermia in monkeys was associated with atypical humoral and cellular immunodefensive responses. The present investigations were exploratory in nature and provided only limited information about each specific immune response studied. Nevertheless, when the present findings were taken in their entirety, two distinct patterns of immunological responsiveness seemed evident. The hypercholesterolemic group of monkeys clearly differed from the control group in various responses to different antigens and to infection itself.

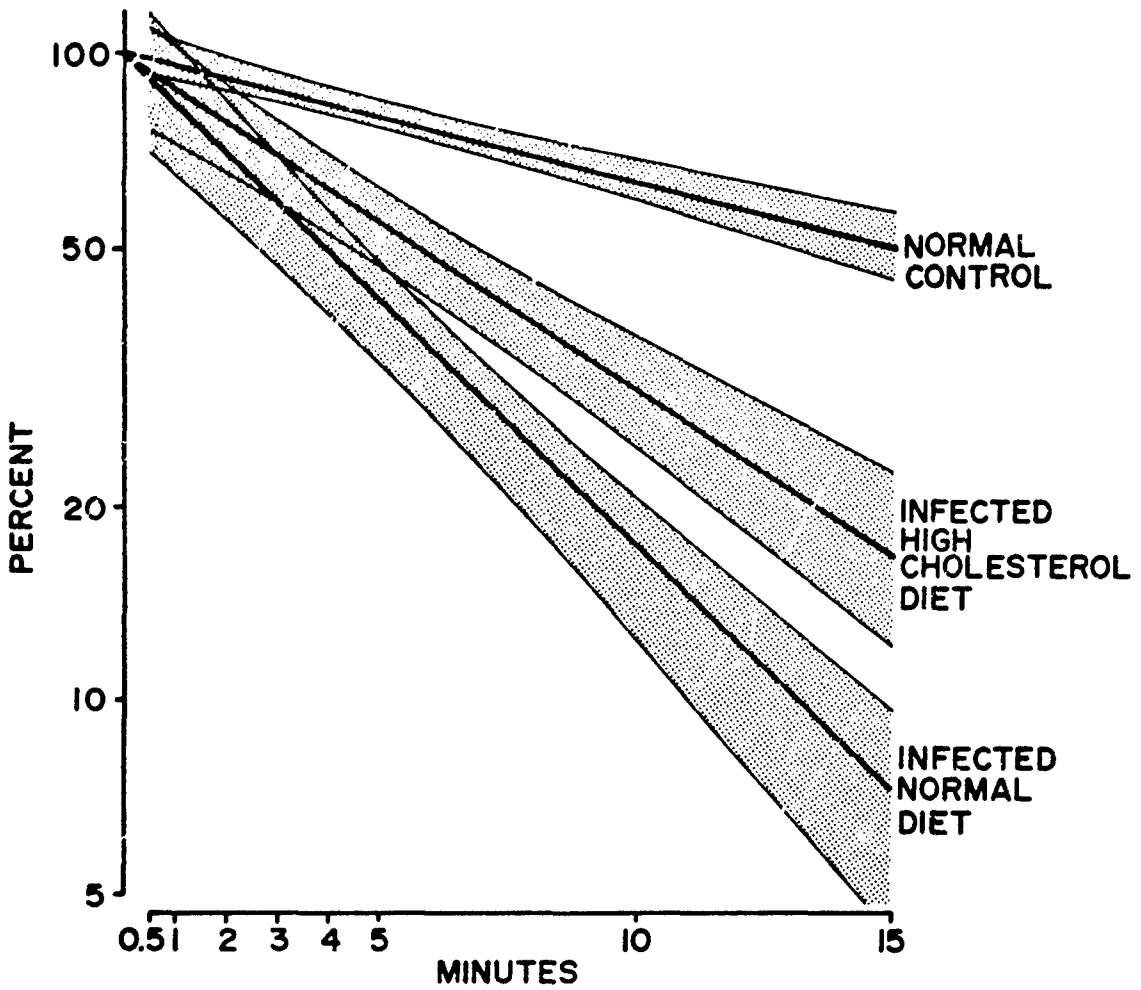

FIGURE 2. CLEARANCE OF COLLOIDAL CARBON FROM BLOOD OF MONKEYS.

A few scattered reports in early literature describe alterations of immune phenomena or phagocytosis that may follow the experimental induction of hypercholesterolemia.[1] Chassin and Bruger[3] suggested in 1939 that the serological responses to typhoid antigens were slightly enhanced in rabbits given a high cholesterol diet. In contrast, our hypercholesterolemic monkeys showed a diminished precipitin response to ovalbumin. On the other hand, the positive PPD reactions in hypercholesterolemic monkeys seemed to relate to an increased sensitivity to the Mycobacterium contained in complete Freund's adjuvant. Another possible cause, active tuberculosis, was eliminated from consideration in these monkeys by histologic examination and by culture. An equal incidence of positive delayed skin test responses to ovalbumin in both dietary groups implied that only certain antigens might produce an increase in delayed hypersenstivity. Differences between the response to tuberculin or ovalbumin could relate to differences in antigen processing.

DiLuzio[4] demonstrated in 1960 that the RES was involved in the clearance of lipids from blood. However, he found no significant impairment of RES function during brief periods of alimentary lipemia in dogs. Stuart and Davidson[5] showed that an intravenous injection of 30 mg cholesterol oleate into mice caused a depression of RES function along with a reduction in antibody formation if an antigen was given during the period of RES depression. The intravenous injection of milligram quantities of methyl palmitate into mice was subsequently found by DiLuzio's group[6] to delay RES clearance of colloidal carbon for many days, thereby depressing the immunological response to particulate antigens.

In contrast to the changes in RES function induced by massive injections of pharmacologic quantities of lipids into the blood stream, the changes in RES function observed by us developed as a consequence of a long-term dietary manipulation. The present findings indicated that chronically hypercholesterolemic monkeys were unable to achieve a magnitude of increase in rates of RES clearance of colloidal carbon comparable to that observed in control monkeys when each group was studied during acute pneumococcal infection.

An increase in rates of RES clearance was reported by Wagner et al[7] to occur during bacterial infections of man. Thus, an increase in phagocytic activity by the RES appears to be an appropriate host response to acute bacterial infection, a response that is blunted by the presence of excess cholesterol in the serum.

Because all our monkeys were killed at a single time for specific lipid studies, no information was obtained concerning the overall effect of hypercholesterolemia on survival from the specific challenge. Accordingly, the observed differences in immune responsiveness could not be related in this study to a change in overall host resistance or to survival. However, altered immune function may contribute to the impairment of host resistance reported in the literature to be associated with an excess of body or dietary lipid.

A sizable portion of the American population has an overt or unrecognized derangement of body lipid metabolism, a familial or acquired abnormality that may range from simple obesity to a complex dysfunction of lipid enzyme and transport systems. Do any of these abnormalities increase the susceptibility of an individual to infection? Do they diminish or inhibit the normal response to antigens used in our vaccination programs? The findings of this study in rhesus monkeys suggest that such questions should be asked, and that an effort should be made to evaluate the actual magnitude and importance of this relationship in man.

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ENDOGENOUS MEDIATORS OF NONFEBRILE HOST RESPONSE

Robert S. Pekarek, Ph.D.*

It has been well established that the inflammatory process, whether induced by invading microorganisms or other particulate or toxic substances, produces significant alterations in the metabolism of many biochemical parameters in the host. In a recent series of prospective clinical studies in volunteers by our Institute, significant alterations in trace metal and amino acid metabolism were observed early in the prodromal period and before the onset of any overt or detectable illness of either acute bacterial or viral infections.[1-4]

Figure 1[5] presents the data obtained serially from a prospective study in a group of volunteers with experimentally-induced sandfly fever (Medical Division Protocol 70-1), a benign self-limited viral infection.[4] Febrile illness started by the end of the 2nd day postexposure and peaked by day 3. Serum Fe and Zn concentrations fell precipitiously just prior to the onset of febrile illness, followed by a slow rise in serum Cu values. Total plasma amino acids, especially those amino acids thought to be essential for protein synthesis, mainly the branched-chain group of valine, leucine, and isoleucine, were significantly depressed by the beginning of the 2nd day. Although anorexia accompanies febrile illness, the decreases in serum Fe and Zn and total plasma amino acids occurred before any decrease in dietary intake. It may be further noted, that maximal depressions in these 2 serum metals and plasma amino acids were reached at the peak of febrile illness.

This study, as well as others, suggests that the observed infection induced alterations in both trace metal and amino acid metabolism may be activated as part of the host's early response to infection or toxemia. Until recently the mechanism, or mechanisms, by which these very early infection-induced metabolic alterations are triggered or mediated have remained unknown. There is now considerable evidence that most, if not all, microbial agents produce fever indirectly by stimulating the liberation of a circulating endogenous pyrogen from tissues of the host.[6] Consequently, we theorized that the observed metabolic changes in trace metal and amino acid metabolism also may be mediated through some humoral mechanism during infection or other stressful conditions. If this is true, then such an endogenous factor could possibly be shown to be present in the serum at sometime after infection or intoxication. In a recent series of studies in a variety of laboratory animals, we reported that an endogenous mediator of serum Zn and Fe depression was present in the serum of these animals 2 hr after infection or endotoxin intoxication, and in high enough concentration such that its Zn- and Fe-depressing effect could be transferred to

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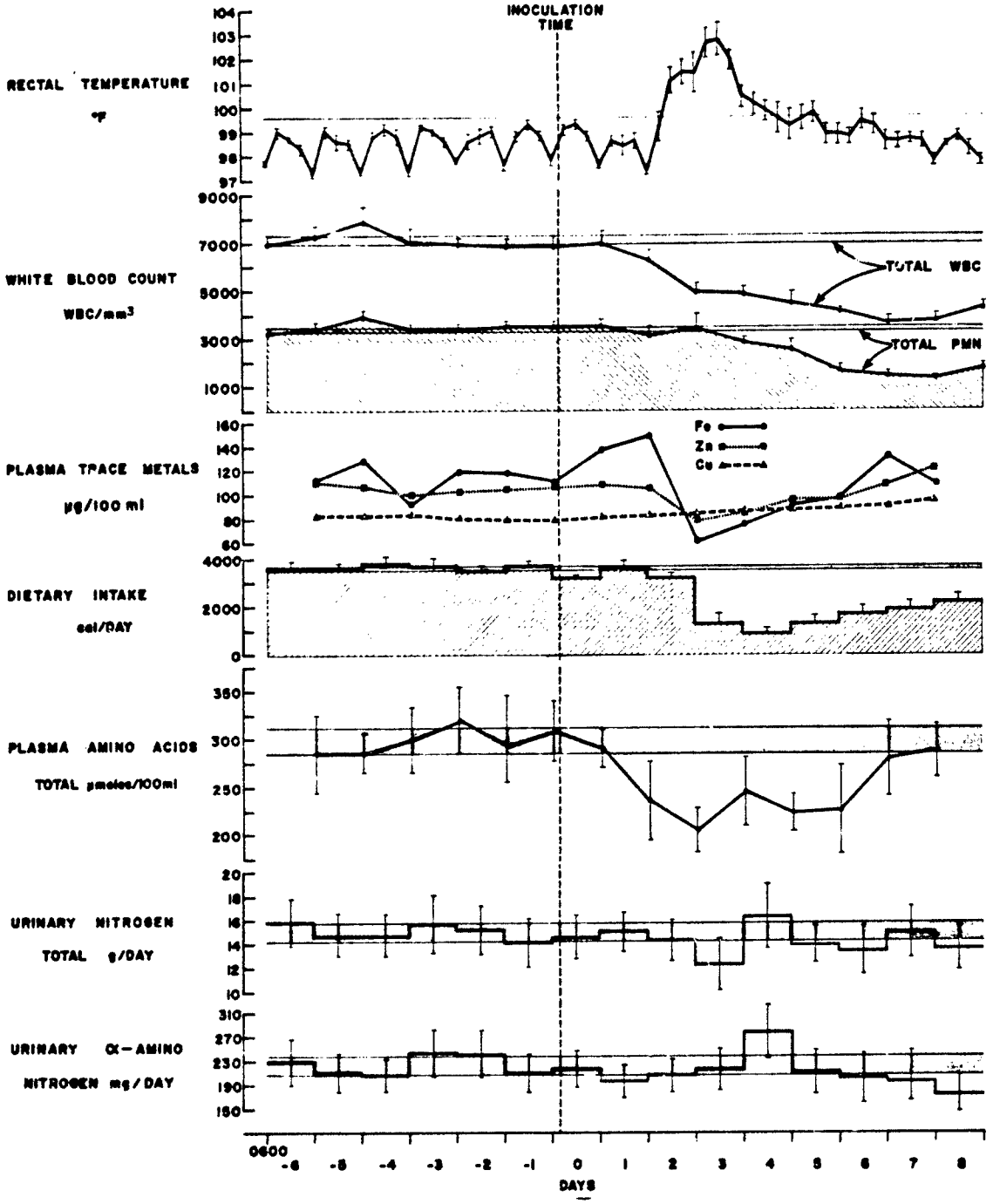

FIGURE I. SEQUENTIAL CHANGES DURING SANDFLY FEVER IN MAN.[5]

normal recipient animals of the same or different species.[7,8] Dr. Wannemacher of our Institute also demonstrated that such an endogenous factor existing in infected animals altered amino acid metabolism.[9] Since a cross species suscepibility to this factor seemed apparent, we attempted to demonstrate the presence of this endogenous mediator in the serum during a bacterial infection in man. While doing a cooperative study with personnel of the University of Maryland (Contract DA49-193-MD-2867),[10] control serum samples and samples obtained on the first day of febrile illness in a group of volunteers infected with Salmonella typhi were Millipore-filtered and injected intraperitoneally (IP) into rats.

As shown in Figure 2, the control, or preinfected human serum produced no significant depressions in either serum Zn or Fe concentrations in the recipient rats when compared to NaCl sham-inoculated rats (band mean ± 1 SEM). However, the postinfected human serum produced significant depressions in both serum metal concentrations. When this postinfected serum was heated at 90 C for 30 min, the depressing effects were lost. Thus, this heat-labile endogenous Zn- and Fe-depressing factor, or factors, appears to be present in the serum of a febrile individual; the fact that this effect can be transferred to a laboratory animal may prove to have potential diagnostic value. Dr. Wannemacher made a similar observation in regard to the flux of liver amino acids of the rat.[9]

In pursuing this problem further, we have found and reported that this endogenous mediator is released in part by polymorphonuclear leukocytes (PMN).[7,8] To demonstrate this, rats were infused IP with glycogen solution; sterile peritoneal exudates were obtained about 21 hr later. The PMN were harvested and washed with normal pyrogen-free saline, centrifuged and resuspended in normal saline; they were then incubated at 37 C for 2 hr in order to allow the cells to excrete the factor into the saline medium. The supernatant represented our crude leukocytic extract or mediator. Before testing the effect of varying doses of the extract, test animals as well as controls were made endotoxin-tolerant in order to exclude the possibility of serum Zn and Fe reductions being produced by contaminating endotoxin.

Test animals were divided into 4 groups receiving 30, 60, 120, or 180 μg of extracted leukocytic protein. Each dose was given IP in 1 ml of normal pyrogen-free saline; Zn and Fe concentrations were determined at 3-hr intervals for each group and controls over a period of 24 hr. As shown in Figure 3, the responses in this experiment not only show that the leukocytic extract mediates alterations in both serum Zn and Fe metabolism, but also that the decreases in serum Zn and Fe concentrations were linear to the logarithm of the doses of extract administered. Although not shown here, the mediator caused a rise in serum Cu concentrations and ceruloplasmin. Preliminary studies by our laboratory on the purification and characterization of the mediator show it to be a low molecular weight protein, which when heated at 90 C for 30 min or when treated with a proteolytic enzyme, loses its activity.

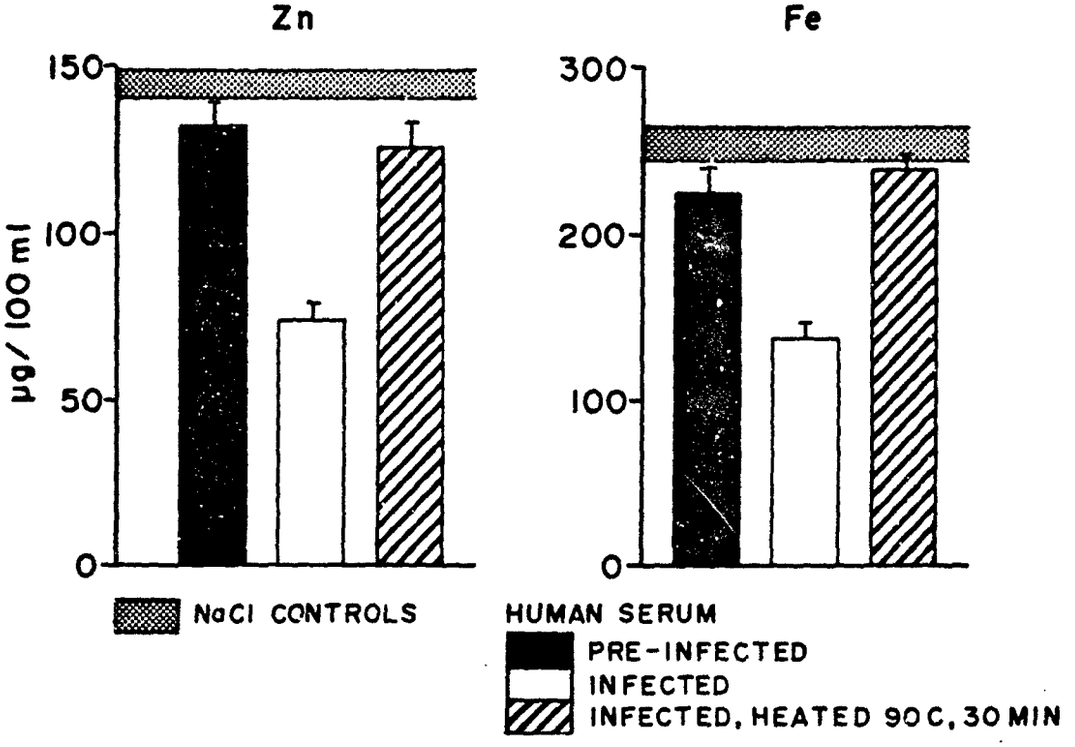

FIGURE 2. EFFECT OF SERUM TRANSFERRED FROM HUMANS INFECTED WITH *S. TYPHI* ON SERUM Zn AND Fe CONCENTRATIONS IN RATS.

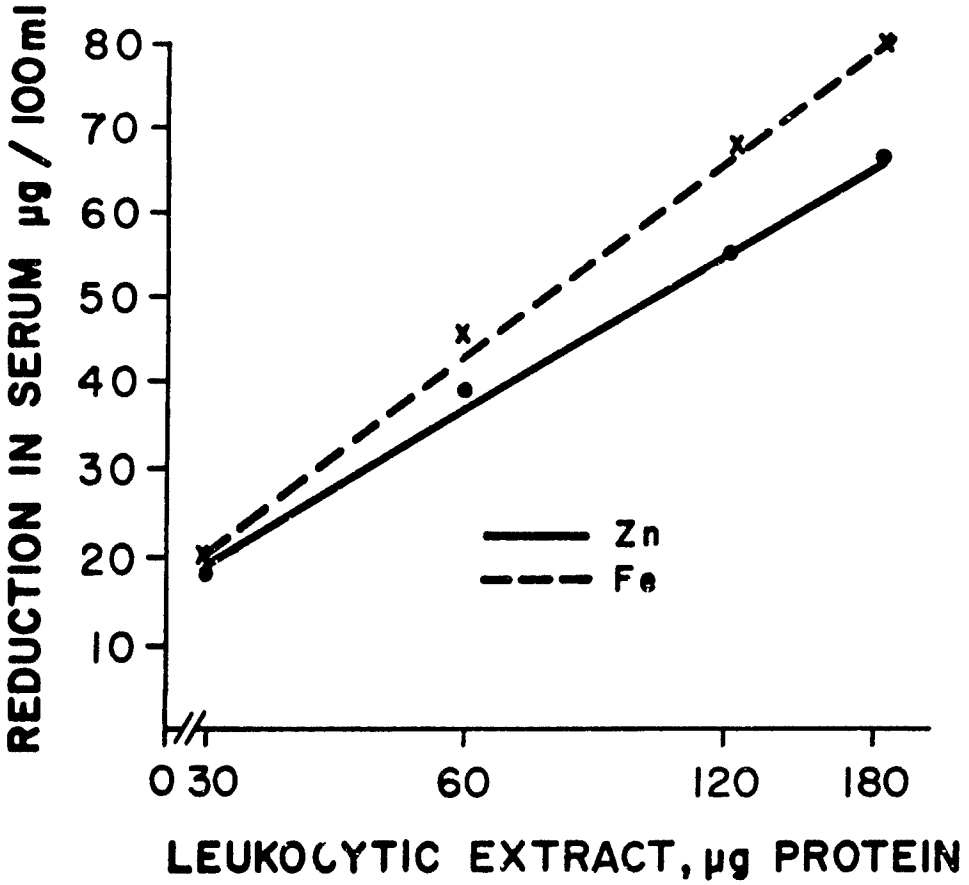

FIGURE 3. RELATIONSHIP BETWEEN SERUM REDUCTIONS AND DOSES OF LEUKOCYTIC EXTRACT ADMINISTERED.

The next questions that arise are, what is happening to these metals or where are they going? Recent studies from our laboratory as well as those reported in the literature, indicated that the very early decreases in serum Fe and Zn concentrations were not the result of increased excretion or decreased gastrointestinal absorption, but were due to a rapid distribution of the two metals. In order to demonstrate this the following experiment was carried out. Rats were pulse-labeled IP with ^{65}Zn, 5 μCi/100 gm body weight. After 18 hr control rats were administered sterile pyrogen-free normal saline IP and test rats were given 150 μg of endogenous mediator IP. After 6 additional hours, both controls and test animals were sacrificed and the distribution of the ^{65}Zn in the tissue, organs, and plasma of the 2 groups was determined.

As shown in Figure 4, no differences between control and test rats were seen in the distribution of ^{65}Zn in the gastrointestinal (GI) tract, pancreas, heart, lungs, or spleen. However, in the kidney, carcass, (mainly bone, muscle, skin and hair), and plasma, Zn was significantly decreased in test animals when compared to controls. More importantly, a significant increase in ^{65}Zn was noted in the livers of experimental rats in relation to controls. Similar results were seen when ^{59}Fe was employed.

Besides these pathophysiologic mechanisms for alterations in trace metal metabolism, other biochemical parameters have been studied with a similar concept in mind. Recently, Dr. Wannemacher has reported that by the use of a ^{14}C-labeled nonmetabolizable amino acid analog (cycloleucine) it was possible to demonstrate a flux of amino acids from muscle to liver in rats infected with Diplococcus pneumoniae.[11] As shown in Figure 5, the free amino acid content of muscle from the infected group was significantly less than pair-fed controls, while a significant early increase in cycloleucine was observed in the livers of infected animals. Dr. Wannemacher has further reported that the endogenous amino acids which moved to the liver were rapidly utilized for the synthesis of serum proteins.[11]

Since transfer experiments from man and infected animals to recipient animals showed that a factor which could mediate an alteration in amino acid metabolism was present in the serum, endogenous mediator was obtained from rat peritoneal leukocytes as previously described and administered IP to normal rats, which were previously pulse-labeled with cycloleucine. Figure 6 illustrates that the endogenous mediator obtained from the leukocytes stimulated a significant increase in the hepatic cycloleucine content of recipient rats when compared to controls. This increase was apparent 1 hr postadministration, peaking at about 3 hr. By 6 hr the cycloleucine content of the liver was still significantly elevated above control values, but had decreased somewhat from the 3-hr level.

Besides eliciting an effect on trace metal and amino acid metabolism, Dr. Winnacker, while at our Institute demonstrated that there was an increased secretion of growth hormone early in the course of infectious illness. Furthermore, leukocytic extracts or mediator prepared from

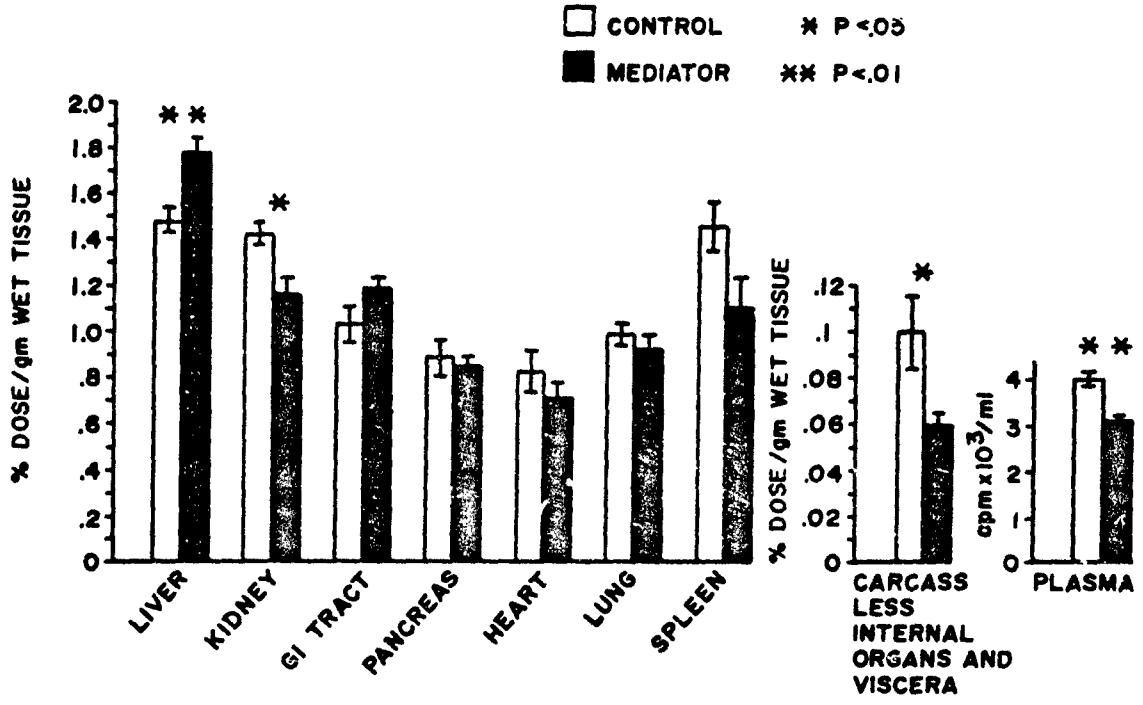

FIGURE 4. EFFECT OF ENDOGENOUS MEDIATOR ON THE DISTRIBUTION OF ^{65}Zn IN THE RAT.

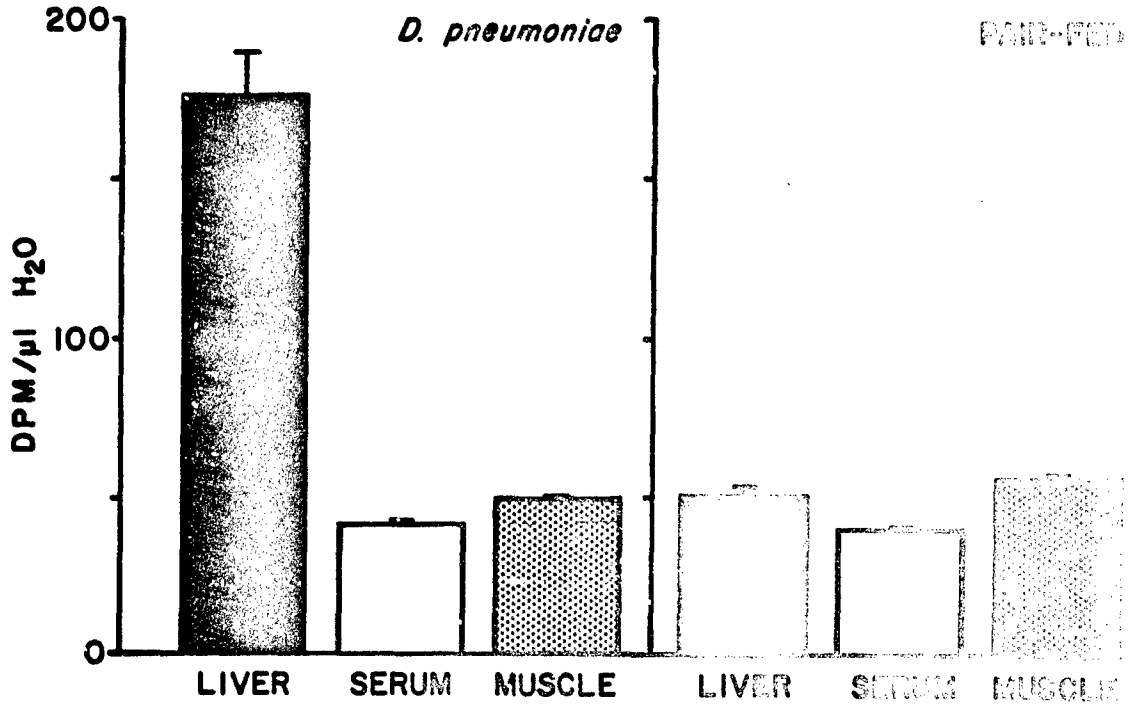

FIGURE 5. EFFECT OF *D. PNEUMONIAE* INFECTION ON THE CYCLOLEUCINE CONTENT OF CELLULAR WATER.

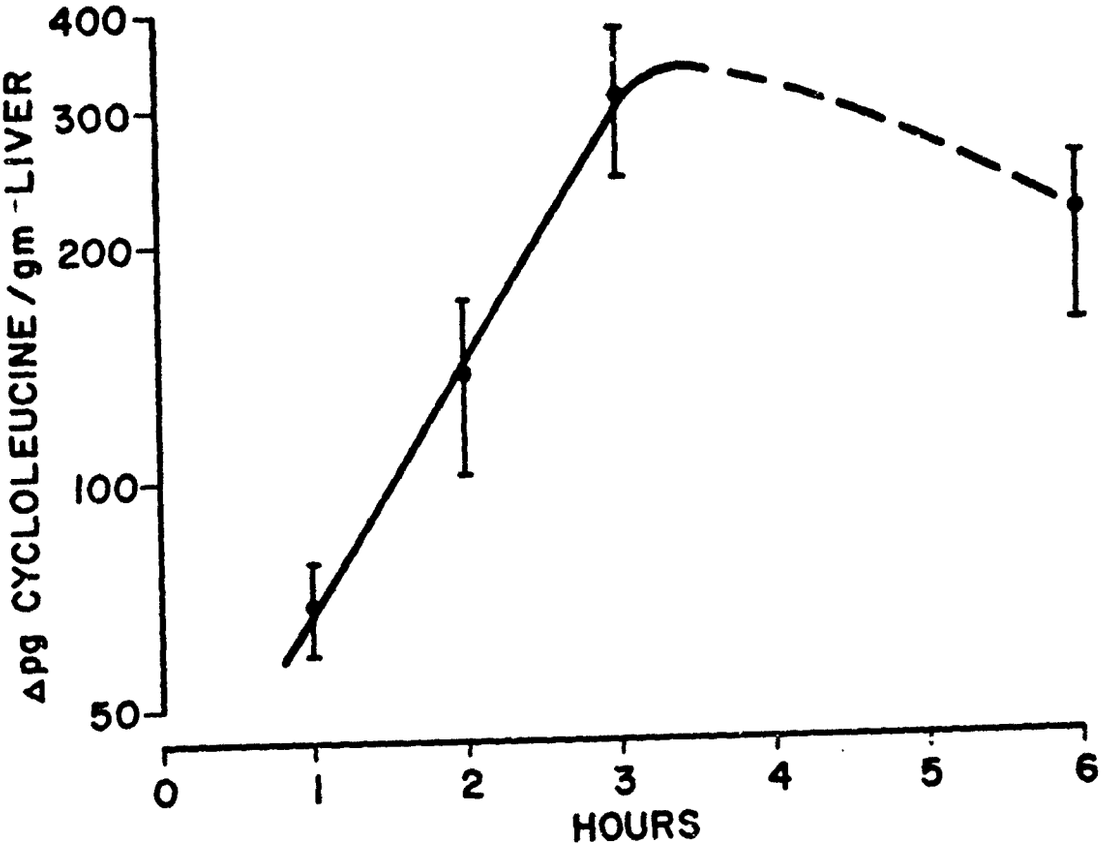

FIGURE 6. EFFECT OF ENDOGENOUS MEDIATOR ON HEPATIC CYCLOLEUCINE.

monkey peritoneal leukocytes stimulated a significant rise in serum growth hormone in the rhesus monkey within 3 hr.[12] Similarly, Kampschmidt's group also demonstrated that the extracts from rabbit leukocytes produced significant increases in the levels of α_1 and α_2 acute-phase globulins of rat within 48 hr.[13]

An attempt to illustrate the current concepts of the endogenous mediator of the nonfebrile host's response to the inflammatory process is shown in Figure 7. As shown on the left, a variety of stimulators have been shown to be able to prime or react with leukocytes and possibly other cells. These cells, in turn, release into the serum an endogenous humoral mediator, or mediators, which, in turn act directly or indirectly on certain target cells, resulting in alterations of a variety of biochemical parameters. There is an influx of Zn, Fe, and free amino acids into the liver. In turn, there is increased output from target cells into serum of Cu, ceruloplasmin, growth hormone, and acute phase globulins.

If we now look at the effect of the endogenous mediator on the sequence of these alterations in the rat model, a pattern seems to evolve (Table I).

TABLE I. EFFECT OF ENDOGENOUS MEDIATOR ON THE SEQUENCE OF ALTERATIONS

PARAMETER MEASURED	TIME OF MAXIMUM EFFECT (hr)
Amino acid flux to liver	3
Serum Zn and Fe depression	6
Serum Cu and ceruloplasmin increase	24-48
Increase of α_1 and α_2 acute phase globulins	24-48

With either infection or the administration of endogenous mediator, there is a rapid influx of amino acids into the liver. This in turn is quickly followed by a decrease in serum Zn and Fe with a significant liver uptake of these metals, both of which are important co-factors for a variety of enzymes and have been shown to be important for protein synthesis and for maintaining the integrity of the ribosomes. This early flux of amino acids and trace metals into the liver is thus followed by an output into the serum of Cu and its newly synthesized carrier protein ceruloplasmin, and the α_1 and α_2 acute-phase globulins.

The synthesis and output of these serum proteins lend further support to the role of the liver during the early stages of the inflammatory process. Further, it appears that this endogenous mediator, or mediators, may play an important role in the host stimulation of these nonspecific host defense mechanisms.

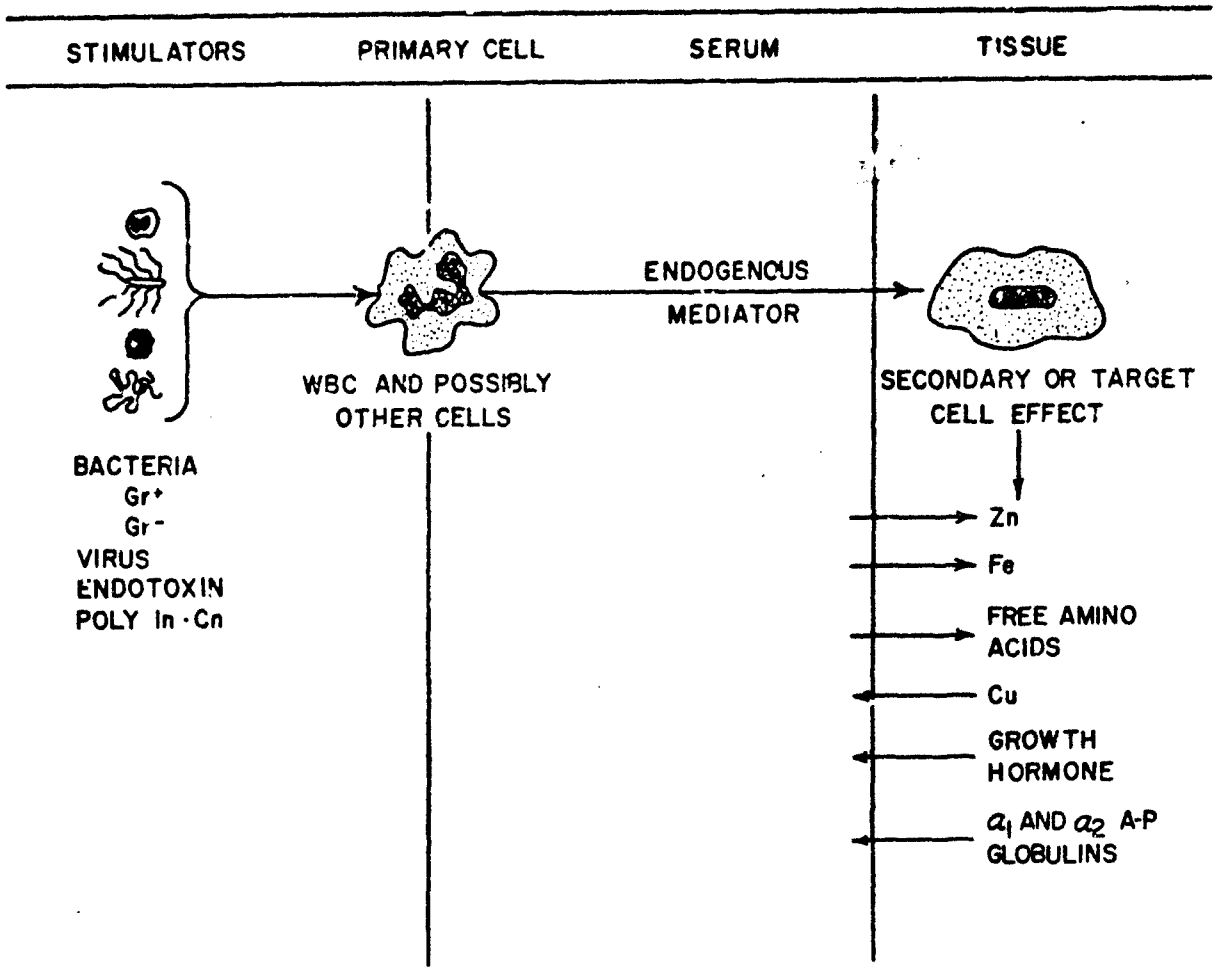

FIGURE 7. CURRENT CONCEPTS ON THE ROLE OF ENDOGENOUS MEDIATOR(S).

Appearance of the endogenous mediator in the serum may be one of the earliest detectable responses of the host to invasion by infecting microorganisms. Studies are now in progress on the further purification and characterization of this endogenous mediating factor.

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SPOTTED FEVER VACCINE

Richard H. Kenyon, Ph.D.*

Of the members of the spotted fever group of rickettsiae, a vaccine has been developed for only one, Rocky Mountain spotted fever (RMSF). Cox[1] developed a formalinized RMSF vaccine prepared from an infected chick yolk sac suspension. In 1945 Craigie[2] modified this technique to include ether extraction to reduce the amount of yolk lipid and protein in the final product. Accumulated experience indicates that this yolk sac vaccine produced by Lederle confers an unsatisfactory level of immunity in man. Hornick[3] studied the yolk sac vaccine in volunteers, and concluded that it produced no substantial protection against the disease. The present work is concerned with the development of an improved RMSF vaccine and its comparison with the Lederle yolk-sac grown vaccine.

The Shelia Smith strain of Rickettsia rickettsii was used. This strain was found to grow readily in duck embryo cells (DEC) to titers of 10^7 yolk sac median lethal doses (LD_{50}). The rickettsiae, after 13 passages in DEC, remained infectious for these cells. The production of toxin for mice as described by Bell and Pickens[4] could also be detected with cell culture grown rickettsiae, but, in our early work, not in as great a quantity nor as consistently as with yolk-sac-grown rickettsiae. Toxin production was not enhanced by the use of various cell growth media or the addition of coenzyme A.

Similarly, pathogenicity for guinea pigs was maintained after passage in DEC. However, as with toxin production, pathogenicity for guinea pigs was obtained with much less consistency in DEC than with chick yolk-sac-grown rickettsiae. These inconsistencies, which could not be attributed to differences in number of organisms, have disappeared and toxin production and virulence are routinely observed with DEC-grown rickettsiae.

For vaccine production we infected DEC with a suspension of R. rickettsii-infected chick yolk sacs. The postinfected DEC suspensions were collected with a syringe and 21 gauge needle and then treated in a Branson Sonifier for 15 sec to disperse clumps of rickettsiae. This suspension was centrifuged at 150 x g for 20 min to remove much of the cell debris and the supernatant fluid was used for vaccine experiments. When formaldehyde treatment and irradiation were to be compared as methods of inactivation for vaccine production, both preparations came from the same lot of cell culture rickettsiae. For the irradiated vaccine, the suspensions were exposed to 500,000 R of gamma radiation from a ^{60}Co source while in the frozen state. Preliminary experiments indicated that this dose was at least twice that required for complete inactivation.

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For formaldehyde-treated vaccine, formaldehyde was added to a final concentration of 0.1%; this suspension was placed at room temperature for 48 hr and then stored at 4 C until used. To compare the immunogenic potency of the vaccines, the preparations were each diluted to the indicated concentration in Hanks' balanced salt solution just prior to injection into guinea pigs, 4 for each dilution of vaccine. The animals were immunized by the intraperitoneal (IP) route with 1 ml of the appropriate dilution of vaccine and were challenged IP 13 days later with approximately 10^8 yolk-sac-grown rickettsiae. Temperatures were recorded daily.

The following figures are a compilation of the results of several replicate experiments comparing the efficacy of the cell culture vaccines prepared by formaldehyde or irradiation inactivation, and comparing these vaccines with the Lederle yolk sac vaccine. Previous workers have used the magnitude and duration of fever in guinea pigs as a measure of susceptibility to RMSF. A guinea pig temperature of $\geq$ 39.8 C was considered elevated. The smaller the areas > 39.8 C in the figures, the greater the protection against RMSF. The points plotted in Figure 1 are the mean temperatures of at least 16 guinea pigs. It is apparent that undiluted Lederle vaccine protects; there is perhaps a small amount of protection from use of a 1:8 dilution. By 14 days after challenge, 95% of unvaccinated controls had died; 5% of those immunized with undiluted Lederle vaccine had died; 44% of 1:8 dilution had died, and 75% of 1:25 dilution had died.

Figure 2 compares formaldehyde and irradiation as methods of killing for vaccine production. No sharp "antigenicity" endpoint for either vaccine is obvious, but it can be seen that guinea pigs immunized with a 1:100 dilution of formalinized vaccine are significantly better protected than those immunized with the same dilution of irradiated vaccine. A dose dependent response is obvious for both vaccines. None of the guinea pigs immunized with the various dilutions of either DEC-grown vaccine had died by 14 days after challenge.

Figure 3 relates a statistic representing the magnitude and duration of fever with the dilution of vaccine injected. As mentioned earlier, the areas under the curves were taken to be inversely related to the degree of immunity these animals possessed against RMSF. It can be seen that the DEC-grown, formalinized vaccine presented here offers better protection to guinea pigs when diluted 1:900 then does the old undiluted yolk sac vaccine. The infectivity prior to formaldehyde treatment is not specified for the yolk sac vaccine, and accordingly no comparison of antigenicity per initial unit of infectivity can be made. It seems improbable that the initial infectivity values of the cell culture preparations were sufficiently greater than those of the yolk sac suspensions to account for the difference in antigenicity of approximately 3 orders. It is possible, however, that nonviable or noninfectious rickettsiae present initially in the cell culture suspensions contribute significantly to the antigenicity of the derived vaccine.

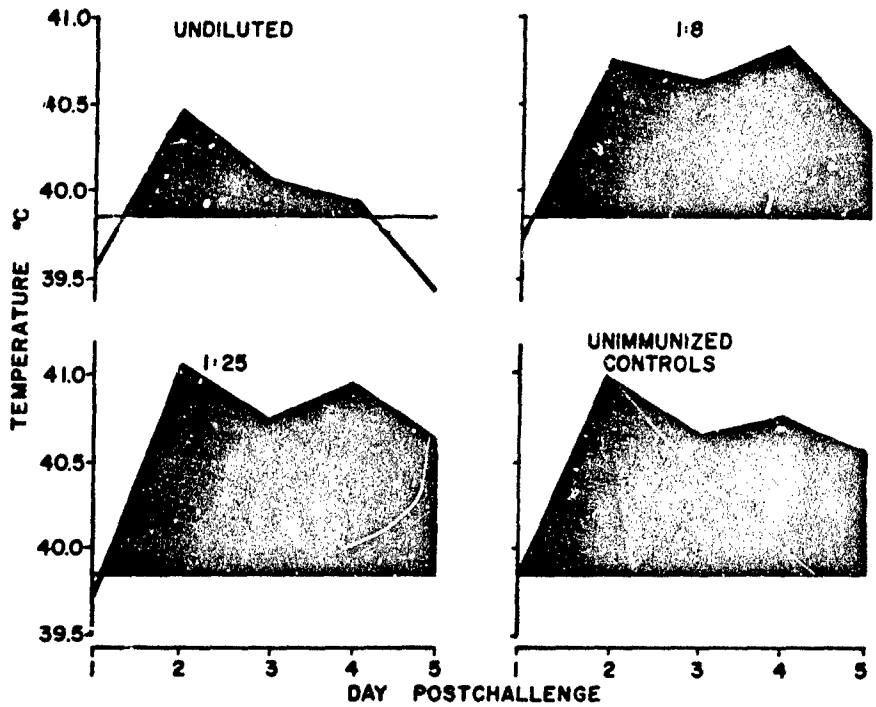

FIGURE 1. FEVER RESPONSES TO CHALLENGE AFTER IMMUNIZATION WITH YOLK SAC VACCINE.

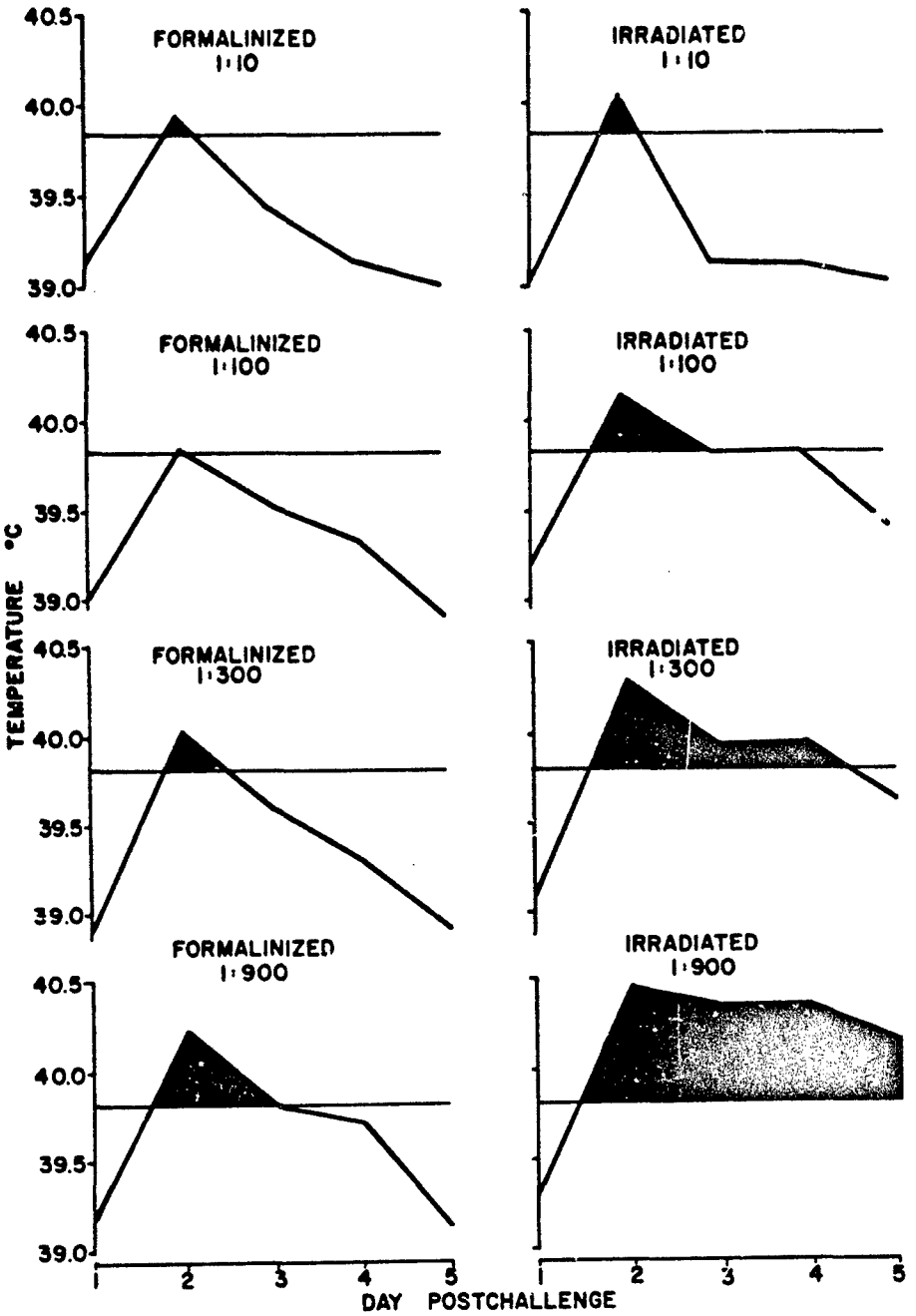

FIGURE 2. FEVER RESPONSES TO CHALLENGE AFTER IMMUNIZATION WITH FORMALINIZED AND IRRADIATED TISSUE CULTURE VACCINES.

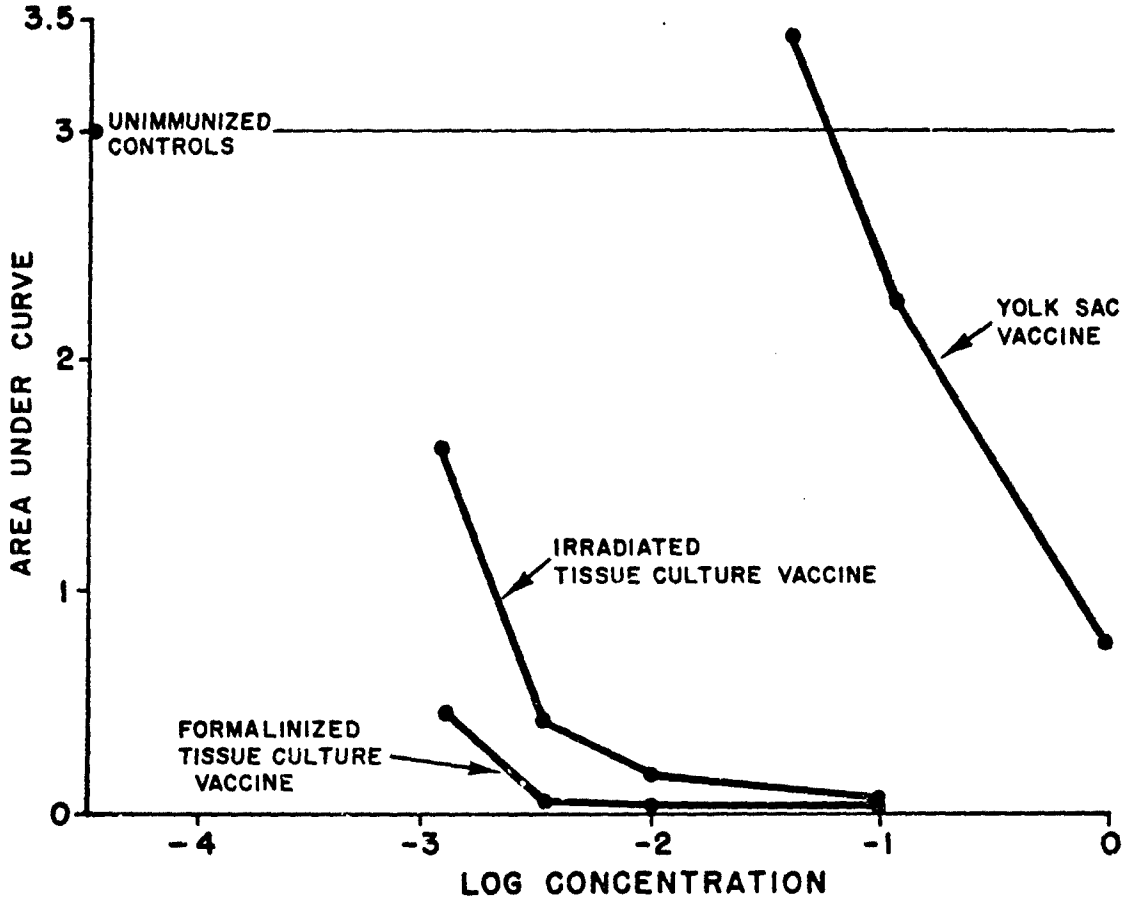

FIGURE 3. FEVER RESPONSES OF IMMUNIZED ANIMALS AFTER CHALLENGE WITH VARIOUS VACCINES.

It was shown by infectivity titrations in eggs that no viable rickettsiae could be detected in the irradiated or the formalinized vaccines. However, it was necessary to eliminate the possibility that viable organisms were present in these vaccines and their propagation in the vaccinated guinea pigs contributed to the antigenicity. To evaluate this possibility guinea pigs were injected IP with 15 mg of chloromycetin for 5 days starting one day before vaccination. After challenge, no differences could be detected in immunity between chloromycetin-treated and untreated guinea pigs.

Complement fixation (CF) tests were performed in an attempt to detect the presence of RMSF antibody. The antigens for these tests were purified DEC-grown rickettsiae or commercial RMSF diagnostic antigen. CF activity could be detected only in sera from guinea pigs that had manifested clinical signs of RMSF. None of the previously described vaccines elicited the production of detectable CF antibody in guinea pigs, even though these same animals became refractory to RMSF challenge.

After establishing the efficacy of the DEC-grown, formalinized vaccine in guinea pigs, we tested efficacy in rhesus monkeys. The vaccine was diluted 1:5, 1:20, or 1:100 in Hanks' balanced salt solution just prior to use in 4 monkeys for each dilution. Each group was administered 2 subcutaneous injections 3 weeks apart. One group of 4 monkeys was immunized with undiluted Lederle vaccine. All were challenged with 0.5 ml of citrated whole infected guinea pig blood 6 weeks after the last injection. The infectious blood had been withdrawn from guinea pigs on the 2nd day of temperature elevation after challenge with approximately 10^6 yolk-sac-grown rickettsiae.

TABLE I. SURVIVAL RATIOS AFTER CHALLENGE OF MONKEYS IMMUNIZED WITH YOLK SAC-GROWN OR DEC-GROWN R. RICKETTSII VACCINE

VACCINE	DILUTION	SURVIVORS[a]/4
Yolk sac	Undiluted	3
DEC, formalinized	1:5	4
	1:20	4
	1:100	4
None, controls		0

a. 30 days after challenge.

All 4 unvaccinated monkeys died 9-11 days after challenge, exhibiting typical spotted fever rash. One monkey which had been immunized with the commercial, undiluted yolk sac-grown vaccine also died 11 days after challenge and exhibited a spotted fever rash. None of the 3 remaining monkeys in this group and none of the monkeys immunized with the DEC-grown vaccine

died or exhibited a rash. It appears that the DEC-grown vaccine offers some protection to monkeys, at least when undiluted. Further studies in monkeys with DEC-grown vaccine are planned.

None of the sera from the vaccinated monkeys showed CF antibody titers prior to challenge; 21 days after challenge CF antibody titers were 1:64-1:512, suggesting that inapparent infection occurred in these monkeys.

Although our primary concern has been with RMSF, studies with other members of the spotted fever group have been initiated. R. conori, akari, australis, siberica, and parkerii have been successfully grown in DEC, with with titers of 10^8-10^7 yolk sac LD_{50} being attained. The cross reactions between these members of the spotted fever group of rickettsiae have been studied by other workers. Lackman and co-workers[5] report that guinea pigs convalescent from RMSF are immune to challenge with R. parkerii and R. conori as well as R. rickettsii, but are not immune to the other members of the spotted fever group. Guinea pigs vaccinated with yolk sac-grown R. rickettsii vaccine are protected against R. parkerii and R. rickettsii infections but not against R. conori. We have confirmed this using the DEC-grown R. rickettsii vaccine.

TABLE II. CROSS-PROTECTION TESTS IN GUINEA PIGS

RICKETTSIAE USED FOR CHALLENGE	RICKETTSIAE USED FOR IMMUNIZATION		
	Live	Commercial Yolk-Sac Vaccine	DEC-Formalinized Vaccine
R. rickettsii	+[a]	+	+
R. conori	+	-[b]	-
R. akari	-	-	-
R. australis	-	-	-
R. parkerii	+	+	+

a. Protection.
b. Less than significant.

In summary, the DEC-grown RMSF vaccine offers much better protection in guinea pigs than does the yolk-sac-grown vaccine. Formaldehyde treatment is superior to irradiation as a method of inactivation for vaccine production. On the basis of the dilution of the vaccines allowing development of similar extents of fever, the irradiated vaccine was more than 300 times and the formaldehyde-treated vaccine, more than 900 times as active as the commercial yolk-sac-grown vaccine.

We are presently in the process of making a large quantity of DEC-grown formalinized vaccine for immunization trials in man. We also plan the development of formalinized vaccines against each member of the spotted fever group and perhaps a multivalent vaccine offering protection against all spotted fever rickettsiae.

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LIVE, ATTENUATED Q FEVER VACCINE

David M. Robinson, MAJ, VC*

The Virology Division of the U. S. Army Medical Research Institute of Infectious Diseases has been investigating the properties of the M strain of Coxiella burneti for the past year and a half. This strain was developed in Russia in the 1950's and used there, as well as in other European countries, as an attenuated live vaccine.[1] It has been given by subcutaneous, endermal (scarification), and oral routes. Table I summarizes the results published in the Russian literature on the use of the vaccine in man.[2,3]

TABLE I. EFFECTS OF M-44 STRAIN Q FEVER VACCINE IN MAN

ROUTE	LOG_{10} DOSE IDE_{50}	REACTIONS		CONVERTED AT 1-2 MON
		Local	General	
		%		%
Subcutaneous[2] (0.5 ml)	4-5	5.5	4	80-91
Scarification[3] (0.1 ml)	7-8	51-80[a/]	None	51-85[a/]
Oral (1 ml)[3]	8-9	None	None	80

a. Yolk sac suspension: 10% low number
50% high number

The Russian vaccine had been produced by diluting heavily infected yolk sacs in sterile skimmed milk and removing larger particulate matter with low speed centrifugation. Therefore, it contained appreciable amounts of egg protein; the local reactions listed for the scarification route of administration may or may not be caused by the rickettsia. The general reactions referred to were limited to fever, malaise, and headache of 2-3 days duration.

The original seed received at USAMRIID had no history of having been tested for viruses of the avian leucosis complex. The heat resistance of the organism was the property used to free it from possible contamination by these adventitious agents. A 1:10 dilution of a sample of the first passage material, labeled 0-1 (original-first pass), produced at the

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Walter Reed Army Institute of Research (WR-E1) was divided into 2 aliquots. One aliquot was placed at 4 C; the other was heated to 56 C for 30 min in a water bath. Both samples were immediately titrated in embryonated eggs obtained from SPAFAS, Inc. (Norwich, Conn.); eggs were also inoculated with the heated material to produce a yolk sac pool. No loss in infectivity titer was detected after heating. The yolk sac pool produced from this material was labeled 0-1-H-1 (original-one pass-heated-one pass) and formed the master seed. A further passage of this master seed, labeled 0-1-H-2 (second pass of the heated material) was produced to serve as a working seed. Both of these materials were free of avian leucosis viruses when tested at the Division of Biologics Standards, National Institutes of Health. The strain was redesignated R-M (rif-free-M) at this time, and the vaccine lot to be discussed is Lot 1 of a series of 5 consecutive lots, produced as first-pass material from the working seed.

The vaccine is intended for use by the endermal route; a single dose contains a very small amount of egg protein. However, we felt that the problem of significant numbers of local reactions occurring in individuals with and without preexisting antibody necessitated purification of the vaccine. The procedure developed utilized only physical methods, and consisted of 2 cycles of differential centrifugation followed by centrifugation in 10% sucrose onto a 70% sucrose "cushion." A comparison of titers and N levels is shown in Table II. The titers were approximately

TABLE II. MEDIAN EGG INFECTIVE DOSE AND N CONTENT OF R-M STRAIN MATERIALS

MATERIAL	LOG_{10} IDE_{50}	µg N/ml
Master seed	11.6	
Working seed	10.9	
Yolk sac pool	11.1	620
Purified vaccine pool	11.1	39
Vaccine[a/]	10.4	0.5

a. 1:75 dilution of the purified vaccine pool.

equal in all cases but the N levels decreased greatly. The N level given for the vaccine is below the sensitivity of our assay and was determined from the N level of the purified vaccine pool and the dilution factor. The reported human dose is 0.1 ml; it has been estimated that perhaps 1/10 of this actually enters the skin. Therefore, the N level of a single dose would be about 0.005 µg N. Figure 1 presents the febrile responses of groups of 6 guinea pigs which were inoculated subcutaneously with 0.5 ml of

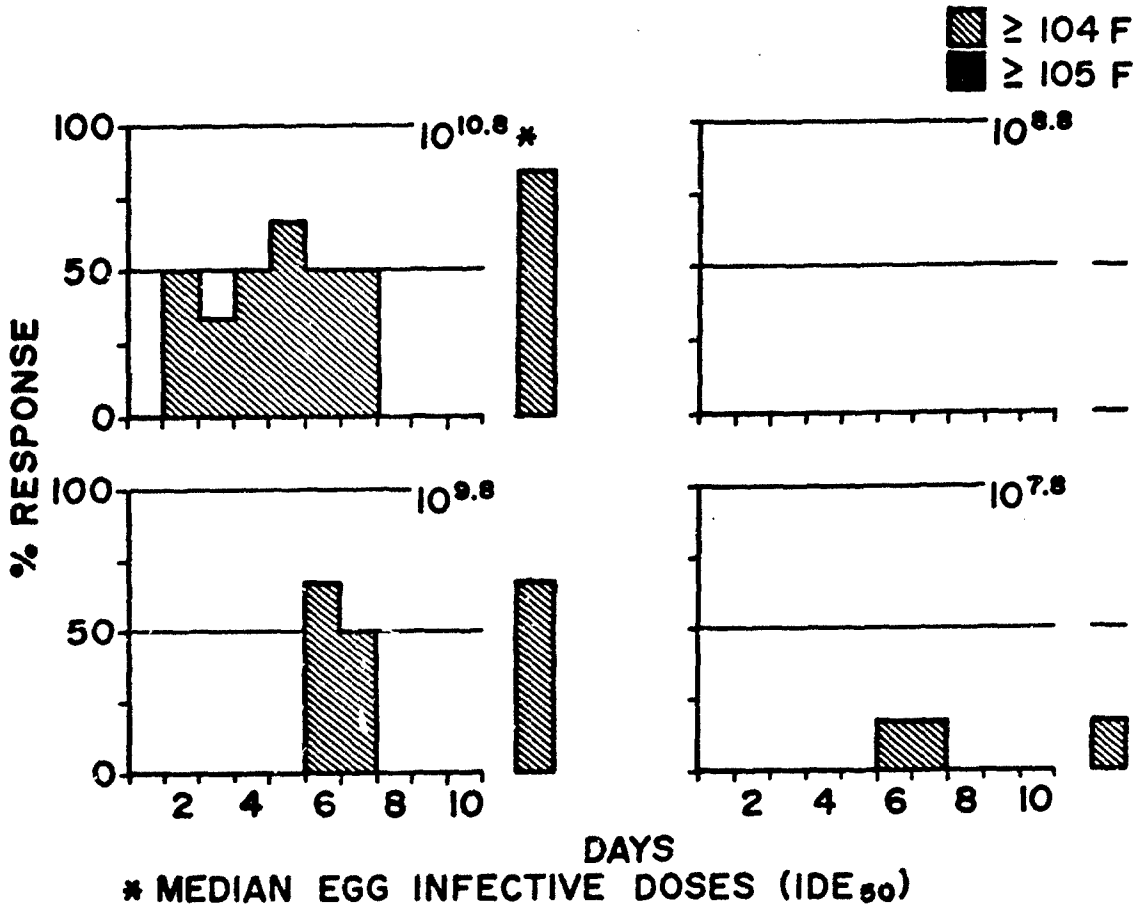

FIGURE 1. FEBRILE RESPONSES OF GUINEA PIGS TO VARYING DOSES (IDE_{50}) OF *C. BURNETI* R-M STRAIN (YOLK SAC, LOT 1).

serial 10-fold dilutions of yolk sac pool Lot 1. No temperatures > 104.8 F were detected and no animals were visibly ill. No temperatures > 103.8 F were detected at doses < $10^{6.8}$ median infective dose for eggs (IDE_{50}). When this same experimental procedure was used for the final purified vaccine 0.5 ml of undiluted material ($10^{10.1}$ IDE_{50}) did not produce any temperatures > 103.8 F.

Immunized guinea pigs which had had no fever, were back-challenged with 10^4 median fever doses (FD_{50}) of C. burneti, strain EP-88 (a phase II organism). The febrile responses of the animals which had received the highest dose of vaccine ($10^{10.1}$ IDE_{50}) illustrate the prompt intense febrile response with an equally prompt remission which has been seen in all animals challenged with 20% yolk sac material (Figure 2). Not all dilutions are shown, only those representing an abbreviated response to challenge ($10^{10.1}$) a medium response ($10^{7.1}$) and a response not different from control values ($10^{5.1}$). Table III is a compilation of 4 potency assays using the 50% fever suppression dose as published by Ormsbee et al[4] as an indication of protection (PD_{50}). Throughout these 4 assays no fevers were detected when guinea pigs were vaccinated subcutaneously with freeze-dried vaccine. The PD_{50} values following challenge do not vary radically from each other; there is no difference in the protection afforded against the 2 challenge levels of phase I organisms.

TABLE III. POTENCY ASSAYS OF R-M SUBSTRAIN VACCINE, LOT 1, IN GUINEA PIGS

CHALLENGE		Log_{10} PD_{50}
PHASE	Log_{10} IDE_{50}	
I	10	4.3
	6	5.4
II	10	4.8, 5.3

While searching for an improved substrate for the growth of C. burneti we tried several cell cultures which were being produced in the laboratory for other purposes. Since penicillin had been used in diluents for the production of Q fever rickettsia[5] we incorporated 100 units of penicillin/ml in the cell culture media and diluent fluids. Briefly, our results showed a progressive number of infected cells up to a maximum at 7-8 days at which time the cultures degenerated. Refeeding decreased the number of infected cells, and of course increased the length of time the cell cultures could be kept without degeneration. When both penicillin and streptomycin were incorporated, no rickettsial growth could be detected. This fortutious finding was used as the basis for the cell culture testing of the vaccine for adventitious agents.

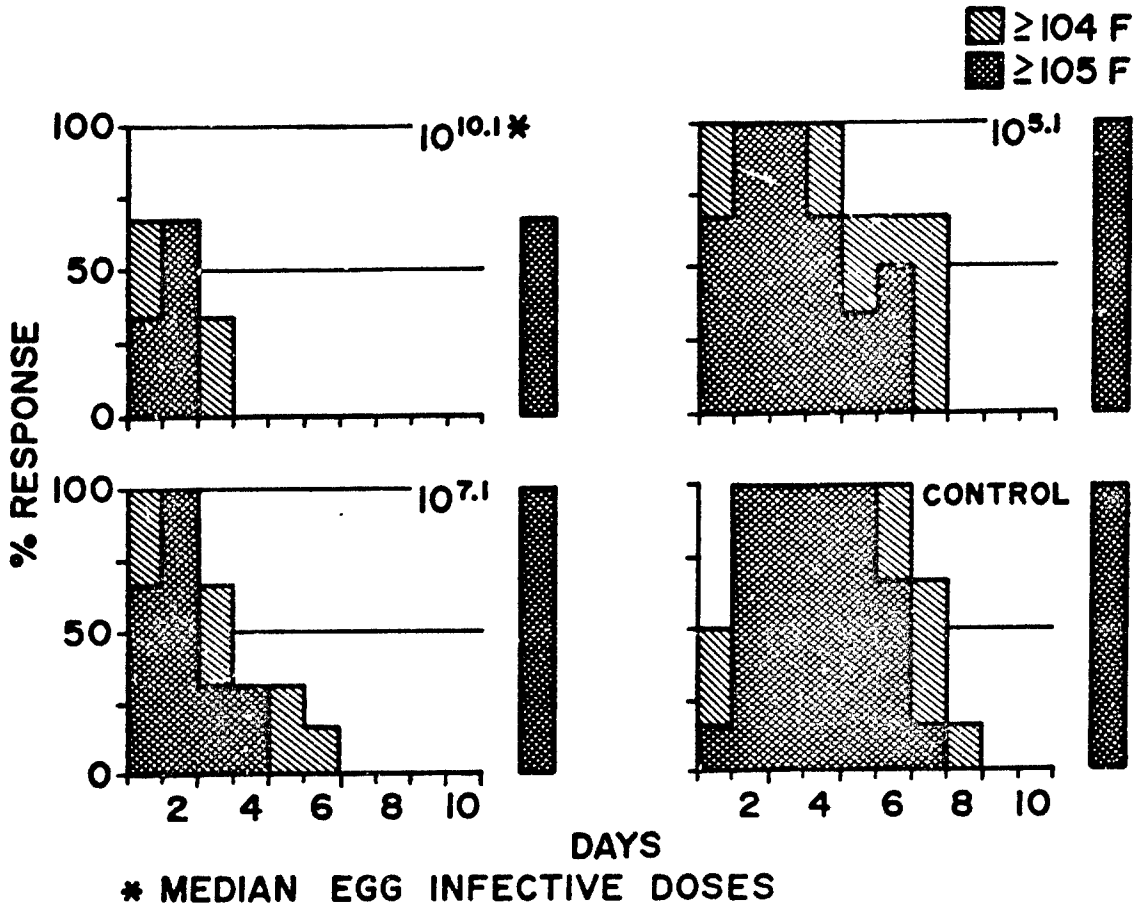

FIGURE 2. FEBRILE RESPONSES OF R-M EXPERIENCED GUINEA PIGS TO CHALLENGE WITH 10^4 FD_{50} OF EP88 *C. BURNETI.*

A list of the testing procedures used in the production of the vaccine is presented in Table IV. Many of these tests are prescribed by regulations.[6]

TABLE IV. TESTING PROCEDURES

STERILITY	
	Bacteria
	Fungi and yeasts
	Mycobacterium spp.
	Mycoplasma spp.
QUALITY	
	Nitrogen content
	Titer
	Potency
	General safety
	Residual moisture
ADVENTITIOUS AGENTS	
	Mice - suckling and adult
	Cell cultures
	Embryonated eggs (duck and chick)
	Avian leucosis agents
IDENTITY	

Not all of these tests were conducted on all materials generated in the production of the vaccine. However, all the adventitious agent tests, except the rif and fluorescent antibody tests for avian leucosis, were conducted on the purified vaccine pool prior to filling and freeze-drying. A single blind passage was conducted 14 days postinoculation with half of the suckling mice originally inoculated. The confluent cell cultures (WI-38, duck and chick fibroblasts, and Vero cells) were refed with material from the purified pool diluted with an equal volume of maintenance medium containing 200 units of penicillin and 200 μg of streptomycin. As previously stated, these conditions would not support the growth of rickettsia in any of the cell systems. The cultures were examined daily for cytopathic effect and refed at 4-day intervals. The cell sheets were examined at 14 days with guinea pig red blood cells for the presence of agents capable of hemaadsorption. All tests were negative for the presence of adventitious agents.

Naturally acquired infections[7] and experimental infections[8] caused by Phase I *C. burneti* have produced granulomatous hepatic lesions in man. To determine whether the M strain induced hepatic lesions, guinea pigs were inoculated with our M vaccine, M killed Phase II Nine-Mile vaccine, EP-88, and Phase I Henzerling EP-2 strain. Specimens were collected from the onset

of fever until 4 weeks postinoculation. The experimental results are given in Table V. The lesions found were either focal or granulomatous necrosis;

TABLE V. INCIDENCE OF HEPATIC LESIONS IN GUINEA PIGS

GROUP	NO./TOTAL	%
Control	2/20	10
Q Fever Vaccine (Killed Phase II)	2/10	20
M Strain	2/10	20
EP-88	4/10	40
EP-2	6/10	60

no organisms were seen. The high percentage of hepatic lesions (10%) in control animals, inoculated with 0.5 ml of Snyder's 1 buffer, indicates the incidence of disease in any group of guinea pigs not derived and maintained under germ-free conditions. The incidence of lesions in the animals given the killed vaccine and the M strain were the same.

Up to 1967 a total of 26 cases of endocarditis[9] had been reported in persons with preexisting valvular lesions. While important, the lack of an adequate animal model has prevented us from examining this facet of Q fever.

It might be desirable to discuss the potential advantages of the attenuated R-M strain as a vaccine over the presently available inactivated phase I and II products. These presently available vaccines have proven to be effective, but as with other inactivated products repeated inoculations of µg-amounts of material are necessary to maintain detectable antibody levels. However, with repeated injections the incidence of sterile abscesses increases. A delivered dose of the R-M strain contains protein levels in the order of hundredths of a µg, and has been used in Europe in persons with and without preexisting antibody with the same percentage of local reactions, limited to transient erythema and induration.

A given amount of raw material, be it yolk sac or cell culture, can be used to produce more live vaccine than the inactivated product. This allows many doses of vaccine to be produced in a short period of time with large lot sizes, thus decreasing the number of quality and safety tests conducted in the laboratory.

In conclusion, we have derived a rif-free strain of C. burneti from the M strain which we have designated the R-M strain. A series of lots of vaccine suitable for human use have been produced and tested. The dose capable of protecting 50% of the guinea pigs inoculated contains a calculated amount of 0.00005 μg N. The incidence of hepatic lesions was not increased over that observed with the presently available killed phase II vaccine. A request for the testing of this vaccine in humans will be submitted to the Army Investigational Drug Review Board.

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HEMORRHAGIC FEVER IN COCHABAMBA

Clarence J. Peters, M.D.,* and Ralph W. Kuehne, B.S.**
Presented by R. O. Spertzel, LTC, VC**

From January to March 1971, 6 cases of hemorrhagic fever occurred in Cochabamba, Bolivia. They were investigated subsequently by the Middle America Research Unit, Balboa, CZ, (MARU) and the Bolivian Ministry of Health. MARU and the Institute of Infectious Diseases are currently engaged in a joint project to isolate and characterize the causative agent.

The first case of hemorrhagic fever in the Cochabamba outbreak (Figure 1) occurred in a 20-year-old student nurse, vacationing with her family in a small settlement called Fortaleza, in Beni Province. She was hospitalized at Seton Hospital, Cochabamba, on the day of her return and subsequently died. She was attended by at least 15 nurses, as well as relatives. The intensity of contact by these nurses was variable, but all were presumably exposed to aerosols and, undoubtedly, fomites. Three relatives assisted in her care, embraced her, and kissed her. Parenteral routes of exposure were not identified. About 9-13 days later, 2 nurses and 2 relatives became ill. Case No. 2 was the father of Case No. 1. He died in Trinidad, Bolivia, with a hemorrhagic fever-like illness; however, hospital records were not available and few details are known. Case No. 3, the 33-year-old aunt of Case No. 1, was hospitalized in Cochabamba at Viedma Hospital and subsequently died. Initially, she was treated on an open ward and later placed in isolation. Case No. 4, a 28-year-old nurse who cared for Case No. 1, was hospitalized in Seton Hospital, and at least 24 persons were directly involved in her care. Case No. 5, a 24-year-old nurse who was exposed to Case No. 1 on a single day, subsequently became ill and was hospitalized in the town of Tarija. She was the only survivor in this series of cases. The 6th case, a 32-year-old pathologist at Seton Hospital, represents the only instance of known parenteral exposure in the entire outbreak. While performing an autopsy on Case No. 4, he cut his finger and immediately responded by plunging it into a nearby formalin bottle. Two of his assistants remained well. Six days later, he had onset of fever and chills. He was admitted to Seton Hospital, placed in isolation, but died. The apparently shortened incubation period could be related either to the route and/or size of inoculum, or to previous infection from specimens submitted to the clinical laboratory.

It is impossible to assess accurately the actual number of people exposed, the frequency, or magnitude of their exposures; however, further clinical cases were not detected, although the entire town of Cochabamba

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** U. S. Army Medical Research Institute of Infectious Diseases.

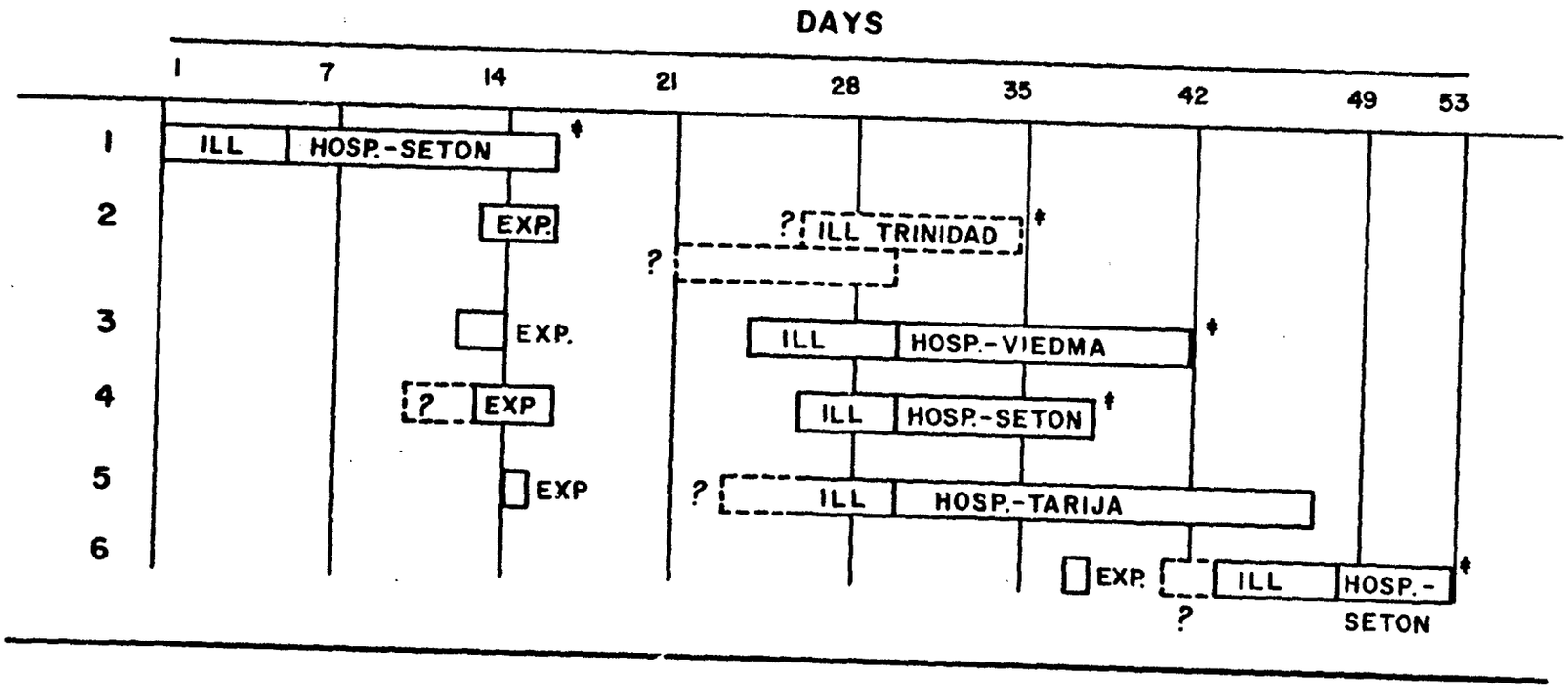

FIGURE 1. EPIDEMIOLOGY OF 6 CASES OF BOLIVIAN HEMORRAGIC FEVER.

was acutely aware of the outbreak of hemorrhagic fever. Severely ill persons in Seton and Viedma Hospitals were examined for evidence of the illness; no compatible cases were found.

Clinical disease in each of the 5 cases for which we have records was remarkably similar. An early febrile period with extreme malaise and myalgia preceded hospitalization. On admission, the patient was found to be acutely ill, febrile, mildly hypotensive, and leukopenic with pharyngeal and conjunctival injection. These findings were often followed by severe prostration, frank shock, hemorrhage from the gums, rectum and/or bladder, petechiae, ecchymoses, moderate icterus, delirium, and convulsions. Three cases died in refractory shock, with central nervous system signs; the 4th patient apparently was recovering from acute illness when death, attributed to thrombosis of the superior vena cava intervened. Renal failure was not a feature of the primary syndrome. Three of the 5 patients were definitely icteric, but the mechanism of jaundice was not clear and no evidence could be obtained for exposure to a common toxic factor which might have contributed to the illness that occurred. The disease resembled severe Machupo virus infection except for the occurrence of jaundice, which is uncommon in either Machupo or Junin infection.

Machupo virus infection of the rodent Calomys callosus, and of humans, characteristically occurs in the lowland savannahs of Beni Province in the region of San Joaquin, Magdalena and Orobayaya, Figure 2. Human infection is related to the occurrence of infected C. callosus in the homes of infected persons. Available evidence strongly supports the hypothesis that the primary route of human infection is via contamination of the environment by infectious secretions or excretions of these rodents. Evidence for arthropod transmission of Machupo virus is nil; the person-to-person transmission, although possible, is uncommon.

Patient No. 1 had been in Fortaleza for 4 weeks prior to her illness; there is no firm evidence of travel to other towns in Beni Province. The incubation period of Machupo and Junin virus infections, as well as the incubation period of secondary cases of the Cochabamba outbreak, implicated Fortaleza as the site of her infection. Although Fortaleza is located in this province, it lies outside areas of known Machupo virus infections. It should be noted that trapping 2 mon later in Fortaleza failed to yield a single C. callosus. Cochabamba is located at an altitude of 5,000 feet on the slopes of the Andes. While there are certain similarities between Machupo and Cochabamba infections, there are marked differences, Table I. Thus, there appear to be significant differences between Machupo infection and the disease in Cochabamba.

FIGURE 2. MAP OF BOLIVIA (DOTTED AREA INDICATES MOUNTAINS).

TABLE I. COMPARISON OF MACHUPO INFECTION AND THE OUTBREAK AT COCHABAMBA, BOLIVIA

MACHUPO INFECTION	COCHABAMBA OUTBREAK
Occurred in a particular area of the Beni Province; always associated with the presence of infected C. callosus.	Index case from Beni, but not from a known endemic area; no C. callosus could be trapped in village of index case; ecologically distinct region outside natural habitat of C. callosus.
Person-to person transmission uncommon; doctors and nurses caring for patients not at excess risk.	Four of 5 secondary cases person-to-person; 5th case autopsy accident; 2 nurses ill.
Jaundice common.	2 of 5 cases with jaundice, with no other explanation.
Spleen or liver from autopsy cases has high titer of virus which produces typical illness in suckling hamsters.	Liver from Case 4 produced equivocal results in suckling hamsters.
CF,[a] IFAT,[b] and neutralizing antibodies appear roughly in parallel titers.	30-day serum specimen from Case 5 had high titers of group-specific CF and IFAT antibodies, but low titer of type-specific neutralizing antibodies.

a. Complement-fixing.
b. Indirect fluorescent antibodies.

Machupo virus, the causative agent of Bolivian hemorrhagic fever, is a member of the Arenovirus group, Table II. Arenoviruses are 100-150 μg in diameter, possess an outer membrane within which are small, sand-like particles (hence the group name "Areno"), contain an RNA core, and are inactivated by chloroform. Usually, but not exclusively, these viruses occur in nature as infections of a specific rodent or rodents and, in many instances, persistent asymptomatic viremia is a prominent feature of this host-parasite relationship. The first 2 members of the group contain related CF antigens. The other viruses also have cross-reacting CF antigens and will be referred to as the "Tacaribe complex." A relationship between the Tacaribe complex and lymphocytic choriomeningitis (LCM) virus can be demonstrated by sensitive IFAT methods. Type-specific homologous antiserum, however is required for virus neutralization. In humans, LCM virus is associated with a variety of clinical manifestations, the most common being aseptic meningitis. Lassa viru causes a severe febrile illness,

TABLE II. THE ARENOVIRUS GROUP

VIRUS	GEOGRAPHIC DISTRIBUTION	HUMAN DISEASE
LCM	Wide-spread	Aseptic meningitis, other syndromes
Lassa	Nigeria	Fever, shock, multisystem involvement
Tacaribe	Trinidad	None known
Junin	Argentina	Hemorrhagic fever
Machupo	Bolivia	Hemorrhagic fever
Amapari	Brazil	None known
Pichinde	Colombia	None known
Parana	Paraguay	None known
Tamiami	United States	None known (antibodies found)
Latino	Bolivia	None known

while Junin and Machupo viruses induce hemorrhagic fever. Neutralizing antibody to Tamiami virus has been detected in human sera, but illness has not been attributed to it.

Some specimens for virus isolation and/or serology were obtained by the Bolivian authorities, Table III. Blood specimens from Cases 3 and 4, collected under less-than-ideal conditions, arrived unlabeled at MARU. One

TABLE III. RESULTS OF VIRUS TESTS AT MARU

CASE	SPECIMEN	ADEQUATE PRESERVATION	SUCKLING HAMSTERS	SUCKLING MICE	GUINEA PIGS
3, or 4	Blood	?	Neg	Neg	Neg
3, or 4	Blood	?	Ill, not typical	Neg	Neg
4	Liver	Yes	Ill, not typical	Neg	ND

			MACHUPO TITERS		
			CF	IFAT	Neut.
5	Serum, 30 days	Yes	1:32	1:64	1:4

of these specimens induced illness in suckling hamsters following simultaneous intraperitoneal and intracerebral inoculations. Neither illness nor pattern of death were typical of Machupo virus infection in suckling hamsters. Hepatic tissue, obtained from Case No. 4 at autopsy, was preserved in liquid N. Inoculation of this material into suckling hamsters produced findings atypical of Machupo virus. Infected suckling hamster brain had low levels of Tacaribe complex CF antigen. Four- and 7-week-postillness sera from Case No. 5 had high titers of CF and IFAT antibodies, but relatively low levels of neutralizing antibodies against Machupo virus. Neutralization tests against other Tacaribe complex members, including Junin, were negative.

Subsequent work, in gas-tight biological safety cabinet systems, has been conducted in our facilities at USAMRIID. A variety of animal hosts and tissue culture lines have been inoculated, and the CF and IFAT techniques have been employed to detect the presence of Tacaribe complex antigen. Table IV summarizes the results.

TABLE IV. PRELIMINARY USAMRIID TEST RESULTS ON COCHABAMBA OUTBREAK

CASE	SPECIMEN INOCULATED	SUCKLING HAMSTERS		TISSUE CULTURE		
		sick	CF (brain)	WI-38 CPE	MA-111 CPE	VERO Fluor.
6	Spleen	+	+	+	+	+
	Blood	+	-	+	+	ND
	Throat swab	+	-		Contaminated	
4	Liver[a]	+	+	ND	ND	ND
3, or 4	Blood[a]	+	+	ND	ND	ND

a. Passage material.

Although there is evidence for the presence of a Tacaribe complex virus, the characterization has not proceeded to specific identification. It will be necessary to compare the serological reactions, particularly in the neutralization test, and biological properties of our isolate with those of Machupo virus.

We have been working with this virus approximately 6 weeks and have inoculated almost 1,000 tissue culture tubes, and at least that many animals. We are currently titrating pooled samples from these animals and cell cultures and have completed preparation of antibody pools for neutralization tests. Parallel studies with Machupo virus are in progress.

It is not yet possible to make any definite statements about the properties and host range of the Cochabamba isolate. The animal host range and behavior in tissue culture seem similar to those of Machupo virus. The differences so far noted may be related to passage level or titer.

In summary, a severe, apparently contagious, fatal human infection with a hemorrhagic fever syndrome has been described. The etiologic agent has been isolated and shown to be a member of the Tacaribe complex of the Arenovirus group, with properties similar in some respects, but differing in others, to those of Machupo virus. Additional studies are in progress.

STAPHYLOCOCCAL ENTEROTOXOID PROGRAM OF PATHOLOGY DIVISION

Joseph F. Metzger, COL, MC*

Staphylococcal toxins are effective in producing illness by aerosol, intravenous, and oral routes. In addition, they are formed in large quantities in suitable culture media, are of low molecular weight and highly stable. They, therefore, must be considered as potential biological warfare agents. Significant studies have been done on monkeys utilizing staphylococcal enterotoxin B (SEB) especially by the aerosol route. Recently developed cultural techniques and isolation of new toxin-producing organisms make possible the production of large quantities of crude concentrates of enterotoxins A and C. New purification procedures have made possible the production of pure lots. Thus, all 3 major enterotoxins must be considered in the development of a polyvalent toxoid for protection against the enterotoxins of Staphylococcus aureus. Though the polyvalent enterotoxoid would be developed primarily as a protection against biological warfare situations, it should also protect against the usual oral toxicity of S. aureus enterotoxins. Production of various types of staphylococcal enterotoxoids has been going on for approximately 30 years. These enterotoxoids have all been produced utilizing formalin and have varied from using whole culture filtrates to purified SEB. All attempts at production of an immunizing agent have more or less centered on use of formalin for toxoiding. Though immunological studies such as precipitins have been studied against the toxoids formed in this way, few, if any physicochemical studies have been done. Silverman, et al.[1,2] reported a decreased precipitin reaction during toxoiding. Charles S. Pfizer Co., produced under contract with USAMRIID a toxoid of SEB. The enterotoxoid was produced by formalization of purified enterotoxin by the addition of 0.8% of formalin. The toxoiding was carried out from 30-40 days at a temperature of 37 C depending on safety tests. This material was tested in a large number of experiments within USAMRIID over a period of approximately 3 years. The usual immunization schedule consisted of 0.5 ml given subcutaneously in 2 injections. Animals immunized in this manner were protected from illness at the 10 μg/kg intravenous dose and protected against death at the 300 μg/kg dose by the same route. Repeated studies utilizing this material were carried out over a 2-year period. Although there appears to be a slight decrease in protection in this time, it is interesting to note that animals immunized 2 years after the toxoid was produced failed to develop toxin precipitating antibodies. The Pfizer enterotoxoid when it was approximately 2½ years from production was injected into rhesus monkeys by a modified schedule of 0.1 ml intradermally, followed by the usual two 0.5-ml subcutaneous doses. This regimen produced satisfactory immunizations against a 10 median illness dose challenge by the oral route. Very little is known about the physicochemical

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properties of this toxoid at the time it was produced. At this time the toxoid consists of a major and minor component as determined by Sephadex filtration. The major component is large and appears in the void volume of a G-100 Sephadex column. The large major component is stable; when rechromatographed it remains pure. The minor component is slightly larger than unmodified enterotoxin; on rechromatography it shifts partially in size to develop the large component as well as maintaining a small amount of the small component. There appears to be an equilibrium in the unstable minor component with a shift to the larger size. The enterotoxoid when electrophoresed shows a shift in charge and migrates approximately as serum albumin, whereas native enterotoxin migrates as gamma globulin.

The program within the Pathology Division is divided into several phases. The first phase is a repetition of a formalin toxoiding with slight alterations in pH for enterotoxins A, B, and C. These will be toxoided with formalin at 0.8%; physicochemical properties such as molecular heterogeneity, molecular size, and electrophoretic mobility, will be studied throughout the period of toxoiding. After suitable safety testing of the individual preparations the 3 enterotoxoids will be mixed, resafety tested, and then used as a polyvalent immunizing agent in monkeys. The immunization schedule will be 0.1 ml intradermally followed by two 0.5-ml subcutaneous injections. These monkeys will be challenged both by the intravenous and oral routes for protection against the 3 enterotoxins.

Phase II will consist of a program to study enzymatically, or otherwise, chemically fragmented SEB to determine if it is possible to isolate a fraction or fragment of the enterotoxin molecule which will maintain its immunogenicity but which would have lost its toxicity. If these studies of chemical fragmentation are successful for SEB they may lead directly into work on enterotoxins A and C. It is hoped that by following the toxoid reaction by physicochemical methods as well as immunologically that a better understanding of the role of formalin toxoiding reactions will occur. In addition, it is hoped that there will be a physical or chemical endpoint for toxoiding which would eliminate the trial and error of repeated safety tests. Finally, the possibility of a development of a fragment of the enterotoxin molecule as an immunizing agent which would not require formalin toxoiding and therefore might well have enhanced immunogenic qualities.

In summary, a program is being developed to study the chemical alteration of a pure, well characterized peptide undergoing the chemical change of formalin toxoiding. This system should furnish information to improve the understanding of formalin-toxoiding in general.

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PATHOGENESIS AND CONTROL OF ENTERIC INFECTIONS IN VOLUNTEERS

Richard B. Hornick, M.D.*

Acute diarrheal illness remains one of man's most vexing problems and more often than not a specific enteropathogen cannot be isolated from affected patients. There is growing evidence that Escherichia coli strains not previously classified as enteropathogenic and unidentified viruses may be responsible for at least a proportion of acute disease. We have been interested in the pathogenesis of enteric infections due to diverse etiologies with the ultimate goal of developing suitable means of disease control through immunologic approaches. Studies conducted at the Maryland House of Correction employing prison volunteers have helped us to understand basic mechanisms whereby enteric pathogens produce illness. Most of these studies have dealt with bacterial pathogens.

Pathogenic potential of microorganisms. In general, the dose, virulence and tissue predilection of the pathogen determine how many individuals become ill, how ill they may be and the nature of their illness. Due to the remarkable stability of the intestinal tract, ingestion of many thousands of organisms is usually necessary to produce disease. Table I shows the doses of virulent bacteria necessary to produce illness in healthy adult volunteers. Three virulent Shigella spp. each produced dysentery in volunteers

TABLE I. DOSAGES OF VARIOUS ENTERIC PATHOGENS REQUIRED TO INDUCE ILLNESS IN MAN

SPECIES	NUMBERS
Shigella	10^2
Salmonella typhosa	10^5
Escherichia coli	10^8-10^{10}
Vibrio cholerae	10^{10}

following ingestion of less than 200 viable cells. That so few organisms can cause disease probably explains why shigellosis is generally transmitted from person-to-person and also why the disease is frequently a hyperendemic and recurrent problem among institutionalized populations. On the other hand, other enteric pathogens such as E. coli, S. typhosa, and V. cholerae which require larger inocula to induce disease, are usually transmitted by food or water.

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Table II lists various bacterial pathogens according to their interaction with the intestinal mucosa and mechanisms whereby diarrhea results. As shown by Sprinz et al,[1] Formal et al,[2] LaBrec and others[3] there are three important anatomic localizations of pathogens in relation to the intestinal epithelial lining. The first group typified by V. cholerae and certain E. coli strains cause disease without tissue penetration. Nonfebrile, watery diarrhea occurs secondary to multiplication in the small intestine and release of an enterotoxin which leads to exsorption of fluid and electrolytes.

TABLE II. PATHOGENESIS OF BACTERIAL DIARRHEAS

PATHOGEN	BACTERIA-INTESTINAL MUCOSAL INTERACTION	PATHOGENIC MECHANISM
V. cholerae E. coli	No invasion	Enterotoxin production
Shigella spp. E. coli	Penetration, intra-epithelial multiplication	Mucosal inflammation with destruction
S. typhosa	Complete penetration of epithelial cell	Inflammation of the lamina propria

The second group of pathogens characteristically invade epithelial cells and lead to mucosal destruction and ulceration. Virulent Shigella and other nontoxigenic invasive E. coli strains produce disease following invasion of the intestinal mucosa and subsequent multiplication. In contrast to the first group, these bacteria produce illness by invasion of the colonic mucosa; the small bowel in generally spared. The intense colitis leads to a febrile illness with frequent bloody mucoid stools of small volume with urgency and tenesmus.

The third important group exemplified by Salmonella strains, differ from the other two by the occurrence of epithelial cell penetration without destruction or ulceration. Here an inflammatory reaction in the lamina propria can be found; the nature of which determines whether the infection will become localized or systemic. For reasons which are not clear a mononuclear inflammatory response is elicited in typhoid infection, and blood stream invasion with systemic symptoms is the rule. On the other hand, nontyphoidal Salmonellae usually stimulate a polymorphonuclear (PMN) leukocyte reaction and the infection is contained in the mucosa with a clinical expression of acute gastroenteritis with low grade fever and watery diarrhea with some mucus occurring.

With the exception of poliovirus infection, the pathogenesis of viral enteric infection has not been well studied. With infection due to wild poliovirus, implantation and replication of virus occurs in the lymphoid tissue of the lamina propria and in Peyer's patches. The virus then seeds the blood via the lymphatics and may reach susceptible extraneural tissue. Sabin[4] has implicated the superficial epithelium in the gastrointestinal multiplication of poliovirus yet immunofluorescent studies are needed for definite localization.

In the early 1950's, Reimann[5] performed rectal biopsies on two patients with mild "nonspecific" diarrhea of presumed viral etiology; each showed mild inflammatory infiltrates with plasma cells and PMN cells. Pathologically, the lesions resembled those of shigellosis; in reading the report one wonders if these two patients did not have bacillary dysentery. Sheehy et al[6] have documented the occurrence of jejunitis with blunting of the villi and inflammatory cell infiltrates in patients infected with infectious hepatitis virus and type 4 adenovirus. Other investigators have shown deranged small bowel histology in individuals infected with other viral agents including measles, varicella, herpes simplex and poliovirus. Each of these viral pathogens has been implicated as a cause of transient diarrhea.

Examination of stools for leukocytes is helpful in determining the anatomic site and nature of an intestinal infection. Mr. John Harris, a senior medical student working in our laboratory, found that the presence of leukocytes in fecal smears of patients with infectious diarrhea invariably indicated pathogenic invasion of colonic mucosa. Patients with infections due to pathogens in Group I (noninvasive) that is, V. cholerae and toxigenic E. coli were uniformly negative for stool leukocytes while those in Group II (Shigellae or invasive E. coli pathogens) had intense PMN responses. Group III infections or those due to Salmonella strains also gave positive results by this test although the number of cells was more variable. In typhoid infection the leukocytes were nearly all mononuclear while in Salmonella gastroenteritis more than half of the cells were PMN. We have examined serial stools of 10 volunteers with viral induced diarrheal illness; leukocytes were not observed. Such a finding further raises doubts as to the similarity in histopathology of viral diarrhea and bacillary dysentery as suggested by Reimann.[5] Disease seen in volunteers with the Norwalk agent and that described in the literature due to supposed viral agents resembles a true gastroenteritis. The short incubation period followed by vomiting, then mild transient watery diarrhea without blood or mucus and without leukocytes implicates an upper gastrointestinal tract disease.

Immune mechanisms of the host. Susceptibility and immunity in gastrointestinal infection are complex concepts which are poorly understood. While general host factors such as age, debility and nutritional status

doubtless have importance to susceptibility as with other infectious diseases, probably of greater consequence in diarrheal disease are the local host factors at the gastrointestinal level.

Prior to infection, the organisms must be swallowed. The first line of defense, other than personal hygiene, consists of gastric trapping of the bacteria in a strongly acidic environment. In general enteric bacteria are quite susceptible to the low pH and many swallowed organisms never reach the small bowel as viable bacteria. Gastric function alteration with antacids or compromised gastric function by disease or prior surgery appears to alter host susceptibility. An increased attack rate of induced shigellosis, cholera and E. coli infection have been demonstrated in volunteers when the organisms are ingested after oral administration of sodium bicarbonate. We had previously thought that the reason for the marked difference in dose required to produce disease with various pathogens might relate to relative acid sensivity. Dr. Calia of our Division has examined acid sensitivity of various enteropathogenic bacteria and, with the possible exception of V. cholerae which is extremely sensitive to low pH, has been unable to correlate the dose response as determined in volunteers with acid sensitivity of the corresponding bacteria. Also, studies in volunteers have shown viable S. typhosa in the stomach for at least 30 min following ingestion while in an occasional individual virulent Shigellae may be obtained up to 20 hr after oral intake, despite a low pH. Both of these organisms are acid sensitive. Doubtless, gastric function is important to susceptibility in enteric infections, however, pH alone does not appear to be the sole explanation. Also, it is known that enteroviruses are resistant to low pH and Dolin and co-workers[7] found that the Norwalk agent similarly resisted acid treatment.

Normal upper intestinal motility which is of a constant nature in contrast to the more sluggish motility pattern of the colon has a cleansing role and probably accounts for the relative lack of microbial growth in this region of the gut. In order to infect guinea pigs with enteric pathogens, it is necessary to first restrict intestinal motility with opiates. Also, the development of systemic salmonellosis with bacteremia has been shown occasionally to follow the use of opiates in patients with mild gastroenteritis. If a pathogen exerts local toxicity or if intestinal transit is inhibited by a drug such as an opiate, the bacteria may localize and multiply. By retarding small intestinal transit, there is an increased contact time between the mucosal cells and the potential pathogen.

In addition to gastric acidity and intestinal motility there are other local immune factors at work. The bacterial flora of the intestine exerts a strong homeostatic influence and deters the potential invader perhaps by competition for space and nutrients or by elaboration of antibacterial catabolites. Many intestinal bacteria produce enough volatile short chain fatty acids such as acetic and butyric acids to inhibit growth of virulent enteric pathogens. Alteration of enteric flora with the accompanying fatty

acid decreased by, for example, antimicrobial therapy, increases susceptibility in experimental animals and man to enteric pathogens.

Intestinal or coproantibodies may play a role in the regulation of intestinal flora and their lack may be associated with local infection. Children with agammaglobulinemia often have persistent gastroenteritis with ileocolitis. The importance of coproantibodies to intestinal immunity seems almost certain in the defense against many enteric bacterial and viral pathogens. In experimental cholera, coproantibodies have been shown to prevent adherence of vibrios to the intestinal mucosa and, in experimental animals, there is a definite relation of resistance to V. cholerae and copro- but not circulating antibody levels. Live, orally administered Shigella and typhoid strains have been shown to induce immunity in man wherein humoral antibody cannot regularly be detected yet there is found a measurable intestinal antibacterial effect on swallowed virulent bacteria and alteration in disease progression. Locally produced immune proteins also have been shown to be important in viral infection. Parenteral immunization with killed poliovirus vaccine protects against the viremia and paralytic disease, while only attenuated orally administered strains give protection against intestinal infection by mild virus. In such individuals poliovirus neutralizing IgA coproantibodies can be detected.

Assuming that a sufficient dose of virulent organisms are swallowed by an individual and host defense mechanisms are not capable of fully dealing with the pathogen, how is diarrhea produced? There are four important pathologic mechanisms which have been implicated in diarrheal illness:

(1) Impaired intestinal absorption;
(2) Increased mucosal or vascular permeability resulting in fluid and electrolyte exsorption;
(3) Presence of intraluminal, nonabsorbable osmotically active solutes; and
(4) Abnormal intestinal motility.

These factors rarely occur alone in a patient with acute diarrhea. In general, each of these factors are stimulated in some way in enteric infection. This is the reason why clinical signs and symptoms are often so similar regardless of etiology.

The intestine can react to injury in a limited number of ways. Small intestinal tissue obtained from individuals with acute diarrhea due to many causes often shows similar changes which include lengthening of the crypts, decrease in villous height and increased mucosal cellularity and inflammation. It has been shown that following recovery from intestinal infection due to a variety of pathogenic organisms, that these characteristic changes of small bowel injury may be found; at the same time

significant impairment of absorptive function can often be detected. In most cases absorptive function returns to normal within a week following infection yet occasionally small bowel dysfunction may persist for longer periods. It is quite likely that viral agents which are capable of inducing gastroenteritis through intestinal mucosal invasion could exert a diarrheal influence by altering absorptive function or by exerting an influence on intestinal motility patterns. Cohen et al[6] studied adults with "acute gastroenteritis" from whom no known pathogens were recovered; they had high counts of viable enteric bacteria in jejunal aspirates, although the upper intestinal tract normally supports only minimal bacterial growth. The fecal flora then could proliferate in the small bowel with subsequent changes in intestinal permeability, absorption and motility.

Now that we have a reproducible model of infection when bacteria-free stool filtrates from individuals with acute gastroenteritis are fed to volunteers, it will be important for us to concentrate on pathogenic mechanisms of infection. Specifically, we will need to document histologic changes of the small and large bowel mucosa as well as measure small intestinal permeability and absorptive functions through intubation studies, and finally to determine the presence and importance to disease pathogenesis of proliferation of fecal flora in the small bowel.

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MECHANISMS OF INFLAMMATORY RESPONSE IN MAN

Sheldon E. Greisman, M.D.*

In 1970, Salomon, Tamlyn, and Grieco,[1] after studying a patient with Escherichia coli pneumonia, summarized the present state of knowledge regarding human inflammatory responses to gram-negative bacteria as follows: "Pneumonia caused by either E. coli or bacteroides is characterized surprisingly by infiltrates containing predominantly mononuclear cells, the other pneumonias, by mixed infiltrates of polymorphonuclear leukocytes and mononuclear cells. In this case the intra-alveolar infiltrate contained mononuclear cells and rare polymorphonuclear leukocytes. Gram-negative rods were present in all lung sections, and in the areas with the radiographic evidence of pneumonia, the cell response was mononuclear.

"The early tissue response in most acute infections is characterized by an infiltrate containing polymorphonuclear leukocytes. The reason for a predominantly mononuclear response in acute pneumonia due to E. coli or bacteroides and a mixed polymorphonuclear and mononuclear response in pneumonia due to other gram-negative organisms remains unclear. Although it is possible that polymorphonuclear leukocytes are quickly destroyed, in the present case portions of the lungs with the earliest pneumonia still exhibited a mononuclear infiltrate.

"It has been suggested that an inadequate polymorphonuclear leukocyte response may occur as a result of underlying disease. Peripheral polymorphonuclear leukocyte counts, however, might be increased in these patients, and debilitated patients will evoke infiltrates containing polymorphonuclear leukocytes in the presence of gram-positive pneumonias.

"...At present the reason for the mononuclear response remains an enigma, but it does appear to be a characteristic of pneumonia caused by E. coli."[1]

The present studies provide an explanation for the "enigma" of the mononuclear response of gram-negative bacterial infection. These studies were initiated to define the human inflammatory response to purified bacterial endotoxins derived from gram-negative bacteria and to compare this response with that elicited by the whole gram-negative organism. The striking similarity of the response to purified toxin and its parent organism, and the difference from that produced by gram-positive bacteria permit this basic inference: gram-negative bacteria evoke predominantly mononuclear inflammatory responses on the basis of their content of highly reactive endotoxin, whereas gram-positive bacteria, lacking endotoxin,

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evoke the classical polymorphonuclear (PMN) response. The subsequent report documents the evidence that supports this hypothesis and additionally, demonstrates the similarity of the endotoxin response to that evoked by purified tuberculin derivative (PPD) in sensitive volunteers.

No febrile or untoward responses were produced following intradermal (ID) inoculation of any of the bacterial preparations, probably because the quantities employed never exceeded 3 log concentrations of the minimal inflammatory dose. This dose falls between 10^7 and 10^8/ml for all the whole bacteria used, and between 0.001 to 0.01 μg/ml for the endotoxin preparations, except for Piromen where the minimal inflammatory dose approximated 0.1 μg/ml. The inflammatory response to serial dilutions of each bacterial preparation was studied in 2-4 volunteers, PPD responses were studied in 8.

In initial studies, it became apparent that if differences in inflammatory responses to bacterial preparations were to be detected, the early inflammatory response, i.e. 3- to 6-hr reaction must be chosen. By 24 hr at the dosages employed, virtually all inflammatory responses appeared qualitatively similar. Thus, regardless of whether pneumococci, staphylococci, E. coli, Salmonella typhosa, Shigella shiga, PPD or purified bacterial endotoxin was injected, the 24-hr response was stereotyped. Mononuclear cells, predominantly lymphocytes, were observed as focal collections in perivascular distribution. The collagen stroma at this time was usually minimally infiltrated with inflammatory cells. The striking metamorphosis from a 6-hr inflammatory response consisting predominantly of PMN scattered diffusely throughout the stroma to the predominantly mononuclear perivascular response at 24 hr is shown in Figures 1 and 2 employing pneumococci as the inflamant. Only when cortisone (10 mg/ml) was mixed with the bacterial preparation could this 24-hr inflammatory response be altered to a predominantly PMN reaction. It appeared that when the anti-inflammatory effect of the steroid wore off, the PMN inflammatory process, which was initially inhibited, now proceeded as usual, the whole response behaving as if it were simply postponed. The steroid alone evoked no inflammatory cellular response at 24 hr.

Since with the dose of bacterial preparations employed, the 24-hr cellular inflammatory responses were stereotyped (at least by light microscopy), in all subsequent studies the inflammatory reaction was studied at 3 hr. At this stage, the early inflammatory responses to the various bacterial preparations were not stereotyped and the findings can be summarized briefly as follows:

Minimal inflammation-producing doses of bacterial endotoxin (approximately 0.001 μg/ml), regardless of their sources, evoked inflammatory responses characterized by predominantly mononuclear perivascular infiltrations. PMN generally comprised < 20% of the inflammatory cell population in the perivascular areas. Small lymphocytes were the most abundant cells, but monocytes were frequent. Minimal collagen infiltration with PMN leukocytes occurred. Tissue necrosis, dermal or epidermal, was absent. Figure 3 shows a typical response to minimal inflammatory doses of endotoxin at 3 hr.

Intermediate doses of endotoxin (approximately 0.01 μg/ml) producing moderate inflammatory responses, evoked increasing PMN infiltration, but mononuclear cells generally remained > 50% of the cell population, Figure 4. The PMN infiltration was primarily seen as infiltrations into the dermal collagen whereas the mononuclear collections were primarily perivascular, as if the former cells migrated more rapidly from their vascular origin. In rare areas, collagen and vascular necrosis appeared and in these areas, PMN predominated.

Marked inflammation evoking doses of endotoxin ($\geq$ 0.1 μg/ml) consistently yielded responses dominated by PMN which infiltrated diffusely all layers of the dermis. Frequent areas of collagen and vascular necrosis, inundated with these leukocytes were characteristic. However, at the periphery of the lesions, where the inflammatory response was mild (presumably because endotoxin concentrations were low) the perivascular collections of predominantly mononuclear cells were evident and resembled the central responses to the more dilute endotoxin preparations.

The inflammatory responses to whole, killed gram-negative organisms were generally similar to those described for bacterial endotoxin. Minimal inflammation-inducing doses of organisms (approximately 10^7/ml) usually elicited predominately perivascular mononuclear responses and minimal stromal infiltration regardless of the species of organism tested. As the dosage of organism was increased logarithmically, polymorphonuclear infiltration became progressively more intense and more diffusely scattered throughout the collagen, resembling the response to the higher concentrations of endotoxin, Figures 5-7.

Minimal inflammation evoking doses of staphylococci and pneumococci evoked responses at 3 hr with predominantly polymorphonuclear infiltrates. These cells generally comprised > 70% of the cell population and were widely scattered through the dermal collagen. The focal perivascular collections of mononuclear cells that characterized the 3-hr responses to bacterial endotoxin and gram-negative bacteria were conspicuously minimal, Figure 1. As the dose of gram-positive bacteria was increased, PMN infiltration increased progressively with focal collagen and vascular necrosis and microabscess formation.

Employing 2 μg/ml of PPD, the cellular inflammatory response at 3 hr in 8 tuberculin positive reactors was minimal or absent when compared to the saline control responses. Of the 3 volunteers exhibiting the minimal responses, the inflammatory reaction was limited to perivascular accumulations of mononuclear cells (predominantly small lymphocytes) in 2 cases, but was predominantly polymorphonuclear leukocytic in one volunteer. This last subject was unique in that he had been tested with PPD the previous week at 4 sites and these had been biopsied at 6, 24, 30 and 48 hr. The possible effect of such "boostering" on the 3-hr responses to PPD requires

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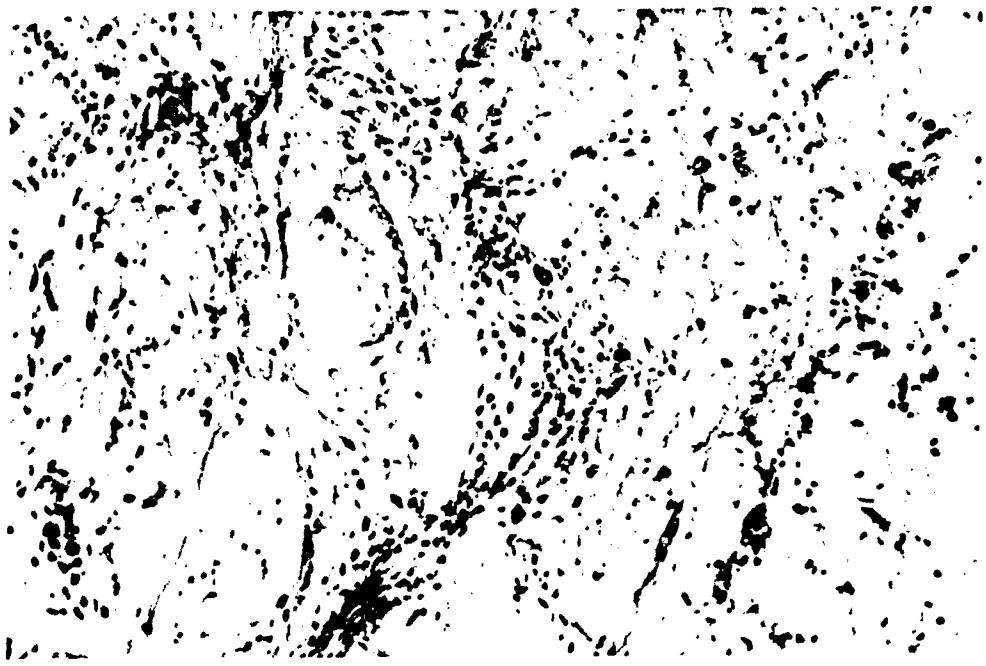

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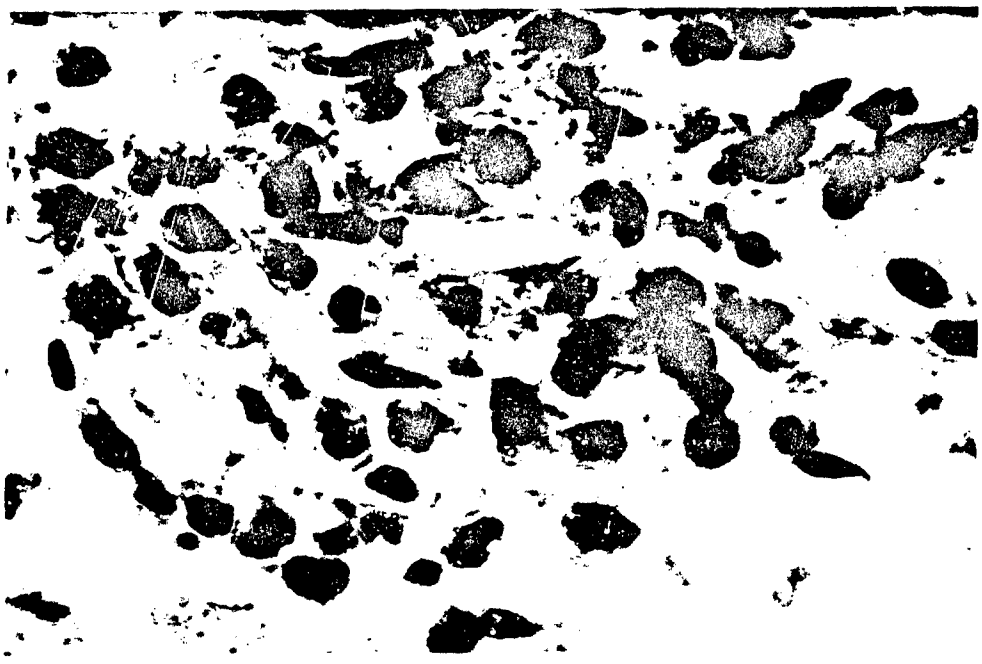

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FIGURE 1. 6-HR INFLAMMATORY RESPONSE TO ID INOCULATION OF 0.1 ml OF 10^8/ml FORMALIN-KILLED, PNEUMOCOCCI. >80% PMN CELLS, SCATTERED THROUGH DERMAL COLLAGEN. A. X270. B. X1620.

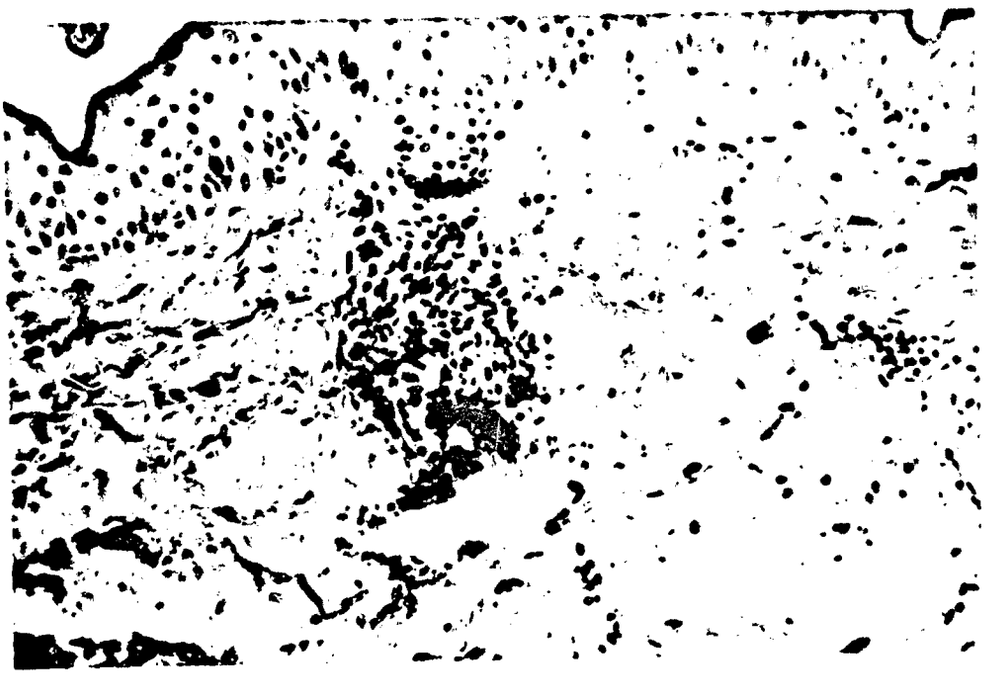

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FIGURE 2. SAME VOLUNTEER AS FIGURE I, AT 24 HR. ≥90% MONONUCLEAR CELLS, MOSTLY PERIVASCULAR. X270

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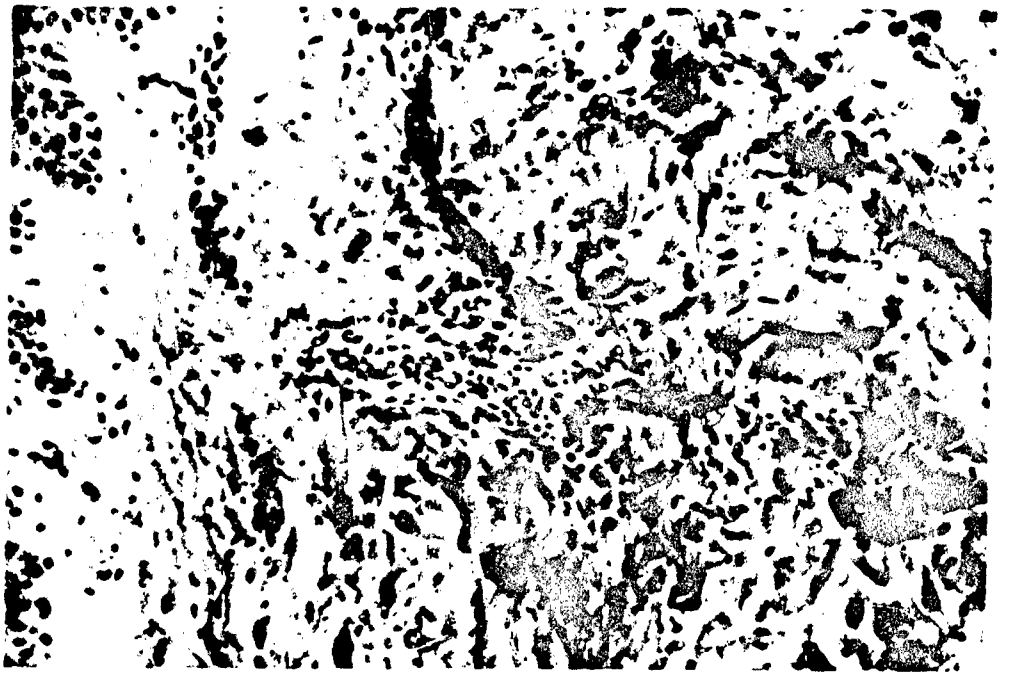

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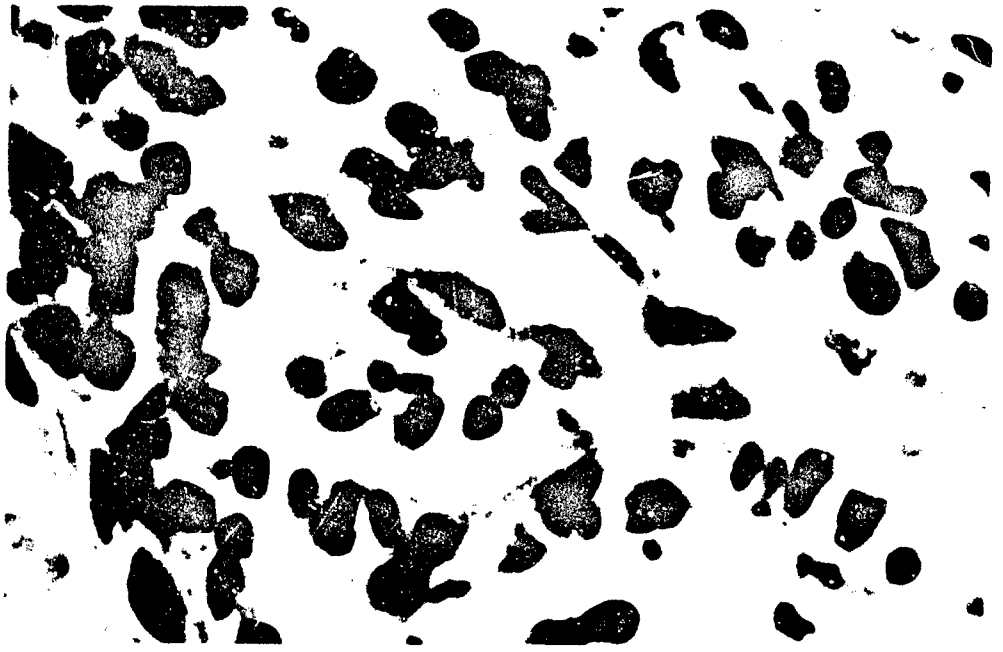
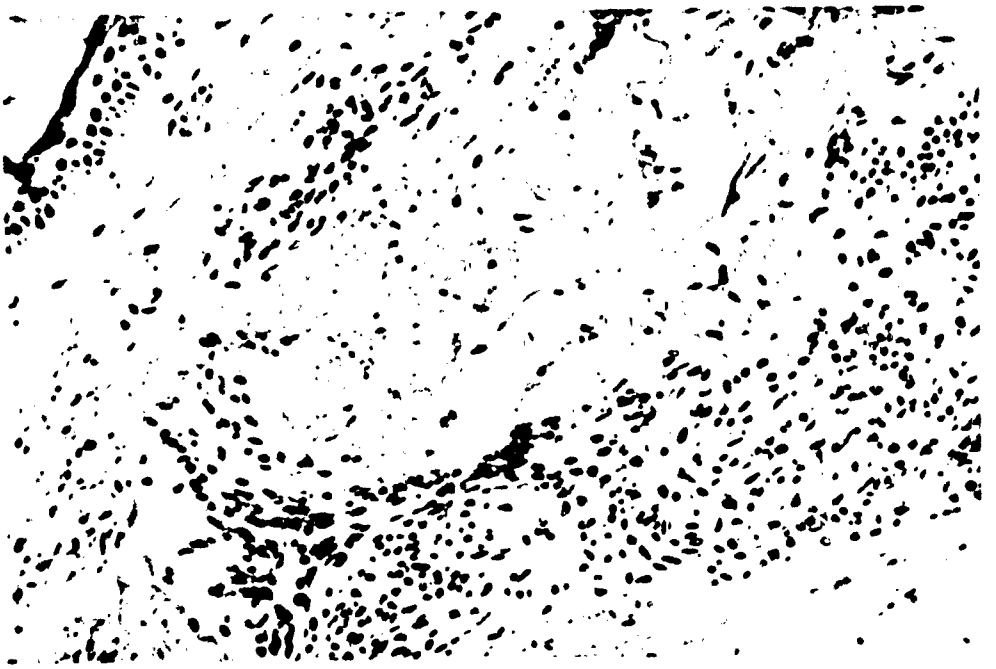


FIGURE 3. 3-HR INFLAMMATORY RESPONSE TO ID INOCULATION OF 0.1 ml OF 0.001 µg/ml *S. TYPHIMURIUM* (ROUGH B MUTANT) ENDOTOXIN. 95% MONONUCLEAR CELLS, MOSTLY PERIVASCULAR. A. X270. B. X1640.



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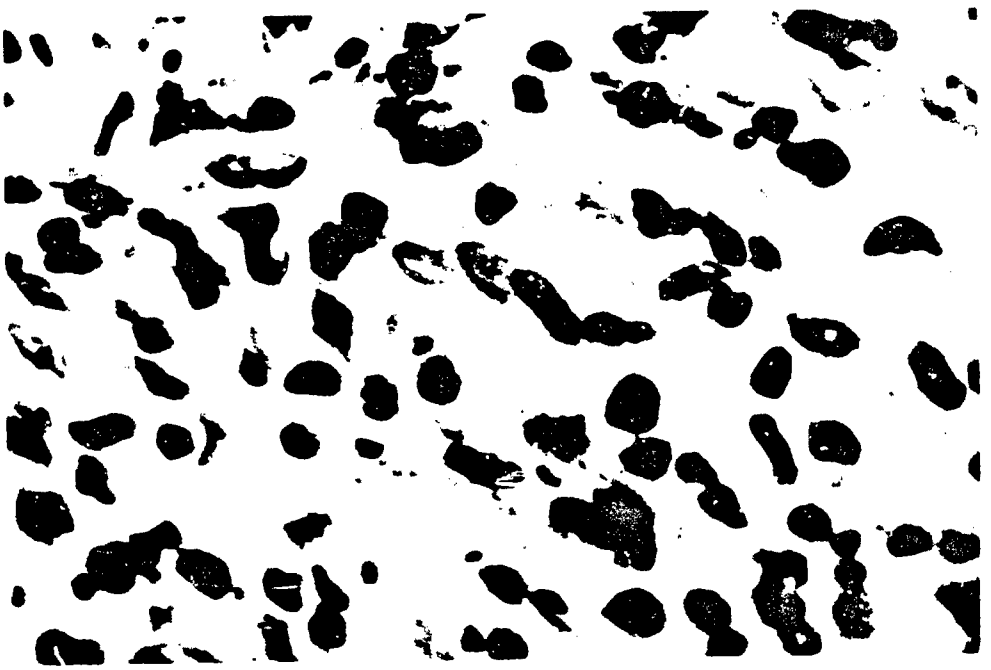

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FIGURE 4. 3-HR INFLAMMATORY RESPONSE TO ID INOCULATION OF 0.1 ml OF 0.01 µg/ml *S. TYPHOSA* ENDOTOXIN. 90% MONONUCLEAR CELLS, MOSTLY PERIVASCULAR. A. X270. B. X1640.

further study. By 6 hr, perivascular accumulation of mononuclear cells was striking in all volunteers, and polymorphonuclear leukocytes generally comprised < 10% of the inflammatory cell population. Minimal cellular infiltration of collagen was seen. The intensity of the perivascular mononuclear infiltration increased progressively, reaching a maximum at 24-36 hr. The few polymorphonuclear leukocytes present in the earlier lesions were almost entirely absent at this time.

For ethanol in concentrations producing a minimal inflammatory response greater than that evoked by saline alone (0.1%), polymorphonuclear leukocyte infiltration of the dermal collagen constituted the major finding at 3 hr. Perivascular infiltration with mononuclear cells was minimal. Higher ethanol concentrations evoked progressively increasing polymorphonuclear leukocytic infiltration.

In addition to the above studies, 3 additional series of investigations were carried out in relation to the pathogenesis of the inflammatory response during typhoid fever:

The inflammatory reaction in a rose spot obtained from a patient with naturally acquired typhoid fever was characterized by a perivascular mononuclear response, consisting chiefly of small lymphocytes but also containing abundant macrophages. Polymorphonuclear leukocytes comprised < 5% of the inflammatory cell population. No collagen or epidermal necrosis was seen, Figure 8.

Responses to endotoxin and whole S. typhosa during typhoid fever were obtained at 3-6 hr in 4 patients during the 4th to 6th days of overt typhoid fever. The inflammatory responses to S. typhosa endotoxin, E. coli endotoxin, and whole formalin-killed, washed S. typhosa were similar to those described previously in healthy control volunteers. Minimal inflammation-inducing quantities of these preparations evoked the predominant perivascular mononuclear responses with minimal infiltration of the dermal collagen with polymorphonuclear leukocytes. As dosage was increased, infiltration with PMN increased progressively. Preillness baseline data are not available to determine if the dosage of endotoxin and whole bacteria required to elicit the minimal inflammatory responses are altered as a result of illness.

To determine the local inflammatory response to chronic exposure to endotoxin, S. typhosa and E. coli endotoxins were injected ID into the same sites in healthy volunteers once per day for 1 week. Biopsies were obtained on the 8th day, 24 hr after the last injection. Similar studies were performed with whole, formalin-killed S. typhosa and with saline controls. In the dose ranges tested, (0.001-0.01 μg/ml endotoxin and 10^8-10^9/ml S. typhosa) inflammatory responses were characterized by intense mononuclear infiltration, both perivascular and in the dermal collagen, with abundant small lymphocytes and macrophages. PMN leukocytes generally comprised < 5%

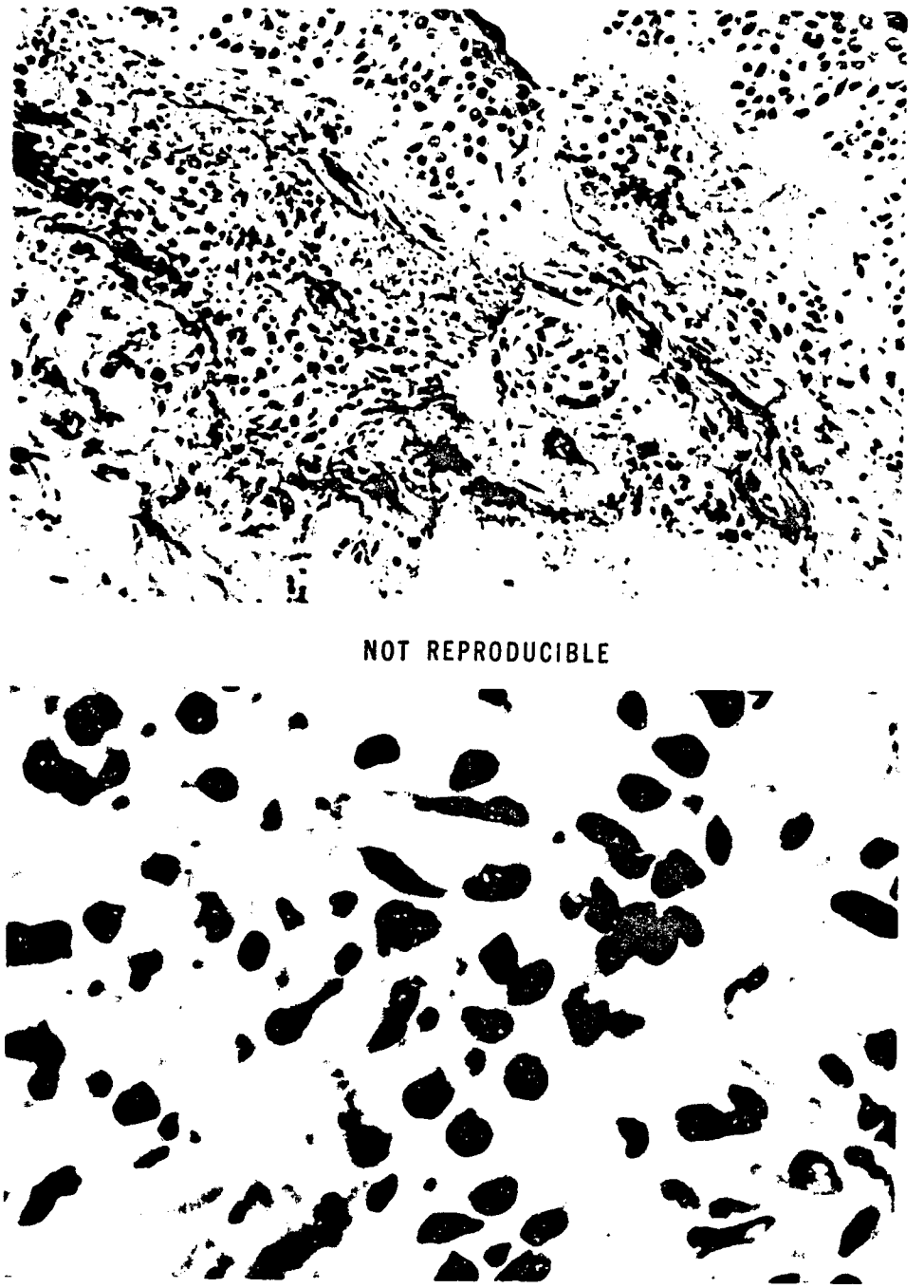

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FIGURE 5. 3-HR INFLAMMATORY RESPONSE TO ID INOCULATION OF 0.1 ml OF 10^8/ml WHOLE, FORMALIN-KILLED E. COLI. >90% MONONUCLEAR CELLS, MOSTLY PERIVASCULAR, SPREADING INTO COLLAGEN STROMA. A. X270 B. X1640.

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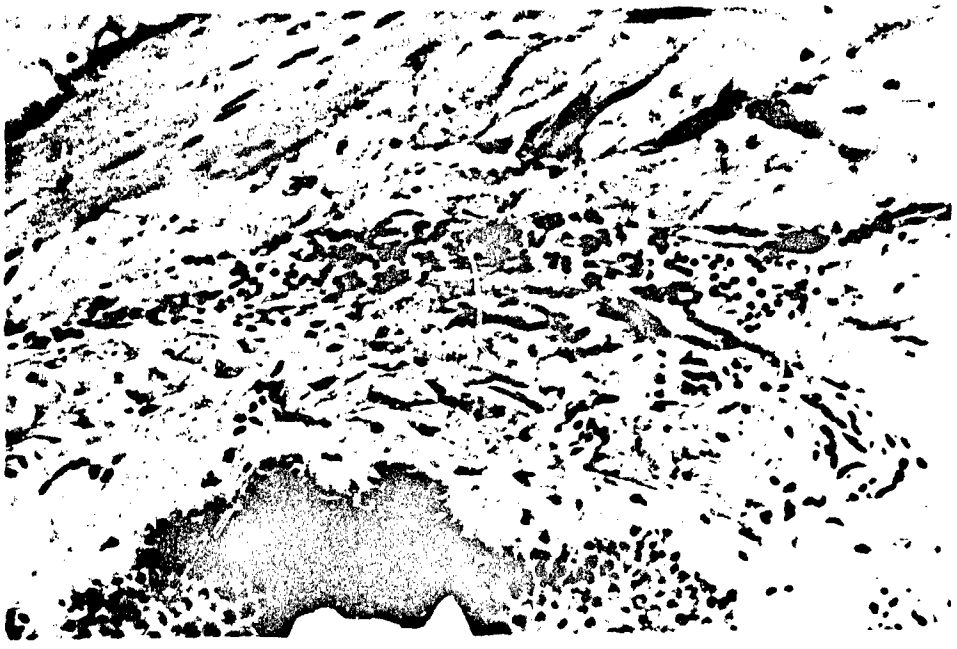

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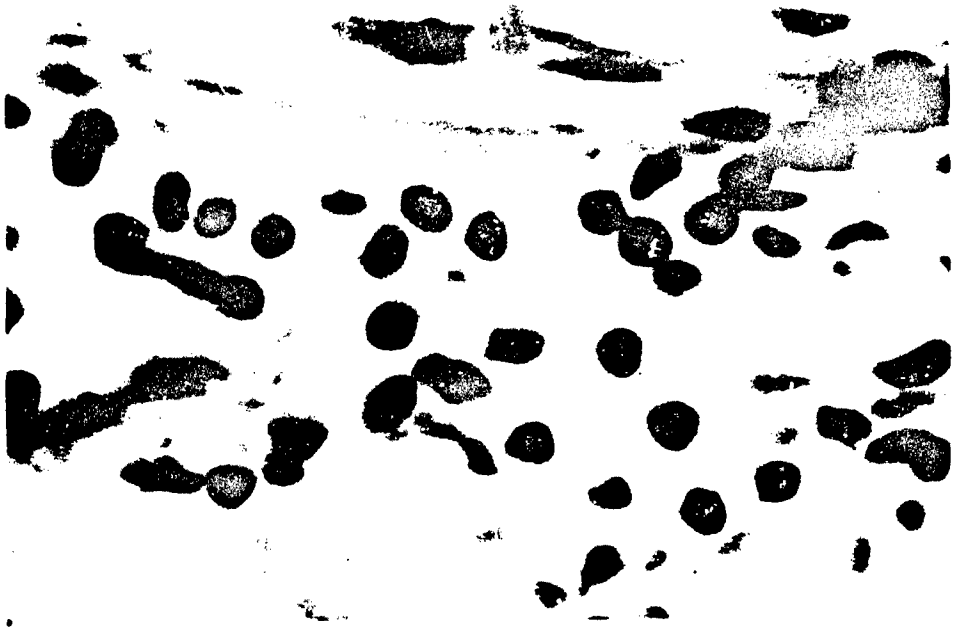

FIGURE 6. 3-HR INFLAMMATORY RESPONSE TO ID INOCULATION OF 0.1 ml OF 10^7/ml HEAT-KILLED *F. TULARENSIS*. >95% MONONUCLEAR CELLS, PERIVASCULAR. A. X270 B. X1640.

A

B

FIGURE 7. SAME VOLUNTEER AS FIGURE 6. 0.1 ml OF 10^9/ ml OF *F. TULARENSIS* GIVEN SIMULTANEOUSLY AT DIFFERENT SITES. REACTION IS DIFFUSE WITH COLLAGEN NECROSIS. 70% PMN CELLS. A. X270. B. X1640.

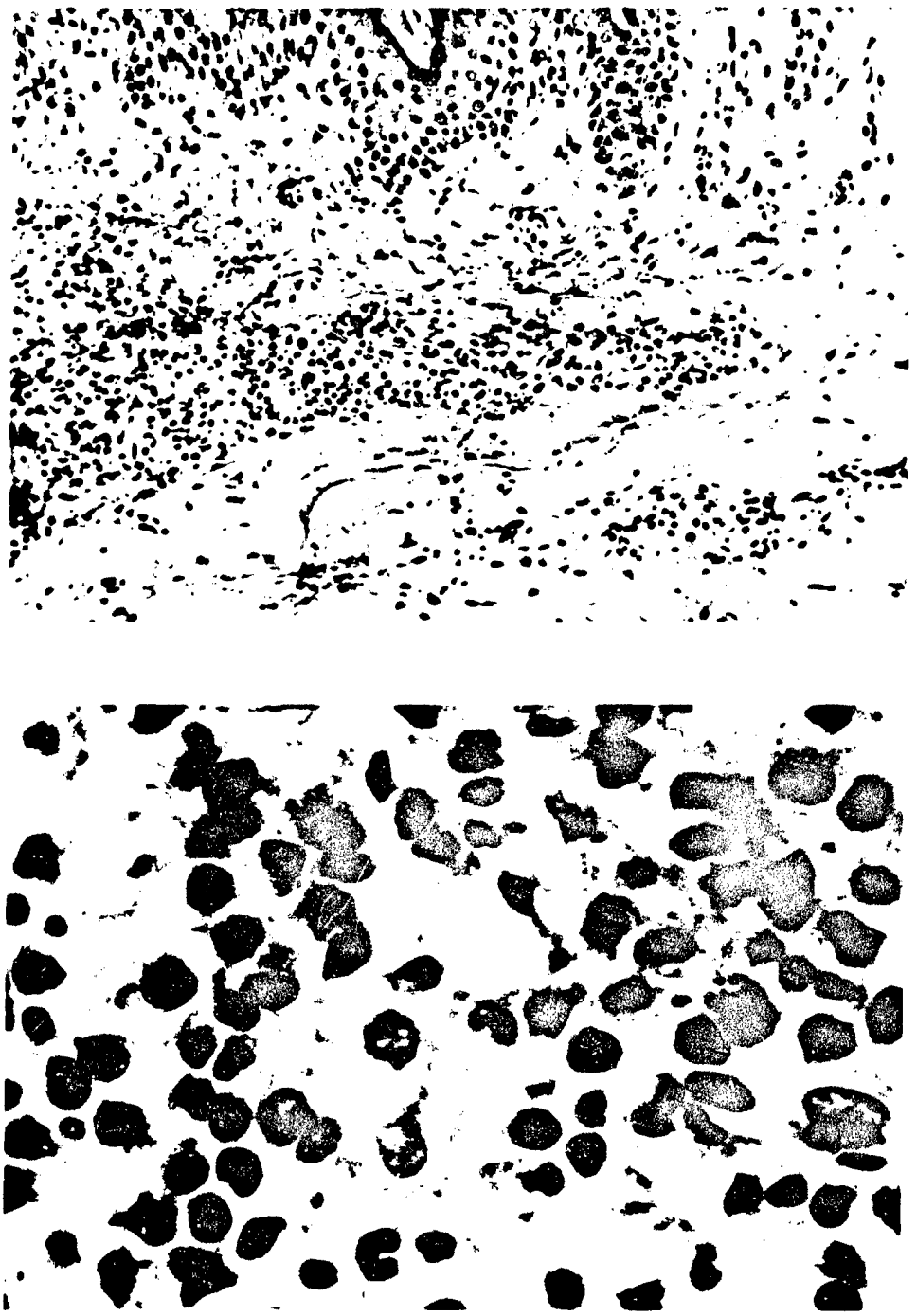

FIGURE 8. FOCAL LESION OF ROSE SPOT IN NATURALLY OCCURRING TYPHOID FEVER. >95% MONONUCLEAR CELLS, A MIXTURE OF SMALL LYMPHOCYTES AND MACROPHAGES. A. X270 B. X1640.

A

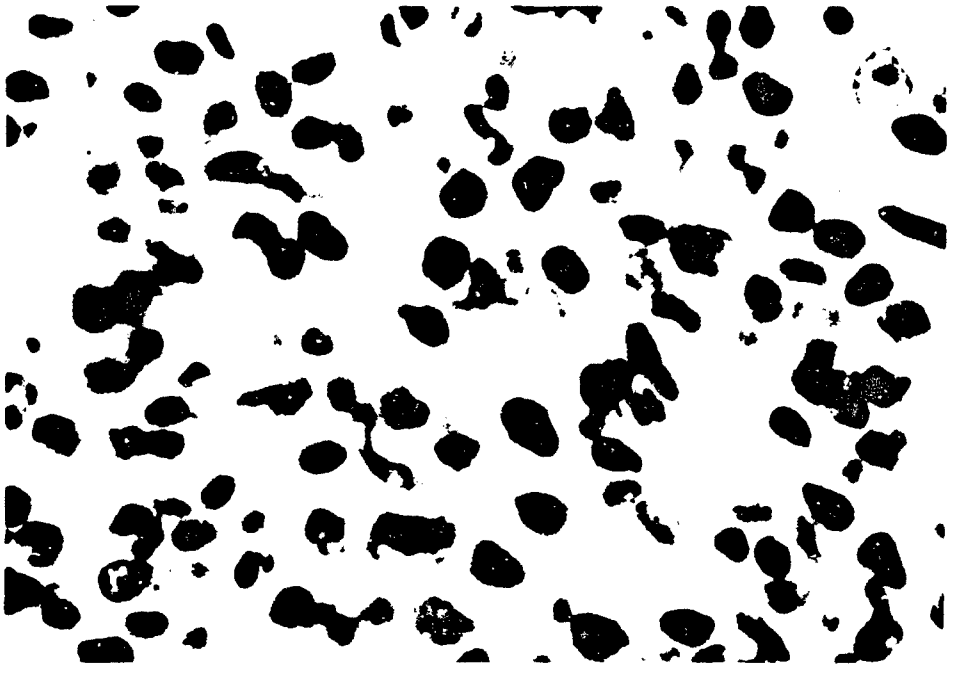

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FIGURE 9. TYPICAL INFLAMMATORY RESPONSE OF HEALTHY VOLUNTEER TO REPEATED ID INOCULATIONS (DAILY FOR 7 DAYS) OF *S. TYPHOSA* ENDOTOXIN (0.1 ml OF 0.01 µg/ml). REACTION ALMOST INDISTINGUISHABLE FROM RESPONSE SHOWN IN FIGURE 8.
A. X270 B. X1640

of the inflammatory cell population. The lesions were virtually indistinguishable from the naturally occurring rose spot, Figure 9. Control sites injected with pyrogen-free saline revealed minimal or no inflammatory responses.

That most, if not all, acute inflammatory infiltrates are initially composed of polymorphonuclear leukocytes, and that this is progressively transformed into a predominantly mononuclear cellular infiltrate has been a generally accepted attribute of the inflammatory response.[2] However one outstanding exception to this rule has caused much interest and continuous controversy---the nature of the early inflammatory response to tuberculin in sensitized animals. Several groups of investigators reported an early, predominantly mononuclear celluiar infiltration distinct from that seen in the usual nonspecific inflammatory responses.[3-8] Other groups of investigators found no such predominant early mononuclear response and concluded that the response in delayed hypersensitivity was nonspecific and had no distinctive histologic features.[9-14] The present studies on the nature of the cellular response during inflammation was stimulated by ongoing investigations related to the pathogenesis of typhoid fever. Histologic studies of the inflammatory response in this illness, even in early stages, have revealed a paucity of polymorphonuclear leukocytes, mononuclear cellular infiltrations predominating.[15-16] That circulating neutropenia is not the simple explanation for this mononuclear typhoidal reaction is evident by the ability of patients with typhoid fever to develop pyogenic abscesses in the presence of complications.[15] Indeed, as emphasized by Paz and Spector, "...certain inflammatory reactions that are commonly designated 'mononuclear', because polymorph infiltration is slight and transient whereas the local accumulation of mononuclear leucocytes is massive and persistent. In this category are the tuberculin reaction and the local lesions of typhoid fever and tuberculosis."[12] Earlier, Mallory had emphasized this point, stating "Histologically, the typhoid process is proliferative and stands in close relationship to tuberculosis...."[17] The present studies were designed to determine if S. typhosa possesses some unique property similar to tuberculin of eliciting mononuclear cellular inflammatory responses and if so, whether the endotoxin component could account for this effect.

In initial studies, the well documented progression from an early predominantly polymorphonuclear leukocytic inflammatory response at 3-6 hr to a predominantly mononuclear response by 24 hr was seen, employing whole washed staphylococci and pneumococci as inflamants. Furthermore, by 24 hr, with the dosages of materials employed, no clear distinction could be made between the cellular inflammatory responses to gram-positive bacteria, gram-negative bacteria, purified bacterial endotoxin, or PPD in sensitive subjects. Only when hydrocortisone was added could the inflammatory response at 24 hr be altered. With steroid use the 6-hr inflammatory response was markedly suppressed, whereas at 24 hr the response was predominantly polymorphonuclear. It appeared as if the steroid had simply suspended the early polymorphonuclear leukocytic inflammatory response for many hours. The steroid alone did not

incite any inflammatory response greater than saline alone. Since the 24-hr phase of inflammation appeared stereotyped, the 3-hr phase was studied in detail. Here definite differences in responses were seen to various bacterial preparations suspended in saline. Injection of formalin-killed, washed gram-positive bacteria in doses that incited mild inflammatory responses, i.e., responses that were slightly but definitely in excess of that provoked by control injections of saline alone, induced responses characterized by predominantly polymorphonuclear leukocytic infiltration. As the number of gram-positive bacteria injected was increased, the PMN infiltration increased progressively, along with increasing vascular and collagen necrosis and microabscess formation. In striking contrast, injection of formalin-killed, washed gram-negative bacteria in doses which elicted similar mild inflammatory reactions evoked responses at 3 hr predominantly mononuclear in nature. Only as the dose of gram-negative organisms was increased did PMN infiltration increase, finally resembling responses produced by the gram-positive bacteria. Of greatest importance, the early inflammatory response to purified bacterial endotoxin was found comparable to that produced by whole gram-negative microbes. Since endotoxin is a constituent of the cell wall of most gram-negative bacteria, the hypothesis is advanced that the difference in the nature of the early cellular inflammatory response produced by gram-positive and -negative bacteria may be based primarily upon the endotoxin content of the latter.

The ability of bacterial endotoxin to elicit an early and predominantly mononuclear inflammatory response in man has, to our knowledge, been reported previously only once, by Braude who found that tuberculin and endotoxin reactions were indistinguishable, being characterized by mononuclear foci in the skin and its blood vessels at 3 hr, reaching maximum levels at 20 hr.[18] The same author, however, in a later study in man reported "At 3 hr endotoxin produced heavy perivascular reactions, composed mainly of polymorphonuclear leukocytes."[19] While no attempt was made to reconcile these apparently conflicting results, the present studies indicate that both responses are entirely compatible with the inflammatory activity of bacterial endotoxin in man and depend upon the concentration of the toxin employed. Stetson, in a study of the inflammatory response to endotoxin in rabbits, was actually the first to suggest this relationship to endotoxin dosage: "Histologically, the components of the cellular infiltrate in the primary reactions to endotoxin appeared to depend, to some extent, on the severity of the reaction. With marked reactions due to large doses of endotoxin, the infiltrate at 18 hours consisted primarily of polymorphonuclear leucocytes, and those in the central portions of the lesions showed more or less severe degenerative changes. With smaller doses of endotoxin, however, it was apparent that mononuclear cells were also present in large numbers, particularly about the periphery of the lesion.... Although direct quantitation of the cellular elements was not accomplished, the presence of considerable numbers of these mononuclear cells seemed to be a consistent finding, perhaps of significance in the light of LaPorte's report indicating mononuclear cells in the characteristic cellular response of the tuberculin reaction."[20] Studies at earlier time intervals in the rabbit would have been of great importance.

The mechanisms underlying the predominantly early mononuclear response to bacterial endotoxin in man is unknown. The response resembles that produced by PPD in sensitive volunteers, but differs by earlier onset. The gross lesions evoked by endotoxin generally become visibly greater than saline controls between 1 and 2 hr and the histologic response is well advanced at 3 hr. With comparable inflammation-producing doses of PPD, similar responses are not generally seen for $\geq$ 4-6 hr. However, whereas the human tuberculin responses continue to progress in size and mononuclear content over 24-48 hr, the endotoxin lesions of comparable size wane after 15-24 hr. Nevertheless, since early mononuclear infiltrations appear characteristic of delayed hypersensitivity responses,[3-8] the endotoxin response in man may represent a peculiar type of such hypersensitivity to a common toxophore antigen as initially suggested by Stetson.[20]

The above concept appears of sufficient importance to warrant further consideration of the existing controversy concerning the mononuclear infiltration in the early inflammatory response of delayed hypersensitivity response is a nonspecific inflammatory reaction characterized by initial predominantly polymorphonuclear leukocytic infiltrations.[9-14] Other groups have demonstrated a distinctive mononuclear infiltration as characteristic of this early inflammatory response.[3-8] Certainly, the early response of man during hypersensitivity, as seen in the present volunteers with appropriate dosages of PPD in preservative-free saline, was not a nonspecific inflammatory reaction, but was characterized by a delayed perivascular collection of mononuclear cellular infilatration with but slight PMN leukocytic infiltration. In only one of 8 volunteers was a definite polymorphonuclear leukocytic predominance observed at 3 hr, and in this volunteer serial biopsies of PPD lesions 1 week previously had revealed predominant mononuclear cellular inflammatory responses. The effect of such "boostering" doses of PPD on the 3-hr histologic responses requires further study. In 2 other volunteers, a mild but unequivocal PMN leukocytic response to PPD was seen at 3 hr. This response was not appreciably different, however, from the saline control sites. Had the saline not been employed, an erroneous interpretation of a predominantly polymorphonuclear leukocytic early inflammatory reaction to PPD might have been proposed. The importance of the diluent control cannot be overemphasized. It seems likely that the divergent conclusions on the mononuclear response in the early phase of delayed hypersensitivity reactions in test animals can be attributed to variations in degree of sensitivity, dosage and nature of test tuberculin, diluent and preservatives, lack of control testing with diluent alone, method of sensitization, and probably concentration and type of circulating antibodies. As emphasized by Kaplan and Dienes: "It seems probable that the more characteristic, or possibly primary, cell response in the tuberculin reaction may be masked by secondary inflammatory factors arising as a result of tissue injury in the absence of detectable necrosis. It is essential for such studies that mild reactions be examined, preferably in the early stages of their development, and in guinea pigs showing a moderate rather than a strong degree of hypersensitiveness."[17] More recent studies have shown that lymphocytes from guinea pigs with delayed-type hypersensitivity to tuberculin,

upon contact with PPD *in vitro*, produce a substance which upon ID injection into normal guinea pigs evoked an inflammatory response at 4 hours consisting almost entirely of mononuclear cells. [21] It appears, therefore, that delayed hypersensitivity responses are characterized by earlier mononuclear cellular predominance than nonspecific inflammation unless complicating factors are also present. If the early mononuclear responses to bacterial endotoxin are indeed mediated by related mechanisms, the finding that such responses are elicited by *Franciscella tularensis* in nonvaccinated volunteers and by endotoxins extracted from a rough B and a glucose-deficient mutant of *Salmonella typhimurium* indicate that it is not a sensitivity to the "O" antigenic side chains of the endotoxin molecule that is responsible for the reaction. Sensitization to a toxophore antigen common to all bacterial endotoxins, as suggested initially by Stetson[20] currently remains a most attractive working hypothesis.

Finally, the question can be raised as to the relation, if any, of the present findings to the cellular inflammatory responses evoked by living microbes *in vivo*. The present studies were conducted in only one tissue, skin, with formalin-killed whole bacteria and with endotoxins extracted by vigorous chemical procedures. Certainly, the factors that contribute to cellular infiltration *in vivo*, in the presence of actively multiplying and invasive organisms, with their release of multiple native toxins must be infinitely more complex than those studied presently. Different tissues may also respond differently to the same toxins. The present studies do, however, provide a partial explanation for one mechanism that may be operative in this complex interplay of the inflammatory response. Thus, in the presence of slowly proliferating gram-negative bacteria, sufficiently small amounts of endotoxin may be present locally to induce a predominantly mononuclear inflammatory response. The inflammatory response during those gram-negative bacterial infections characterized by relative low virulence may therefore at all times be predominantly mononuclear in nature. As virulence increases and more rapid proliferation of the microbe occurs, responses would become progressively more polymorphonuclear, in part at least, by virtue of the contribution of the higher local concentrations of bacterial endotoxin. This thesis was initially promulgated in 1907 by Duval and White, with relation to the histologic lesions of the gram-negative bacterial infection, experimental glanders: "The strong toxins of the glanders bacilli cause degeneration or necrosis of cells and exudation, while the dilute and weak toxins produce proliferation."[22] In 1951 Braude, studying experimental brucellosis, concluded "From these findings, it appears that the good defense of guinea pigs against *Br. abortus* was associated with the presence of nonsuppurative granuloma and marked splenomegaly. The less effective defense against *Br. suis* was associated with the development of widespread suppuration. *Br. melitensis*, intermediate in its destructive capacity, was the most debilitating. This information indicates that another factor, possibly an endotoxin, plays a part in the evolution of the experimental disease.[23] Similar reasoning would extend to the lesions of typhoid fever, where suppuration is uncommon, the inflammatory response being predominantly

mononuclear with a paucity of polymorphonuclear leukocytes.[16,18] The ability of the endotoxin of S. typhosa to evoke an early mononuclear response during overt typhoid fever was shown to occur as in healthy volunteers. The ability to reproduce histologically the rose spot of typhoid fever by chronic administration of trace amounts of bacterial endotoxin, as well as of the whole formalin-killed washed organism is also compatible with an important contribution of the endotoxin component to the focal mononuclear lesions of typhoid illness.

The influence of delayed hypersensitivity to tuberculin on the mononuclear infiltration during the inflammatory response in tuberculosis has received a great deal of attention. The present studies suggest a similar role for endotoxin in the pathogenesis of the cellular inflammatory response to gram-negative bacterial infections and indicate that endotoxin may be considered to be the "tuberculin" of gram-negative bacteria.

In summary, healthy male volunteers were inoculated ID (0.1 ml) with serial dilutions (10^8-10^9/ml) of whole formalin-killed, washed S. typhosa, E. coli, Sh. shiga, F. tularensis, Pseudomonas aeruginosa, Proteus morganii, Staphylococcus aureus, and pneumococci. Other volunteers were inoculated with serial dilutions (10^3-10^{-1} μg/ml) purified bacterial endotoxins derived from S. typhosa, E. coli, Pseudomonas, and wild type rough B and glucose-deficient mutant strains of S. typhimurium. PPD, 2 μg/ml was also tested in sensitive volunteers. All bacterial preparations were suspended in sterile, pyrogen-free saline without preservatives, and were bacteriologically sterile before testing. Each preparation was given to 2 to 4 or more volunteers. Inflammatory lesions were biopsied under local anesthesia and multiple sections studied with H&E and Giemsa stains.

Regardless of the bacterial preparation employed, in the dosages used, histologic responses at 24 hr were similar and composed of predominantly perivascular mononuclear cellular infiltrations. Inflammatory responses at 3 hr, however, were distinctive. Bacterial endotoxin and whole gram-negative bacteria, in doses that elicited a mild inflammatory response (unequivocally greater than that elicited by saline alone) evoked predominantly mononuclear cellular responses. The cell population consisted primarily of small lymphocytes and macrophages. Polymorphonuclear leukocytes generally comprised between 10 and 20% of the inflammatory population. As the dose of endotoxin or whole gram-negative organism was increased, the intensity of the 3-hr inflammatory response increased progressively and became progressively more polymorphonuclear leukocytic in nature.

In contrast to gram-negative bacteria, at 3 hr the gram-positive organisms elicted predominantly polymorphonuclear leukocytic inflammatory responses, even with the smallest doses eliciting an inflammatory reaction greater than that produced by saline alone. PPD in sensitive volunteers resembled bacterial endotoxin and elicited a predominantly mononuclear response that became evident between 3-6 hr and increased progressively over 36-48 hr. Polymorphonuclear leukocytes generally comprised less than 10% of the early inflammatory infiltrate.

The hypothesis is presented that the difference in the early inflammatory response evoked by gram-positive and gram-negative bacteria is related to the endotoxin content of the latter. The relationship of this hypothesis to the histologic changes seen in gram-negative bacterial infections is considered.

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