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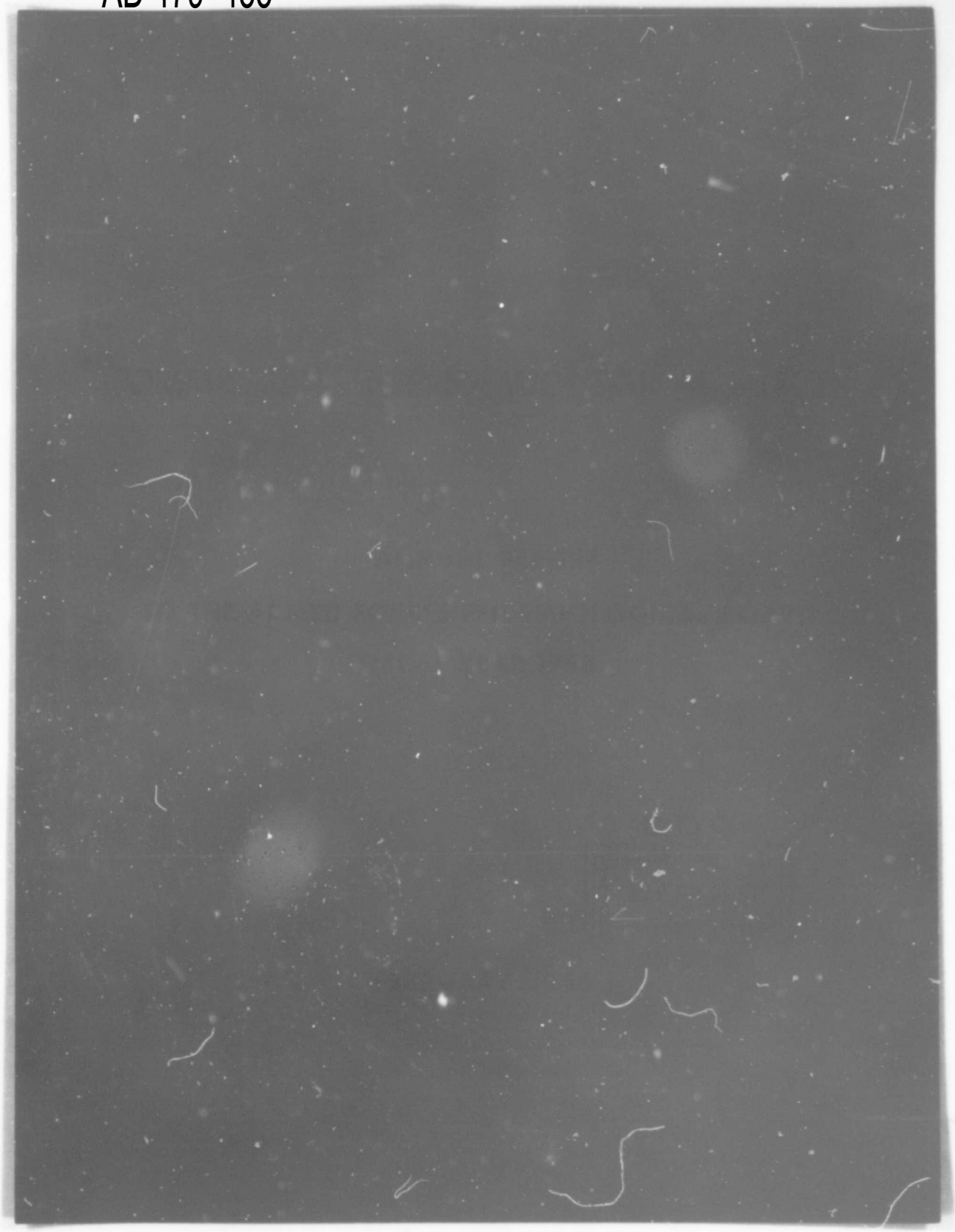

(6) COMMISSION ON EPIDEMIOLOGICAL SURVEY.

(21) ~~ANNUAL~~ REPORT TO THE ARMED FORCES EPIDEMIOLOGICAL BOARD.

~~FISCAL YEAR 1965~~

(9) Annual rept. for Fiscal Year 65,

(11) FEB~~RUARY~~ 1966, (12) 140p.

(10) A. T. Dawkins, Jr., D. S. MacNair, R. F. Jaeger, A. Buzzell and N. R. Blemly.

(16) DA-IC622401A096

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ABSTRACT

Preparing Organizations: Commission on Epidemiological Survey of the Armed Forces Epidemiological Board and U. S. Army Medical Unit, Fort Detrick, Frederick, Maryland

Title of Report: Annual Report to the Armed Forces Epidemiological Board

Director: Dr. Theodore E. Woodward

Principal Investigator: Colonel Dan Crozier, MC

Number of pages, illustrations and date: 140 pages, Fiscal Year 1965

Progress is reported in selected areas of research in medical defense aspects of biological agents by the U. S. Army Medical Unit. A separate report covers research on staphylococcal enterotoxin B.

Key Words: sandfly fever, Q fever, tularemia, Venezuelan equine encephalomyelitis, typhoid fever, anthrax, pneumococcal infections, pasteurella infections, tissue culture, anthrax toxin, vaccines, fluorescent antibody technique, diagnosis identification, electron microscopy, radiation, immunology, endotoxins, biophysics, centrifugation, metabolism, adrenal glands, steroids, thyroid glands, biosynthesis, proteins, liver alkaline phosphatase, hemagglutination, pathology.

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THE DIRECTOR'S SUMMARY REPORT

The annual meeting of the Commission on Epidemiological Survey was held at the Walter Reed Army Institute of Research on 9 and 10 September 1965. Senior representatives of the Departments of Army, Navy, and Air Force, the U. S. Army Medical Unit and the U. S. Army Biological Laboratories attended the meeting. A number of ad hoc meetings were held during the year for discussions of specific problems. The Commission has been strengthened by the participation of Drs. Charles L. Wisseman, Sheldon E. Greisman and Richard B. Hornick as active members. Grateful appreciation was expressed to Captain Sidney A. Britten, USN, Executive Secretary of the Board and Miss Betty Gilbert, Administrative Assistant, for their help in administering the Commission's affairs.

The first day's meeting was devoted to hearing the reports which follow of the work completed or in progress by investigators of the U. S. Army Medical Unit and one of its contractors.

An ad hoc group consisting of members of the Commissions of Rickettsial Diseases and Epidemiological Survey and staff members of the U. S. Army Medical Unit and Walter Reed Army Institute of Research has developed a program extending the studies of Q fever vaccine. Dr. Wisseman directs this Q Fever Ad Hoc Committee which is studying the antigenic and immunologic relationships of Phase I and II components of Coxiella burnetii. Phase I and II antigens, including viable strains, are being appraised as vaccines in volunteers under the University of Maryland contract.

The scope of the program of the Medical Unit and its contractors has been broadened. The fate of viruses within cells is being appraised by immunochemical techniques and the effect of ionizing radiation on susceptibility to Venezuelan equine encephalomyelitis (VEE) infection is under study. Metabolic studies have included appraisal of adrenal gland functions in human tularemia, sandfly fever and Q fever and thyroid function during pneumococcus infection of rats. Alterations of tissue and leukocyte alkaline phosphatase have been appraised in tularemia, sandfly fever and pneumococcus infections. Synthesis of protein and nucleic acid metabolism have been assessed in VEE infections in laboratory animals. There has been good progress in developing a VEE vaccine and an inactivated multivalent Arbovirus vaccine.

Studies of typhoid and tularemia vaccines in volunteers are nearing completion; and the physiologic changes of endotoxin have been extended.

The executive session on the second day was devoted to a general discussion of staphylococcal enterotoxin B and to administrative affairs of the Commission. The former is the subject of a special report to the Commission published separately by the U. S. Army Medical Unit.

In conducting the research described in this report the investigators adhered to the Principles of Laboratory Animal Care as established by the National Society of Medical Research.

THEODORE E. WOODWARD
Director
Commission on Epidemiological Survey

REPORT ON Q FEVER VACCINE STUDIES

Albert T. Dawkins, Jr., M.D.*

Interest in Q fever has been stimulated by the discovery of and the desire to understand the phase variation of Coxiella burnetii. Although the currently available commercial vaccine yields good protection, it seemed advantageous to investigate new immunologic procedures in order to learn what role phase variation plays in immunogenicity. Genig[1] reported results obtained in volunteers immunized with a living vaccine which had had 44 egg passages and, thus, was probably made up of all Phase II organisms. When this vaccine was administered to Russian volunteers, very little reactivity occurred, and excellent serological responses were observed. Phase II organisms have not been used in volunteers in this country prior to the initiation of these studies.

Knowledge concerning the Phase II organism may yield a less reactogenic and equally immunogenic vaccine as that derived from Phase I organisms. Employment of the current Q fever vaccine has been associated with occasional sterile abscess formation, especially in individuals who have had prior experience with Q fever. However, the incidence of these abscesses is not really known.

The Q fever studies at the School of Medicine have been designed with the following purposes in mind:

1. To evaluate the reaction rate and immunogenicity of a single dose of Phase I killed vaccine.

2. To determine whether Phase II C. burnetii can induce disease in man.

3. To evaluate the efficacy of a single dose of Phase I killed vaccine in preventing aerogenically induced disease.

4. To evaluate the efficacy of living Phase II vaccines in preventing aerogenically induced disease.

Preliminary results were reported to the Rickettsial Commission in March, 1965. Review, follow-up, and progress will be presented in this report. In November 1964, sixty-four volunteers were vaccinated subcutaneously with a single dose containing a rickettsial mass of 30 μg of Phase I killed Henzerling vaccine. There were no serious or severe reactions; some volunteers complained of minimal tenderness following the administration of vaccine and experienced mild to moderate erythema and induration at the injection site.

* University of Maryland School of Medicine, Baltimore, Maryland.

No sterile abscesses developed. Employing the microagglutination technique and using Phase I Nine Mile antigen, 78% of this group demonstrated significant antibody rises following the vaccination.

Twenty-four volunteers at the Maryland House of Correction were inoculated using varying dilutions of a Phase II living Nine Mile strain (88th egg passage, EP-88) as prepared by Dr. Lowenthal (Walter Reed Army Institute of Research). The dilutions employed were 10^{-8}, 10^{-6}, 10^{-4}, and 10^{-2}. Symptoms and serologic conversion with this living vaccine varied directly with the strength of the inoculum. For example, in the 6 men of the 10 group, all had symptoms consisting of fever > 100 F, headache, myalgia, malaise, and tenderness and erythema at the injection site. Five of 6 had antibody rises as tested by complement fixation (CF) using Phase II Nine Mile antigen. Testing of these men using Phase I Nine Mile antigen has yielded no antibody titer changes through the 8th and 12th weeks postvaccination. The reaction rates and Phase II antibody responses are presented in Table I. None had any significant hematological aberrations other than elevated C-reactive protein values, and none demonstrated any changes in serial chest x-rays.

TABLE I. RESPONSE OF VOLUNTEERS TO VARYING DILUTIONS OF PHASE II LIVING NINE MILE VACCINE STRAIN (EP-88)

VACCINE DILUTION	NO. OF SUBJECTS	NO. OF REACTIONS	PHASE II ANTIBODY RESPONSE
10^{-8}	6	0	0
10^{-6}	6	1	1
10^{-4}	6	6	3
10^{-2}	6	6	5
TOTALS	24	13/24	9/24

In June, 1965, seven Phase I Henzerling vaccinees (1 dose of 30 μg) and 3 controls were taken from the Maryland House of Correction to Fort Detrick for challenge in the static aerosol exposure chamber. These 7 men had been vaccinated 7 months earlier. Six additional Phase I vaccinees and 3 controls were challenged in an identical manner in late August, 1965. However, the postvaccination period in this group had extended to 10 months. The volunteers were exposed to an aerosol dose of $10^{3.5}$ $GPIPID_{50}$ of AD strain C. burnetii (predominantly Phase II with a significant concentration of Phase I). The challenge material came from the same lot as was previously used in Q fever volunteer studies accomplished some years ago. Dr. Joseph Jemski (U. S. Army Biological Laboratories) was responsible for making the exposure chamber challenges successful.

All men were hospitalized in the University of Maryland Research Ward at the Maryland House of Correction. All volunteers had been examined prior to challenge and declared acceptable for study. After challenge oral temperatures and pulse rates were recorded every 4 hr; the men were examined regularly for 28 days, longer in those men who became ill. Each volunteer had weekly urinalysis and hematological studies including: white blood count, hematocrit, and C-reactive protein determination. Chest x-rays were obtained every 3 days from day 7 through day 19. Daily urines were collected from the controls starting on day 9, continuing for 2 weeks, and frozen at -70 F; they will be used for rickettsial isolation attempts.

The therapeutic regimen established for and carried out in the diseased individuals consisted of tetracycline; 3 gm by mouth as a loading dose and 1 gm every 8 hr until a total of 20 gm had been given. This treatment was instituted 24-30 hr after the volunteer had experienced a temperature > 100 F.

None of the 13 aerogenically challenged Phase I vaccinees became ill. Five of the 6 controls had disease (i.e., temperature > 100 F for 24-30 hr, clinically requiring antibiotic therapy); the remaining control was suspected of being infected (i.e., illness, but not great enough to warrant antibiotic therapy).

Figure 1 is representative of all men who developed Q fever. The control having the most severe illness became sick on day 11. His greatest febrile reaction occurred on day 13 with an oral temperature of 103.2 F. Tetracycline was begun that day; the patient was afebrile by day 16. Along with fever his illness was characterized by the following: severe headache, chills, sweats, malaise, myalgia, conjunctivitis, photophobia, sore throat, pharyngitis, nasal stuffiness, increase in cervical lymphadenopathy, pleuritic-type chest pain, and anorexia. A pulmonic infiltrate was detected on day 14 and was completely resolved several weeks later.

One control was classified as clinically ill; his temperature rose to 100 F on days 8, 11, 23, and 24. This patient was mildly ill, with malaise, moderate headache, productive cough, conjunctivitis, and an increase in cervical lymphadenopathy. This man was given no antibiotic therapy and was subsequently discharged in a healthy state. Examination of serial chest films revealed no changes. Since serologic changes did not occur through the 8th week postchallenge, this man probably will be classified as not having experienced Q fever.

The results of our laboratory investigations among the challenged volunteers show little or no change in hematological studies and in urinalyses. Three of the 5 controls experiencing illness had elevations in C-reactive protein determinations at some point during the disease process.

Thirty-eight of 49 Phase I Henzerling vaccinees demonstrated a significant rise in antibody titer following vaccination as tested by microagglutination using Phase I Nine Mile antigen. To date, prechallenge sera from only 6 of

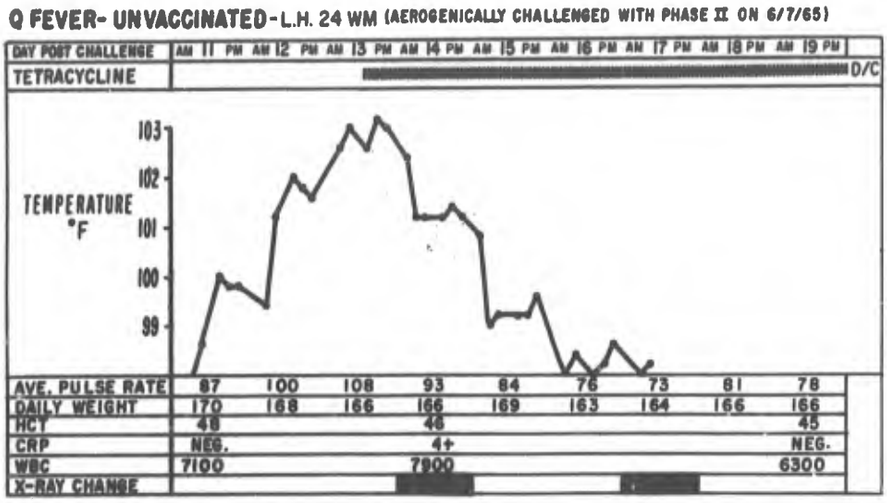

FIGURE 1. CLINICAL SUMMARY OF VOLUNTEER INFECTED WITH Q FEVER.

the Phase I Henzerling vaccinees have been tested by CF using Phase II antigen; 5 of 6 had rises in Phase II titers. This confirms the belief that Phase I Henzerling vaccine contains some Phase II components.

One of 13 Phase I Henzerling vaccinees developed a Phase II CF antibody rise following AD strain challenge, as shown by testing with Phase II Nine Mile antigen. When testing this same group for Phase I antibodies using the microagglutination technique and employing Phase I Nine Mile antigen, we found that 1 of the 13 showed a postchallenge rise in Phase I antibody. To date, only 3 of 6 controls have been serologically studied, and 2 of these have had significant Phase I and Phase II antibody rises. Both were among the 5 controls who became ill with Q fever.

It is interesting to note that one of our challenged Phase I vaccinees did not develop Phase I antibodies following vaccination and aerosol challenge, and also did not become ill. These serological results have not been repeated for validity and are not yet complete with respect to the last specimens obtained.

On August 30, 1965, eight Phase II living Nine Mile vaccinees were exposed by aerosol to $10^{3.5}$ $GPIPID_{50}$ of C. burnetii, AD strain. This is the first challenge trial for the living Phase II vaccine. All 4 dilutions used to vaccinate these men were represented. The preliminary results of this study are presented in Table II.

TABLE II. RESPONSE OF VOLUNTEERS IMMUNIZED WITH PHASE II LIVING NINE MILE VACCINE (EP-88) TO CHALLENGE WITH C. BURNETII, $10^{3.5}$ $GPIPID_{50}$, AD STRAIN

VACCINE DILUTION	SEROPOSITIVE PRECHALLENGE NO. CHALLENGED	DISEASE SERONEGATIVE	DISEASE SEROPOSITIVE
10^{-8}	0/1	1/1	0
10^{-6}	1/3	2/2	0/1
10^{-4}	2/3	0/1	0/2
10^{-2}	0/1	0/1	0
TOTALS	3/8	3/5	0/3

Three men became ill with an average incubation period of 10 days. The clinical picture was consistent with that already described. Prior to challenge, 5 men had developed no Phase II antibodies. Three of the 5 developed Q fever. The remaining 3 vaccinees had Phase II CF antibodies and did not become ill. Postchallenge serology is not yet complete.

One additional aspect of these studies involves the possibility of the oral route as a mode of contracting Q fever. The oral ingestion of organisms has been suggested as a cause of natural outbreaks of Q fever, but no reliable data are available. It has been shown that people living in endemic areas have detectable antibody titers. At times milk is found to contain antibodies to Q fever and, therefore, might contain viable rickettsiae. On the same day as our original aerosol challenge, 7 additional volunteers from the Maryland House of Correction were challenged orally with the same aerosol-challenge slurry. Three of these men received undiluted material (i.e., the same as the aerosol challenge dose), and 4 men received a preparation which had been concentrated 100X. The slurry was placed by syringe into several milliliters of milk to prevent any aerosolization and then swallowed by each volunteer. Clinical and laboratory evaluation were made on each of these subjects similar to those described for the aerogenically challenged group. In addition stools were collected on each man within 24 hr of challenge and daily thereafter for 2 weeks. These specimens were frozen at -70 F; they will be used for isolation attempts for rickettsiae.

None of these men developed infection or disease consistent with Q fever. One volunteer did, however, demonstrate a fever of 103.2 F on day 17 and remained ill for 9 days despite routine tetracycline therapy. Although attempts to characterize his illness yielded no definite diagnosis, it is the opinion of the several clinical investigators who saw this man regularly that he did not have Q fever.

There were no significant hematologic changes or variations in routine urinalyses. One volunteer who received the concentrated dose of organisms developed Phase II CF antibodies which were demonstrable in tests using Phase II Nine Mile antigen. These titers are not from the sera of the volunteer with the unidentified illness. Microagglutination studies for Phase I antibodies are not yet complete.

SUMMARY

1. A group of volunteers was vaccinated with Phase I killed Henzerling vaccine by giving 1 injection containing a rickettsial mass of 30 μg. It appeared that protection is obtained when vaccinating in this manner.

2. Depending upon the dilution of the material used for vaccination, some Phase II living vaccinees were protected when aerogenically challenged.

3. Although no illness was produced by the oral ingestion of C. burnetii, 1 volunteer developed Phase II antibodies.

ACKNOWLEDGEMENTS

Special recognition is due our volunteers at the Maryland House of Correction without whose aid these studies would not have been possible.

The unlimited cooperation of the Maryland correctional authorities and the entire staff at the Maryland House of Correction, especially the warden, Mr. John P. Garrity, is greatly appreciated.

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DEMONSTRATION OF VIRUSES IN LEUKOCYTES BY THE FLUORESCENT ANTIBODY TECHNIQUE

Donald S. MacNair, Major, MC,* and Robert F. Jaeger, B.A.**

The virus of Venezuelan equine encephalomyelitis (VEE) has been used as a model for these studies. The standard approach for determining the presence of viremia has been to inject some of the material in question intraperitoneally into mice. If the mice died between 4 and 10 days after the injection, this was suggestive evidence that the virus was present. This was then confirmed by a neutralization test either of the clinical specimen or the brain of the dead mouse. Thus the specific diagnosis of viremia due to VEE could not be accomplished in less than 10 days. The present study was undertaken in an effort to reduce this time.

The problem was to see if virus particles could be identified in any of the cellular constituents of the blood using a simple fluorescent antibody technique.

Nine beagle dogs were prebled to establish that they neither had viremia by conventional techniques nor hemagglutionation-inhibiting (HI) antibodies to VEE. Blood smears contained no viral particles. The dogs were challenged intramuscularly in groups of 3 with 33, 10,000 or 33,000 mouse median intraperitoneal lethal doses of infectious particles. They were bled daily through day 14 and again on days 17, 21 and 28. To separate the cellular components, 5 ml of blood were mixed in a vaccine bottle with 250 units of heparin; the mixture was incubated at 37 C for 1 hr. By this time, the red cells had fairly well settled out, and the grayish-pink supernatant plasma contained the suspended leukocytes and platelets. The supernatant fluid was placed in a small plastic tube, centrifuged at 800 rpm for 10 min, and decanted. The cell button was resuspended in the small amount of residual plasma; one drop was placed on a microscopic slide. Ten smears could be made from each suspension. Slides were air-dried.

The slides were fixed in formalin buffered to pH 7.3 for 10 min at room temperature and washed in phosphate buffered saline (pH 7.3) for 5 min. The slides were flooded with burro anti-VEE gamma globulin which had been conjugated with fluorescein isothiocyanate. They were incubated in a moist chamber at 37 C for 30 min, then washed 3 times in phosphate buffered saline using 5 min per change. Cover slips were mounted with glycerinated saline buffered with a carbonate-bicarbonate mixture to pH 8.9. The procedure to this point was accomplished conveniently in less than 3 hr using only about 30 min of technician time. Slides were examined through a Zeiss model GFL microscope using a HBO-200 light source, Schott exciter filter BG-12 and barrier filter GG4.

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Normal smears contained relatively few red blood cells. The two elements appearing in greatest concentrations were polymorphonuclear leukocytes (PMN) and platelets. When viewed with fluorescence microscopy, the only cells in the normal smear which showed any fluorescence were leukocytes. Eosinophils, although relatively infrequent, had rather prominent large dull yellow-fluorescing granules. The nuclei of PMN's fluoresced dull gray. The granules in these cells sometimes fluoresced a rather dull yellow-green. Autofluorescence was always at low intensity.

In positive smears, fine granular particles were seen which fluoresced a brilliant apple-green. These particles occurred singly or were numerous, frequently coalescing to form masses of fluorescing material in the cell. They have always been found in the cytoplasm, and, with a single exception, have been seen in PMN's (Figure 1). The single exception was a lymphocyte. In this regard it should be pointed out that differential counts of the white cells indicated that approximately 90% of the cells were PMN's. For some unknown reason, the positive cells seemed to cluster together in a rather limited area of the slide so that the whole slide had to be searched with care.

In an attempt to determine the specificity of this reaction, the slide was studied with several filter combinations in each of which it should have remained positive.

Three controls were usually run. First was a smear of normal cells. For this we used the prechallenge smears; these had to be negative. This ruled out autofluorescence, still a serious problem with human blood. In this particular case it demonstrated a clear-cut difference between viremic and nonviremic blood.

A second control consisted of treating the slides first with ordinary burro anti-VEE serum in an attempt to saturate all the antigen sites with specific antibody. This was then removed and the slide was treated with fluorescein-conjugated burro anti-VEE gamma globulin. In this case no fluorescence was seen or it was markedly reduced indicating that a specific antigen-antibody reaction had been blocked.

The third control consisted of treating the slides with fluorescein-conjugated burro antitularemia gamma globulin. Absence of fluorescence indicated that no nonspecific antigen-antibody reaction was occurring.

All 9 dogs had positive slides every day through day 6. Curiously enough, the first 2 dogs to become negative were in the high dose group; one each became negative on days 7 and 8. The remainder were negative on day 9. By other methods, viremia was detectable until day 2 or 3 but uniformly absent on day 4 and later. Antibodies were first detectable on day 4 in 2 dogs from the high dose group by the HI test. In the other 7 dogs, HI titers did not appear until day 5; neutralization tests became positive on day 9.

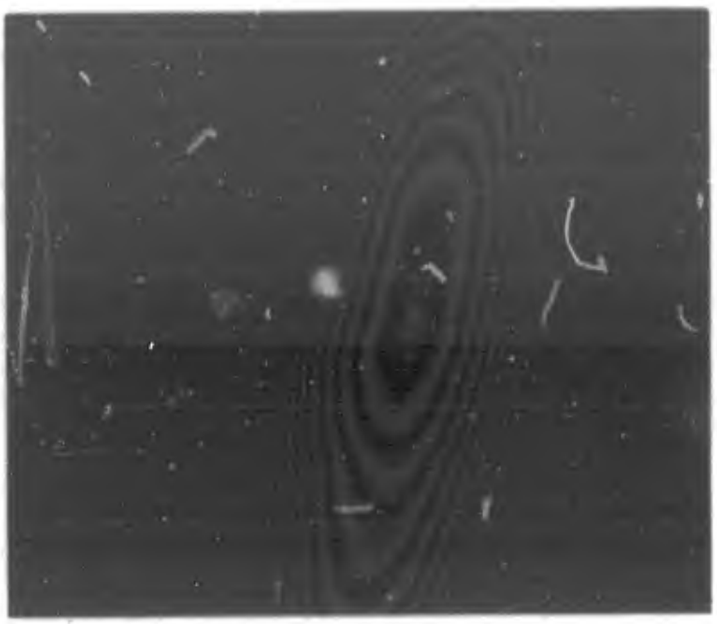

FIGURE 1. FLUORESCING AND NEGATIVE POLYMORPHONUCLEAR LEUKOCYTES.

SUMMARY

By a rather simple and rapid method, it has been possible to demonstrate viremia due to VEE in dogs. The test became positive on the first day after challenge, it was positive longer than viremia was detectable by other methods, and 3 days after antibodies were already detectable. It was positive on a day when neither viremia nor antibodies were detectable by other methods.

In an attempt to determine whether live virus was involved the white cell suspension was diluted as much as 3 logs with rabbit serum buffer and was still lethal for mice. A similar study was performed in burros with comparable results.

Studies will be extended to specimens from humans receiving the attenuated live virus vaccine. Autofluorescence in leukocytes will still be a problem.

RECOVERY OF VIRAL PARTICLES FROM SERUM FOR ELECTRON MICROSCOPY

Anne Buzzell, Ph.D.*

A procedure for counting and identifying in the electron microscope virus particles from unpurified suspensions at low concentration has been under investigation. Ultimately it is hoped that the procedure can be made simple, reliable, and sensitive enough for practical use in early diagnosis.

The first major problem in using the electron microscope to detect virus infection is how to concentrate the virus particles and free them from high concentrations of extraneous matter such as salt and serum proteins, which would mask their presence. The second problem is how to identify the images seen in the electron microscope. A procedure developed by Howard Hegstad and the writer for virus concentration and purification was presented in 1963 to the Commission. A much simpler, more versatile version of that procedure and progress made on the problem of virus identification are presented.

The concentration and purification procedure is based on that of Sharp[1] published in 1949 in which the virus is separated from contaminants by centrifuging onto a block of agar. Small molecules such as salt and protein filter into the agar leaving the virus on the surface. In Sharp's procedure the virus was stripped from the agar with a collodion film which was placed on an electron microscope grid. The film was formed by letting a drop of collodion run over the agar; the film was then floated from the agar, virus-side down, onto distilled water. This procedure had some disadvantages: the virus particles might be washed off the agar or the film and the procedure is tedious since the small pieces of film are hard to see on the water.

The present method avoids the difficulties just mentioned. A formvar film is made by dipping a glass microscope slide into a solution of formvar in ethylene dichloride. When dry the slide is dipped into water to float off the film. The film is then retrieved from the water by touching it with a piece of "saran wrap," tautly stretched over a glass frame made of 3 microscope slides taped together, the middle part of the central slide being cut out. The saran has a centrally placed, circular hole slightly larger than an electron microscope grid (Figure 1).

After virus has been transferred to the formvar the film is transferred to an electron microscope grid as follows: on a large piece of glass a small square cut from a microscope slide is mounted with masking

* U. S. Army Medical Unit, Fort Detrick, Maryland.

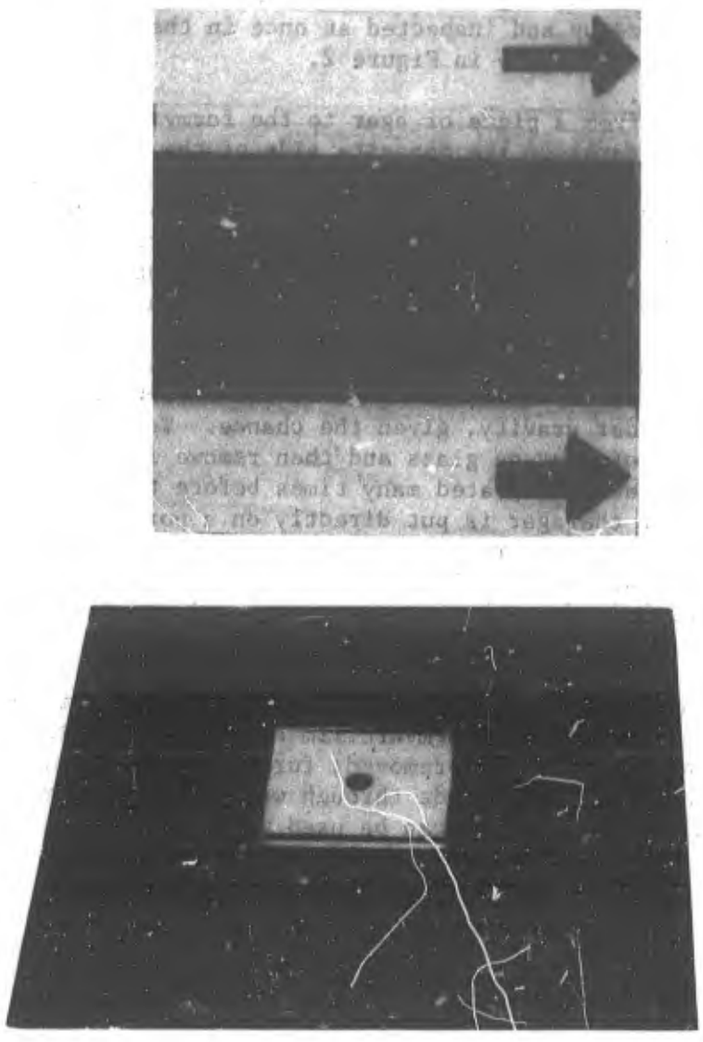

FIGURE 1. DEVICE FOR STRIPPING VIRUS PARTICLES FROM AGAR.

tape. A piece of saran is smoothed down on the glass and the electron microscope grid to be coated is placed on it. The frame is then fitted over the small glass square so the formvar film is stretched over the grid adhering to the surrounding saran. The film severs around the grid which can then be picked up and inspected at once in the microscope. The procedure is shown schematically in Figure 2.

To transfer virus from a piece of agar to the formvar film the agar is placed against the saran on the opposite side of the hole from the formvar. After a few minutes the agar is flipped off and the formvar film can be lowered over the electron microscope grid as described. The similar procedure for transferring virus from a Millipore filter is also shown in the figure. Agar is used in the second procedure merely to wet the Millipore filter which cannot be done directly by water.

Contact between formvar and agar should be avoided since it apparently is not adsorption to formvar but washing frees virus from the agar. Water in agar will settle under gravity, given the chance. To demonstrate this, one can place a block of agar on glass and then remove it; a thin film of water remains. This can be repeated many times before the water film decreases visibly. If the agar is put directly on a porous film, water escapes by evaporation and no free water layer forms. However, if the agar is supported slightly above the film, by placing it on the saran at the opposite side of the hole from the formvar, a water layer forms which lasts for a few minutes.

Fortunately, the same washing procedure works for transferring virus from a Millipore filter. If the formvar film touches the filter it adheres and, unless very thick, tears when removed; furthermore, very little virus is transferred. When contact is made through water, virus is readily transferred and thin formvar films can be used without breakage.

The condition for optimum virus transfer is, fortuitiously, also that for optimum "negative staining." A negatively stained virus is embedded in a matrix of salt opaque to electrons thus enhancing contrast and revealing structural detail which can be used to classify the virus. This simplifies the task of identification.

For best negative staining it is necessary that the virus reach the formvar before the stain and that a thin layer of stain solution dry from a free surface. If the solution dries through the formvar one observes "positively stained" virus images, more opaque to electrons than normal virus but fuzzy with no fine detail visible. The requirements for negative staining, therefore, are met in incorporating the stain solution directly in the agar and carrying out the transfer procedure as described.

The thin layer of solution visible on the formvar as a pale gold film when the agar or Millipore filter is removed, disappears almost instantly. As a result, the distribution of virus is uniform without the distortion

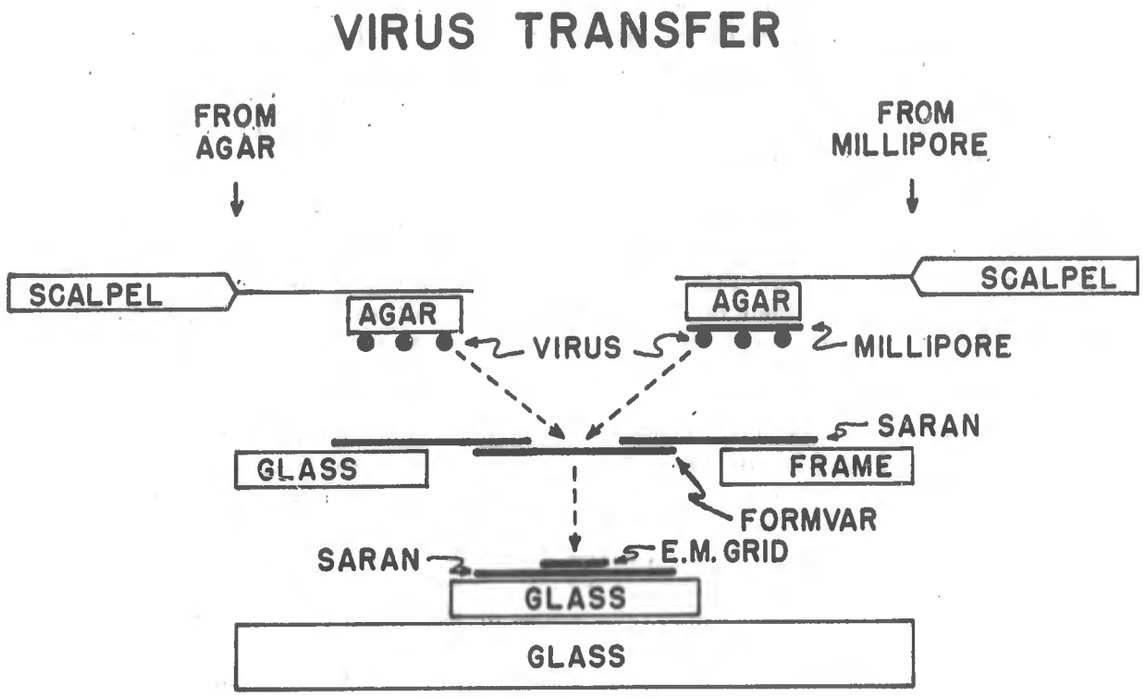

FIGURE 2. METHOD OF VIRUS TRANSFER.

of particle count one normally finds at the edge of a large drop. The negative stain is also uniform and so can be made very thin. Consequently, a low electron beam intensity can be used for viewing and no extra support film of carbon is needed to conduct away heat.

With little stain directly under or over the virus particle, the virus image is very sharp and one can focus on as fine a feature as a bacteriophage tail even at a magnification of 5600, which is advantageous for particle counting.

Figure 3 shows Escherichia coli bacteriophage T4 transferred from a Millipore filter by a sodium phosphotungstate solution which percolated from the agar through the filter for 2 min. Even large bodies such as bacteria can be transferred in this fashion. The phage images are sharp with tails clearly visible with facets on many heads. This electron micrograph, taken of the first partially successful transfer from a Millipore filter, illustrates also the "positive stained" fuzzy images seen where filter and formvar were in contact.

Figure 4, taken at a magnification of 32,000, of the phage transferred from agar shows the uniform staining achieved with the facets of the head and the constriction and fibers of the tail equally clear for all images. Subunits can be discerned on some tails but on others they are obscured by granularity and circular distortions (later found to arise from minute traces of ethylene dichloride in the formvar).

A freshly made formvar film is hydrophobic and causes a layer of stain solution to contract into separate droplets distorting virus distribution and greatly reducing clarity of the images. Elimination of the last traces of ethylene dichloride requires that the formvar films be dried about 30 min before use. Similarly, after the visible film of virus and stain solution has disappeared, the last traces of water left in the formvar evaporate slowly and will interfere with adhesion of the film to saran during transfer to the electron microscope grid unless the film is dried about 30 min more. Thus, the total time required for assay is about 1 hr.

Since virus can be transferred from Millipore filters as well as from agar, new techniques for concentrating the virus and separating it from serum components are possible. In the previous presentation, it was pointed out that a 100-fold greater concentration could be achieved by filtration than by the centrifugation technique of Sharp. Unfortunately, filtration cannot be used for virus in sera, since serum proteins rapidly clog the fine-pored Millipore filter required for retention of small viruses such as that of poliomyelitis. Therefore, means of improving the centrifugation procedure are being explored. A field-aligning capsule which would allow the virus to funnel rapidly onto a tiny area of a Millipore filter might permit detection of virus at concentrations of 100-1000 particles/ml in as little as 0.1 ml of serum. Such a capsule has been made and can be tested now that virus transfer from a Millipore filter is feasible.

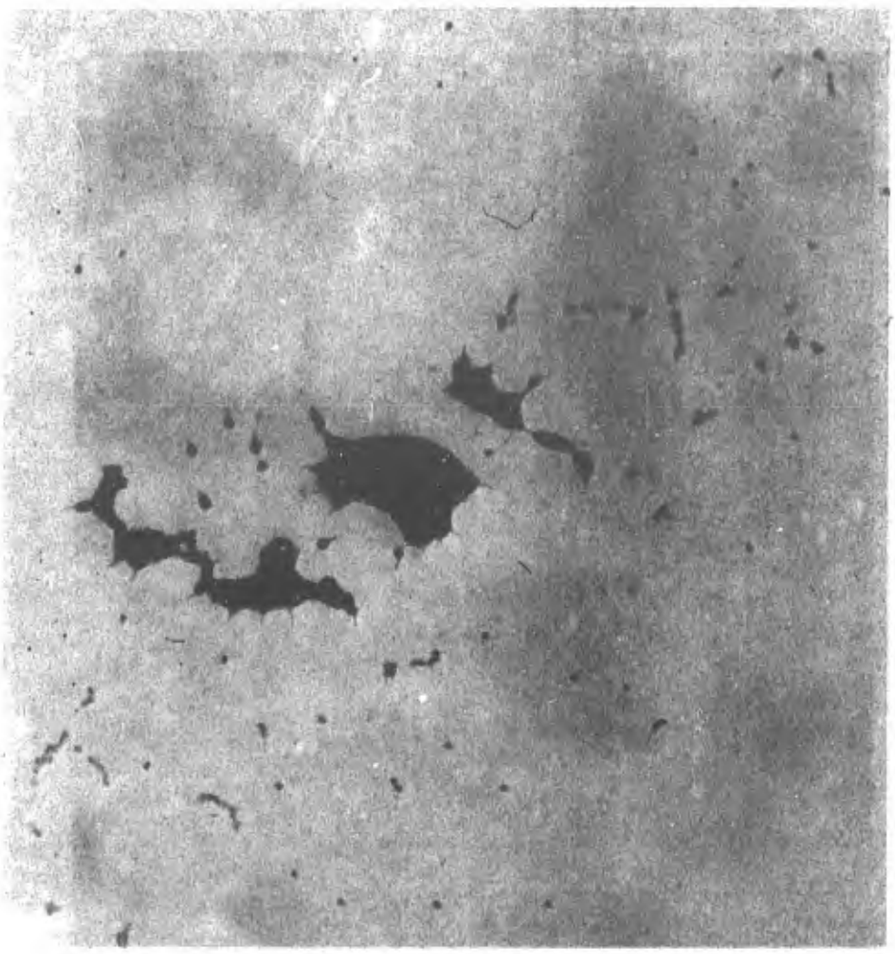

FIGURE 3. BACTERIOPHAGE & BACTERIUM TRANSFERRED FROM MILLIPORE FILTER.

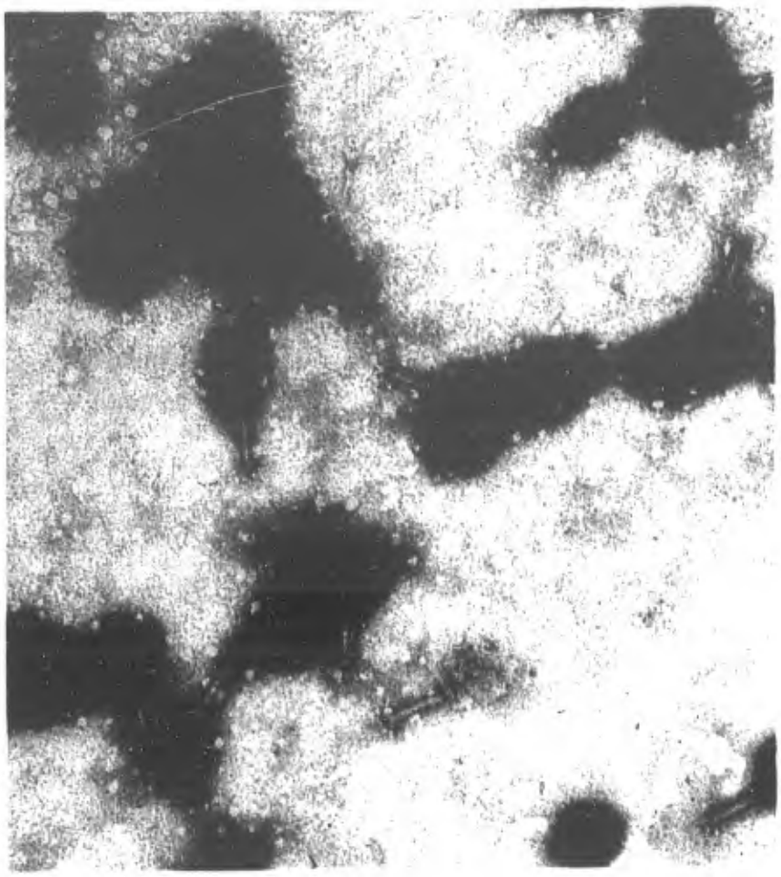

FIGURE 4. BACTERIOPHAGE TRANSFERRED FROM AGAR.

SUMMARY

In its present stage of development the assay for virus particles in serum is capable of detecting virus at concentrations of a 10^6 particles/ml in 1 ml of fluid by centrifuging onto a block of agar in conventional centrifuge cells. The procedure for transferring the virus to an electron microscope grid has been greatly simplified and now incorporates the step of negative staining which is of aid in identifying particle images. Final identification of the virus using specific antibodies with an electron-dense label such as ferritin, may also be simplified since ferritin should be visible in the thin uniform layer of negative stain. An improved centrifuge technique, still to be tested, may make possible detection of virus at concentrations as low as a 10^2-10^3 particles/ml in 0.1 ml of serum with about 1 hr required for the whole assay procedure.

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EFFECT OF IONIZING IRRADIATION ON THE IMMUNE RESPONSE TO VENEZUELAN EQUINE ENCEPHALOMYELITIS

Nelson R. Blemly, Lt Colonel, MC*

In a recent review of the available literature, Stoner, Hess and Bond[1/] compiled abstracts of over 800 articles concerning the effects of ionizing radiation on infection and immune mechanisms. Less than 10% of these articles pertain to the effects of irradiation on virus diseases, an area which has been the concern of the Medical Unit for some time.

Preliminary studies conducted at the Medical Unit were reported to the Commission in 1964. Additional studies have been conducted on the influence of irradiation on immune responses to live viral agents and are presented.

Mice were employed to evaluate the influence of x-irradiation on infection with 2 strains of Venezuelan equine encephalomyelitis (VEE) virus: virulent Trinidad and an attenuated strain of the same virus. This is a unique system in that it affords the opportunity to study the consequences of infection with an attenuated virus as well as to study response to infection by subsequent challenge with a virulent virus of the same strain.

Trinidad virus is lethal for all mice given an infecting dose. The attenuated strain produces an inapparent infection as well as resistance to subsequent challenge with the parent virus. Immunizations and challenge in these experiments were by the intraperitoneal route.

Whole body irradiation was accomplished with a 1 MEV unit with a dose rate of 17 r/min at 200 cm. With one exception, irradiation was delivered as a single acute dose.

The initial experiment was designed to evaluate the effect of irradiation on subsequent infection with attenuated virus. Sixty mice (10/group) were exposed to 500 r whole body irradiation 8, 16, 24, 32, 40 and 48 hr prior to immunization with 10^3 guinea pig median intraperitoneal immunizing doses ($GPIPID_{50}$) of attenuated virus. All animals were challenged 14 days after immunization with 10^3 mouse median lethal doses ($MIPLD_{50}$) of parent virus. It was found that irradiation did not alter infection with attenuated virus; all animals survived challenge. The experiment was repeated with identical results. These findings were in disagreement with those of Berdjis et al, as reported to the Commission last year; however it should be noted that somewhat older mice were used in current experiments.

Since no effect was demonstrable in irradiated animals challenged 14 days after administration of attenuated virus, the next experiment was designed to determine if an effect was detectable if challenge occurred at

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different times. One hundred eight mice were used; 54 animals were irradiated with 500 r; all were inoculated 24 hr later with 10^3 $GPIPID_{50}$ of attenuated virus and challenged with 10^3 $MIPLD_{50}$ of Trinidad strain virus at 1, 3, 5, 7, 9, 11, 14, 21 or 28 days. Results are presented in Table I. There are two

TABLE I. EFFECT OF IRRADIATION UPON ANIMALS CHALLENGED AT INTERVALS AFTER ADMINISTRATION OF ATTENUATED VEE VIRUS

VIRULENT CHALLENGE DAY AFTER ATTENUATED VIRUS	SURVIVORS/10	
	Irradiated	Nonirradiated
1	1	2
3	3	6
5	6	6
7	6	5
9, 11, 14, 21 and 28	6	6

aspects of this experiment that are of interest; first, there are survivors when animals challenged 1 day after immunization, and second, a difference can be seen between irradiated and nonirradiated groups. It appears that irradiation altered the development of resistance as evidenced by the difference in survivors of irradiated and nonirradiated groups 1 and 3 days after administration of attenuated virus. It is also apparent that the effect is transient since similar differences did not occur in groups challenged on or after day 5. The single death that occurred in the nonirradiated group challenged on day 7 was considered to be nonspecific.

In the next experiment mice (10 for each group) were administered 500 r at 7, 10, 11, 12, 13 and 14 days after inoculation with 10^3 $GPIPID_{50}$ of attenuated virus. Challenge with 10^3 $MIPLD_{50}$ Trinidad was conducted on day 14. No effect of resistance was observed, although there was one nonspecific death in the 10-day group.

Results of previous studies from this unit have shown that animals immunized with attenuated virus were refractory to challenge with up to 10^9 $MIPLD_{50}$ of virulent virus. In view of these findings an experiment was performed to determine if irradiation altered resistance to large challenge doses.

Animals were infected with 10^3 $GPIPID_{50}$ of attenuated virus 24 hr after a single dose of 500 r. Groups of animals (10 each) were challenged 14 days later with log increments of virulent virus ranging from 10^3 to 10^6 $MIPLD_{50}$. All animals survived.

Since an effect of irradiation could not be demonstrated by the use of large challenge doses, an alternate experiment of administering small doses of attenuated virus was performed. The conditions of irradiation, time of administration of attenuated virus, and time of challenge were the same as described in the previous experiment. The quantity of attenuated virus was varied, with 3 groups of 6 mice each receiving 10^1, 10^2 or 10^3 $GPIPID_{50}$. Challenge virus was 10^3 $MIPLD_{50}$. The only observed effect was in the irradiated group administered 10^1 $GPIPID_{50}$'s of attenuated virus where 2 died; however, since groups were small, significance of this observation requires confirmation with larger groups of animals.

Since a single acute dose of irradiation in several experiments did not alter development of resistance, it seemed of interest to determine the effect of repeated doses of irradiation. Animals were administered 200 r in a single acute dose. This dosage was repeated every 7th day until dosages of 200, 400, 600, 800 or 1,000 r had accumulated. Irradiation of all animals was completed on the same day; 24 hr later each animal was immunized with 10^3 $GPIPID_{50}$ of attenuated virus. Challenge with 10^3 $MIPLD_{50}$ of Trinidad virus occurred 14 days later. Although irradiation doses of the order used were expected to alter the response to infection no effect was observed.

Since results of previous studies suggested that relatively small quantities of attenuated virus and early challenge following its administration were requisite for demonstrating an effect of irradiation, these two parameters were combined in a single experiment. Ninety mice, administered 500 r whole body irradiation 24 hr earlier, were divided into 3 groups and inoculated with 10^1, 10^2, or 10^3 $GPIPID_{50}$ of attenuated virus respectively. The same quantities of attenuated virus were given to 90 nonirradiated animals. On days 1, 2, 3, 5 or 7 following inoculation of attenuated virus 6 mice from irradiated and nonirradiated groups were challenged with 10^3 $MIPLD_{50}$ of Trinidad virus.

As seen in Table II both the size of the immunization dose and the time between immunization and challenge were factors in the development of protection against challenge with virulent virus. There was a marked decrease in the degree of protection in mice that had received 500 r whole body irradiation before immunization. The effects of irradiation could be overcome, at least partially, by increasing the immunizing dose or by increasing the time between immunization and challenge.

TABLE II. EFFECT OF 500 r WHOLE BODY IRRADIATION ON RESPONSE OF MICE TO ATTENUATED VEE VIRUS

GPIPID$_{50}$ ATTENUATED VIRUS	SURVIVOR/6 (BY DAY OF CHALLENGE)									
	Nonirradiated					Irradiated				
	1	2	3	5	7	1	2	3	5	7
10^1	0	5	6	5	4	0	0	0	0	1
10^2	0	3	4	6	6	0	0	1	3	5
10^3	0	5	6	6	6	0	0	1	5	5

SUMMARY

Attenuated VEE virus produced a solid immunity in mice as demonstrated by resistance to challenge with Trinidad VEE virus 14 days after immunization. Mice infected with attenuated virus showed no ill effects and this state of well-being did not appear to be altered by the added stress of whole body irradiation. The resistance to challenge achieved was not affected by acute whole body irradiation before or after immunization or by repeated doses of irradiation before immunization when standard immunizing doses of attenuated VEE virus were employed. When immunizing doses were reduced and the time between immunization and challenge was shortened an inhibitory effect of irradiation on the resistance to challenge resulted.

The exact cause of the irradiation effect is not fully understood, but it may be hypothesized that there is interference with replication of attenuated virus to a degree that infection and subsequent resistance to challenge are not established and/or that antibody production sites are damaged to a degree that antibodies cannot be produced. Under this hypothesis, if adequate recovery of irradiated tissues has occurred before elimination of virus from the host system, resistance to challenge will become established.

Current and future studies consist in part in a determination of disappearance time of attenuated VEE virus from the host system in irradiated and nonirradiated animals, and if the protection animals have when challenged 1-3 days after immunization is nonspecific, involving interferon or interference phenomena, or whether significant amounts of antibody have been produced so soon after immunization.

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STUDIES ON THE PATHOGENESIS AND CONTROL OF TYPHOID FEVER

Richard B. Hornick, M.D.*

Investigations of the value of typhoid vaccine in preventing induced typhoid fever have continued during the past year. At the last meeting of this Commission it was decided to utilize smaller numbers of organisms for challenge, since ID_{50} or greater inocula caused the same incidence of disease in immunized volunteers as in controls. Selection of the number of typhoid bacilli to be utilized has been based on dose-response data reviewed in Table I. These figures are a summation of the disease rates in control

TABLE I. DOSE RESPONSE FOR TYPHOID FEVER, QUAILES STRAIN, IN MAN (Reed-Muench Method)

CHALLENGE DOSE	NO. REQUIRING TREATMENT/NO. EXPOSED	% INFECTED
10^9	40/42	98
10^8	8/9	94
10^7	15/30	68
10^5	24/84	24
10^3	0/14	0

volunteers ingesting varying doses of Salmonella typhosa, Quailes strain. The ingestion of 100,000 organisms caused disease in 24 of 84 men which corresponded to an approximate ID_{25} for man. Seventy-four volunteers, who received 3 doses of either the acetone-treated (Vaccine K) or phenol-heat killed (Vaccine L) vaccines 3-5 months earlier were challenged with this dose. Table II gives the results of these studies. The disease rates in

TABLE II. COMPARISON OF EFFECTIVENESS OF TYPHOID VACCINE AGAINST CHALLENGE WITH QUAILES STRAIN

CHALLENGE DOSE	VACCINE				CONTROLS	
	K		L			
	No.[a]	%	No.	%	No.	%
10^9	2/3	67	3/4	75	4/4	100
10^7	12/28	43	13/24	54	15/30	50
10^5	2/35	6	3/39	8	20/72	28

a. No. requiring treatment/No. exposed.

* University of Maryland School of Medicine, Baltimore, Maryland.

the vaccinated groups were significantly less than the controls following the 5-log dose of typhoid bacilli: 6 and 8% of the men in the K and L vaccine groups, respectively, as opposed to 28% among controls. This evidence supports the results obtained in the field trials in Yugoslavia[1] and in British Guiana.[2] In those studies significant protection was afforded vaccinated volunteer populations at-risk in endemic areas. Vaccine K proved to be the more efficacious of the 2 vaccines used in those locales. Our results with induced disease imply that naturally-acquired waterborne disease is caused by ingestion of < 100,000 organisms, assuming equivalent strain virulence and host susceptibility and that no ancillary or synergistic agents are swallowed concomitantly with the typhoid bacilli. Furthermore, one can speculate that vaccine-induced immunity will only serve against waterborne disease, that is, a low dose exposure; it was not protective for individuals eating poorly refrigerated contaminated potato salad in which S. typhosa had multiplied to 7 or 8 log per gram of food.

Previous studies have demonstrated that volunteers immunized with Vi antigen had no more immunity to ≥ 1 ID_{50} of typhoid bacilli than controls. As it became apparent that vaccine effectiveness might be demonstrable with smaller challenge doses, interest was renewed in the role of Vi antibody in this protection. A large group of volunteers was immunized recently and will be challenged in the next few months with 1 ID_{25} dose to determine if Vi antigen will induce a level of resistance equivalent to that demonstrated with K and L vaccines. Each of these vaccines gives rise to Vi antibodies, but not as consistently or in as high titer as the purified antigen.

All of the typhoid fever work has been conducted with one strain, the Quailes, isolated from a carrier living in Annapolis who was discovered when her grandchildren were diagnosed as having typhoid fever. Little is known of the virulence of other typhoid strains for man. In addition the importance of the Vi and other antigens of this organism in initiating disease in man is speculative. Part of our mission has been to evaluate other typhoid strains and the significance of antigenic variation in altering virulence potential for man. One study has recently been completed using the standard Ty2 laboratory strain as the inoculum; 16 volunteers were given varying doses. Table III outlines the clinical responses.

TABLE III. DISEASE INCIDENCE IN MAN INFECTED WITH TY2 STRAIN OF S. TYPHOSA

DOSE	NO. CHALLENGED	NO. INFECTED	NO. WITH DISEASE	% WITH DISEASE
10^3	5	1	0	0
10^5	5	1	1	20
10^7	6	2	2	33

demonstrated any significant evidence of disease with this dose. One man developed a mild infection with low grade fever starting on day 10 which lasted 13 days; positive stool cultures were obtained. His O antibody titer rose significantly as it did in 3 other volunteers with no clinical evidence of disease. Blood cultures were negative. Marked changes in numbers of bacteriodes, as well as coliform bacteria, occurred with this antibiotic treatment. However, this aspect of the study was technically difficult to carry out, and no correlations between susceptibility and culture data can be made at the present time. This type of approach appears to offer a new approach in man to gain information as to the role of enteric bacteria as a host-defense mechanism. More sophisticated studies are planned.

In previous reports to this Commission, short statements pertaining to efficacy of various antibiotics, other than chloramphenicol, have been presented. Our model is ideal to evaluate promising drugs, since the response to chloramphenicol is so consistent and predictable that it is possible to determine quickly if an experimental drug is as effective as chloramphenicol. In vitro studies and a few clinical reports suggested the cephalosporin group of antibiotics may be effective in typhoid and other salmonelloses. Therefore, Cephaloridine, the newer and more promising derivative, was given to volunteers in dosages of 4-6 gm daily to treat induced typhoid fever. No clinical response was noted after 48-72 hr indicating less therapeutic effectiveness than chloramphenicol. Colymycin was similarly ineffective despite excellent in vitro bacteriocidal activity against the strain. The tests of these 2 antibiotics again emphasize the lack of correlation between in vitro tube dilution sensitivity results and clinical responses. Chloramphenicol remains the drug of choice in our experimental model.

SUMMARY

Further investigations of the value of typhoid vaccines in preventing induced typhoid fever are reported. Use of challenge doses of 100,000 Quailes strain S. typhosa organisms, i.e., 1 ID_{25}, permitted demonstration of a significant level of vaccine effectiveness. Renewed interest in the role of Vi antigen led to immunization with Vi antibodies. Challenge with 1 ID_{25} will be carried out during the coming year.

The laboratory strain, Ty2, was used as challenge material in 16 volunteers. When 10^7 organisms were used 2 of 6 men became ill, while with 10^3 organisms none of 5 became ill.

Based upon evidence in laboratory animals that endotoxin in proper dosage can enhance nonspecific resistance to infection an acetylated endotoxin was used as a vaccine in volunteers. Following challenge with typhoid or tularemia organisms there was no evidence that immune mechanisms had been enhanced by endotoxin vaccination.

A preliminary investigation into the possible role of colonic bacteroides species in protecting man against typhoid fever was begun.

Colymycin and Cephaloridine were tested in volunteers with induced typhoid fever; neither was as effective as chloramphenicol, the drug of choice, although they were effective *in vitro*.

ACKNOWLEDGEMENTS

We sincerely acknowledge the courage and cooperation of the participating inmates at the Maryland House of Correction without whom these studies would be but mere speculation rather than accumulated results.

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ROLE OF ENDOTOXIN DURING TYPHOID FEVER AND TULAREMIA

Sheldon E. Greisman, M.D., William E. Woodward, M.D.
Richard B. Hornick, M.D., and Theodore E. Woodward, M.D.*

The present investigation described the continuation of efforts to determine the role of endotoxin in the pathogenesis of typhoid fever and tularemia. The studies to be outlined concern the effects of prolonged intravenous (IV) infusions of purified bacterial endotoxins in man; they are based upon the premise that if endotoxemia plays an important role in the pathogenesis of typhoid fever and tularemia, the fever and toxemia characteristically observed during these diseases should be reproduced in normal subjects by such prolonged endotoxin infusions. In addition, continuous IV infusions of endotoxin during overt typhoid fever and tularemia should also result in sustained intensification of the febrile and toxic course.

Figure 1 presents the typical febrile response of healthy volunteers to a continuous IV infusion of Escherichia coli endotoxin. An identical pattern was seen when Salmonella typhosa endotoxin was employed. The lower curve presents the infusion response on day 1; X on the abscissa represents the rate of infusion. Two hours later the infusion rate doubled, and at 7 hr increased 9 times. The temperature increased initially, but then declined and reached baseline in spite of continuing increases in the endotoxin infusion rate. Symptomatic reactions paralleled the febrile response, and each volunteer characteristically expressed disbelief that the infusion was being maintained. This unresponsive state will be designated as the "pyrogenic refractory state" to distinguish it from the classical tolerant state which develops after repeated single daily IV injections of endotoxin. Figure 1 also shows that the pyrogenic refractory state is transient; when the infusion was resumed on day 2, volunteers not only lost the refractory state, but hyperreacted to the endotoxin infusion.

A brief review of present knowledge about the mechanism of this pyrogenic refractory state follows as determined by studies in the rabbit. Figure 2 depicts the effect of varying rates of endotoxin infusion. As the rate increased, rabbits showed increases in mean febrile response which, as in man, returned to baseline despite continuing infusion.

Figure 3 illustrates that blockade of the reticuloendothelial system with Thorotrast, followed 3½ hr later by a continuous IV endotoxin infusion (indicated on the abscissa by X), does not alter the pyrogenic response pattern. It is inferred from these data that the pyrogenic refractory state is not based upon enhanced phagocytic activity of the reticuloendothelial system.

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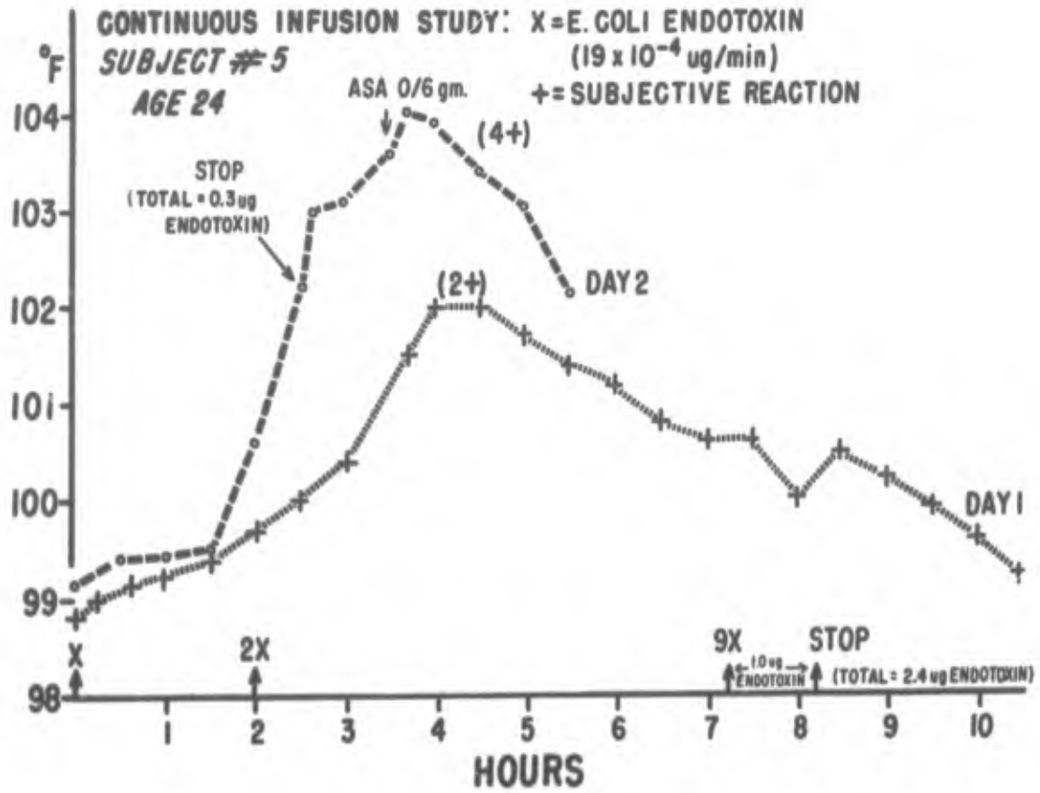


FIGURE 1.

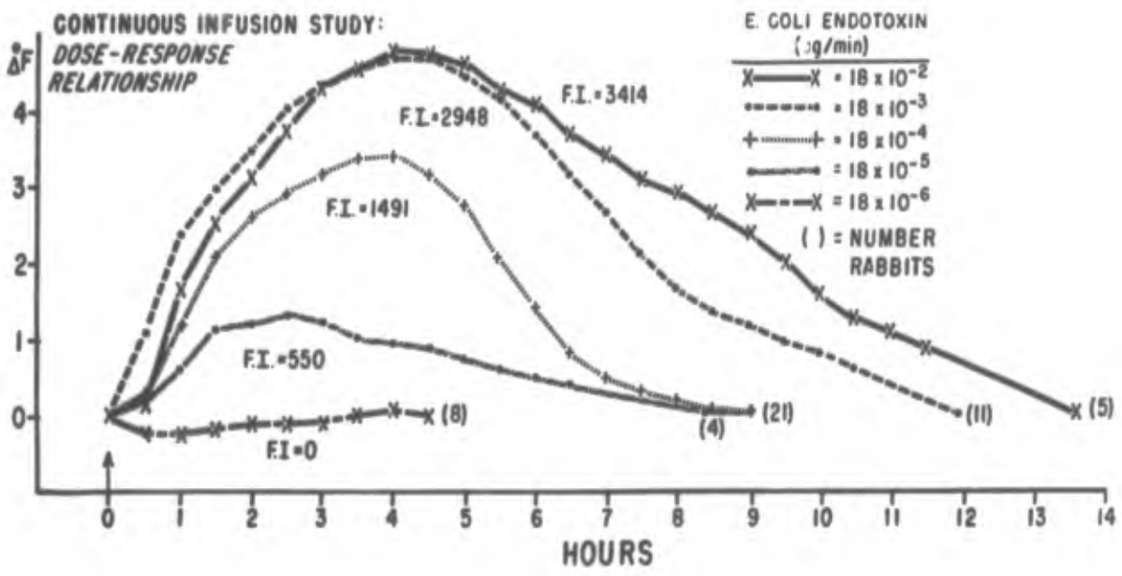


FIGURE 2.

Figure 4 shows that pretreatment of animals with refractory phase plasma 1 hr prior to endotoxin infusion does not blunt the subsequent pyrogenic response. Furthermore, the refractory phase plasma produced no fever in the recipient animals. The pyrogenic refractory state was not mediated by any readily demonstrable protective humoral factors; pyrogenic factors, i.e., endogenous pyrogens (EP), were not readily detectable in the plasma of refractory animals.

Infusion of fresh normal homologous plasma during the refractory state did not reverse this state (Figure 5). This suggested that the refractory phase was not based upon depletion of humoral factors which interacted with infused endotoxin to produce fever.

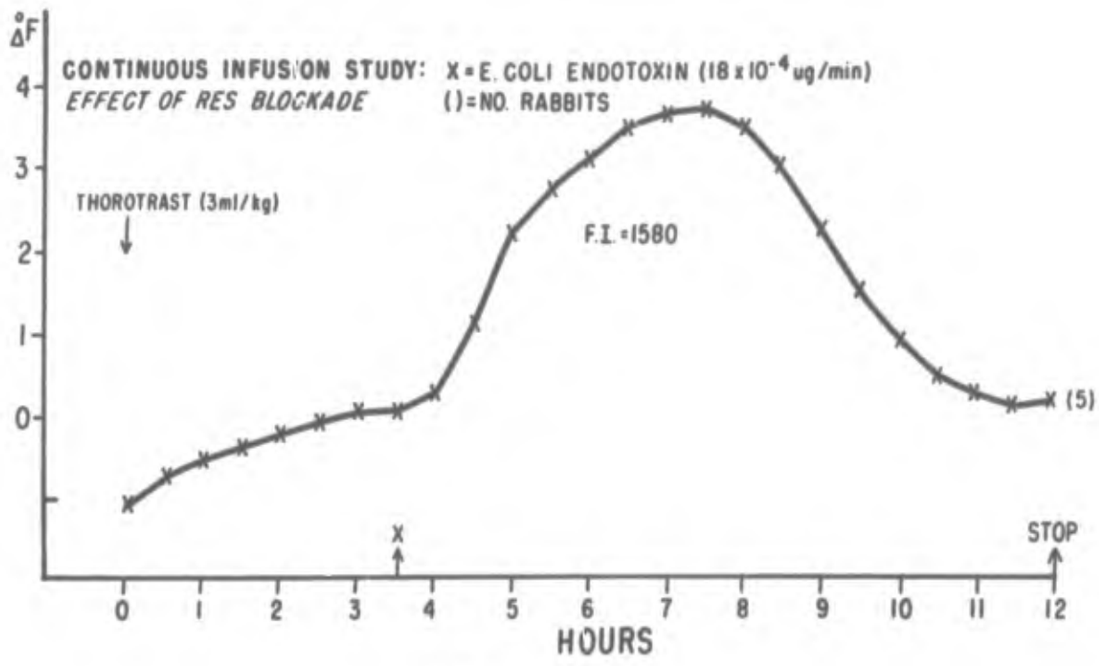
When plasma was obtained during the febrile phase of the endotoxin infusion and infused into recipients refractory to comparable rates of endotoxin infusion, an immediate monophasic febrile response ensued (Figure 6). Such findings indicated that during the refractory state (1) a pyrogenic factor (presumably EP) is no longer present in the febrile phase plasma and (2) the thermoregulatory mechanisms remain responsive.

From these, and additional studies,[1/] it is inferred that the pyrogenic refractory state stems from inability of the host to continue to mobilize EP. Studies currently in progress suggest that this inability to continue to mobilize EP is not based upon nonspecific depletion of EP but rather is mediated by specific desensitization to endotoxin at a cellular level.

Using these data as background, S. typhosa endotoxin was infused continuously into volunteers during overt typhoid fever and tularemia. Figure 7 depicts the febrile reaction pattern during overt typhoid fever. Note that the baseline rectal temperature is > 100 F. The general febrile reaction pattern was identical with that seen in normal subjects. Thus following the initial temperature rise, progressive defervescence to baseline developed despite the continuing and indeed increasing rates of endotoxin infusion. The subjective toxic responses declined in parallel with the febrile responses. The results indicated that desensitization to endotoxin occurred just as readily in patients with typhoid fever as in normal control subjects. The finding that the temperature and toxemia return to, but not below, the febrile baseline level despite desensitization to exogenous endotoxin indicates that sustained endotoxemia does not play the major role in the production of the typhoidal febrile and toxic state.

Figure 8 illustrates the effects of a continuous infusion of S. typhosa endotoxin during tularemia. A reaction pattern identical to that of volunteers with typhoid fever was seen. Note again that following desensitization to exogenous endotoxin, the baseline pyrexia persisted.

Figure 9 shows the reaction pattern of 2 other volunteers on the 3rd day of typhoid illness. These data not only support the preceding conclusions but also indicate that nonspecific depletion of pyrogenic substances is an



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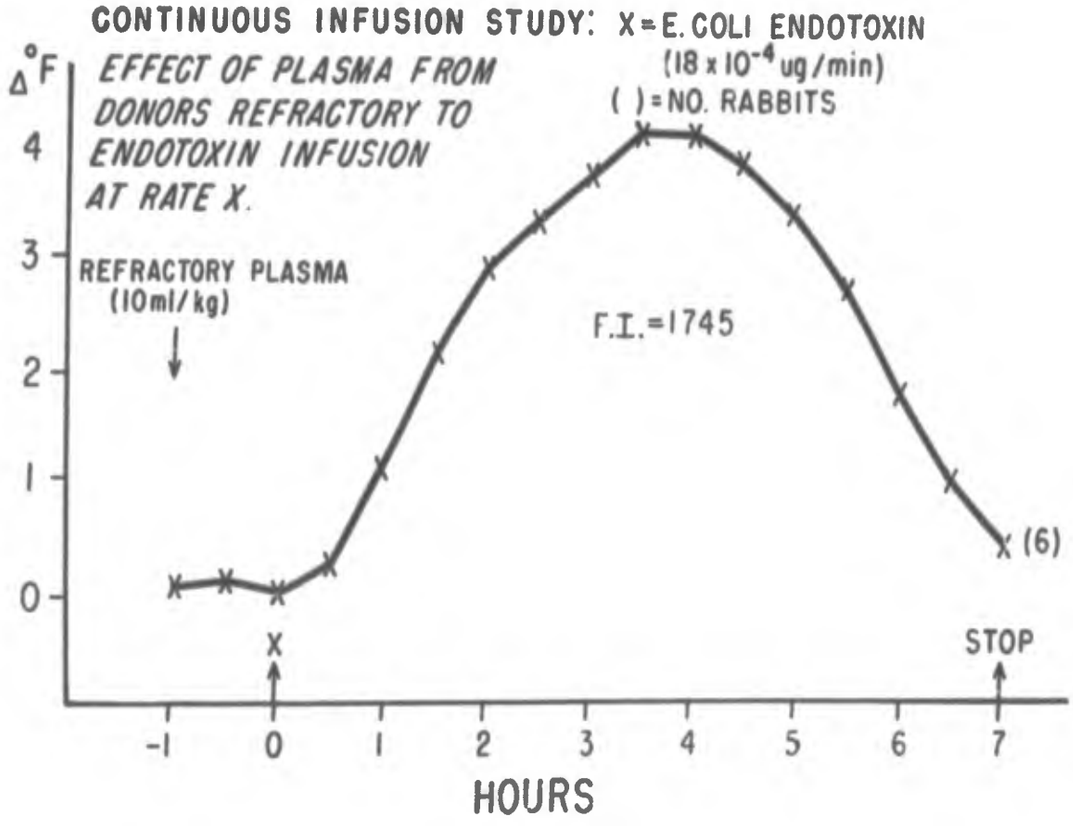

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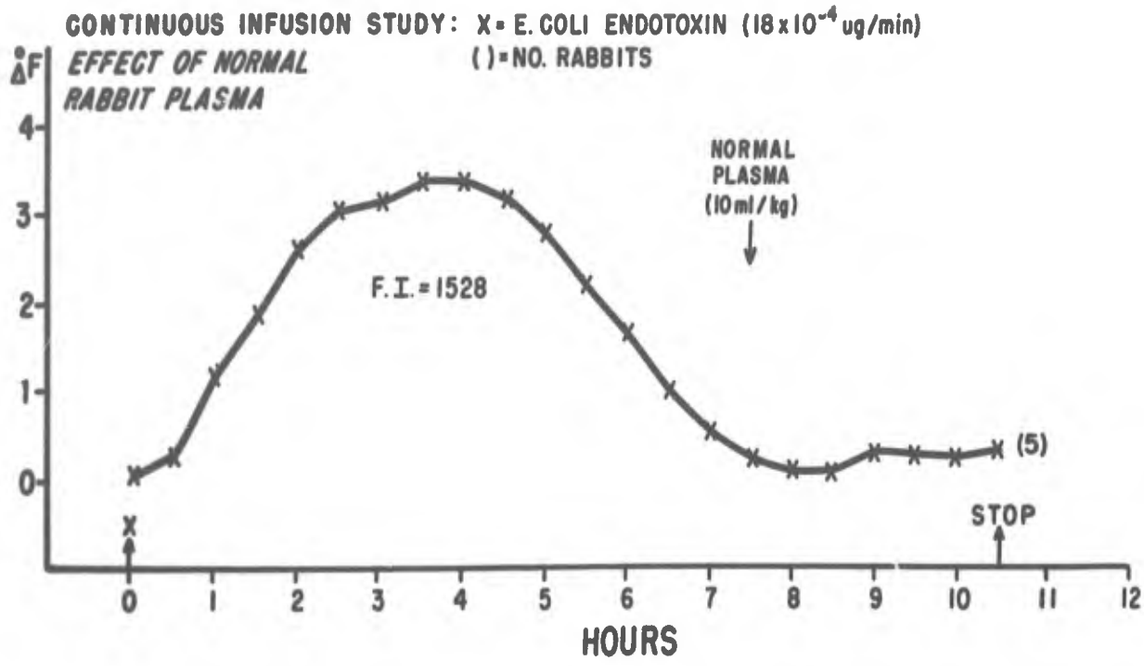

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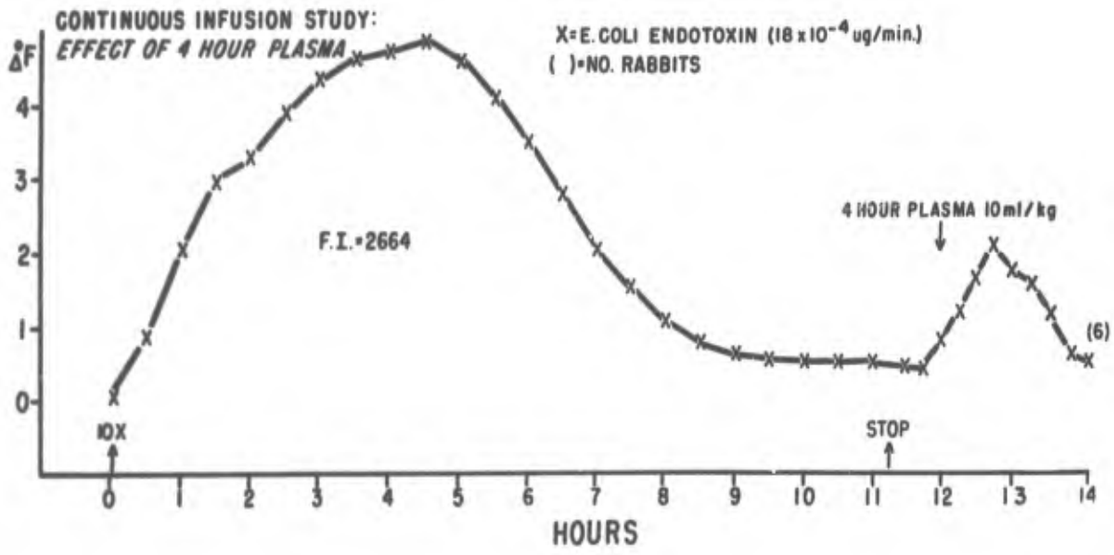

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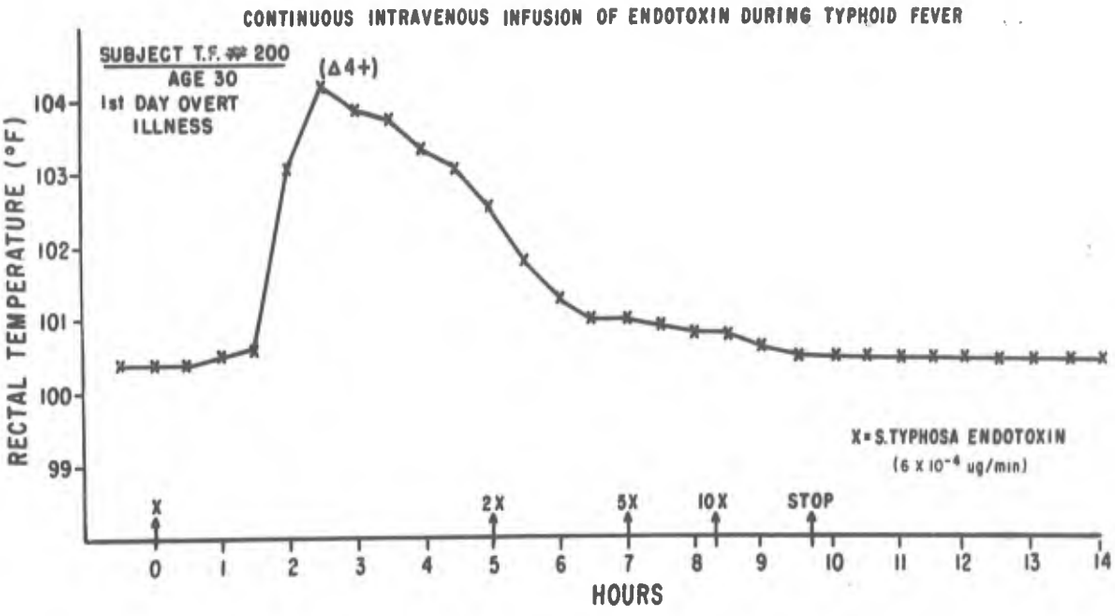

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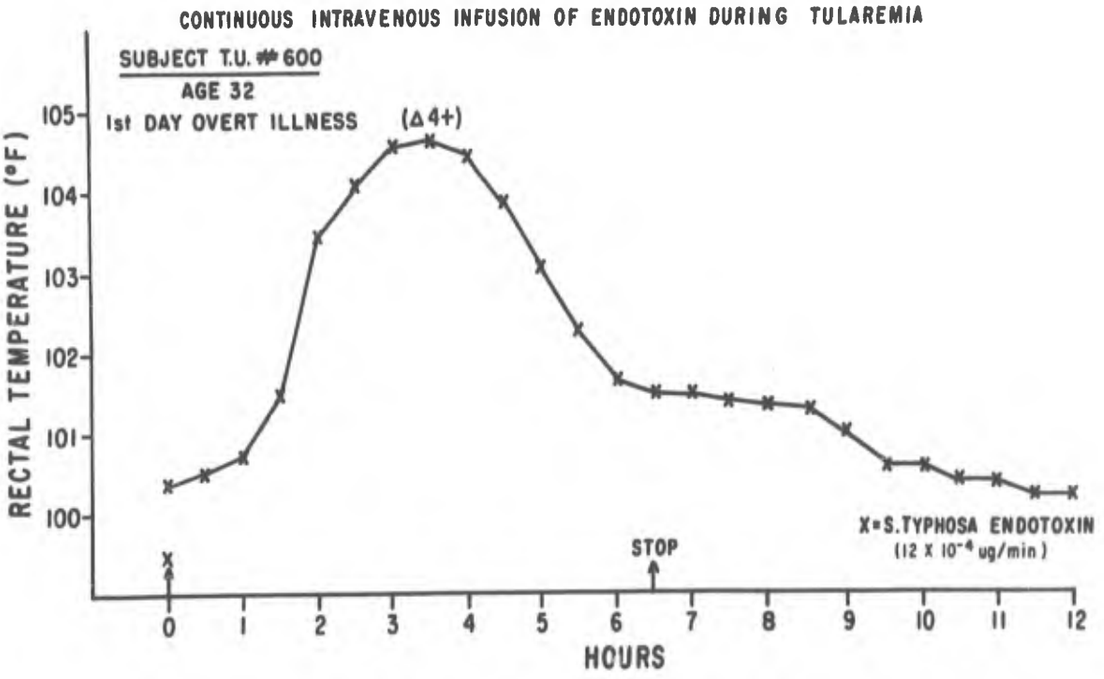

FIGURE 8.

unlikely explanation for the defervescence during the endotoxin infusion since desensitization was achieved readily even though the volunteers responded with minimal febrile reactions.

In other volunteers, the pyrogenic response pattern to S. typhosa endotoxin infusion during typhoid illness was biphasic. Defervescence during the infusion was interrupted by an abrupt 2nd progressive and protracted rise in temperature (Figure 10). This 2nd rise in temperature coincided with the characteristic rises in late afternoon temperature seen in control subjects with typhoid fever. Moreover, when the temperature curve of each volunteer was plotted on the following day in the absence of endotoxin infusion, the occurrence of the spontaneous afternoon rise in temperature was superimposable upon the point of onset of the 2nd rise during the endotoxin-induced fever. These data further demonstrated that the fever and toxemia of typhoid fever cannot be prevented by desensitization to exogenous endotoxin.

Figure 11 illustrates another typical biphasic febrile pattern during the continuous infusion of endotoxin in the course of overt tularemia. From such data, a similar inference as that from the preceding study may be drawn, i.e., the fever and toxemia of tularemia are not prevented by desensitization to exogenous endotoxin.

SUMMARY

The sustained release of bacterial endotoxin into the circulation was not primarily responsible for the continuous febrile and toxic course of typhoid fever and tularemia in man. Rather, other mechanisms, presumably similar to those responsible for sustained fever and toxemia during infections induced by nonendotoxin containing microbes, must have been operative. It is emphasized, however, that while endotoxemia could not account for sustained illness, it could account for abrupt intensification of fever and toxemia at any time during the course of typhoid fever or tularemia. Thus, it could be demonstrated that subjects with typhoid fever and tularemia exhibited remarkably hyperreactive febrile and toxic responses to a single IV injection of endotoxin.[27] Release of a relatively small bolus of endotoxin into the circulation during illness (or upon institution of appropriate antibiotic therapy) could therefore produce an acute febrile and toxic spike, including shock, but such an event would be sharply circumscribed and would be superimposed upon the more basic mechanisms responsible for the characteristic sustained febrile and toxic state.

CONTINUOUS INTRAVENOUS INFUSION OF ENDOTOXIN DURING TYPHOID FEVER

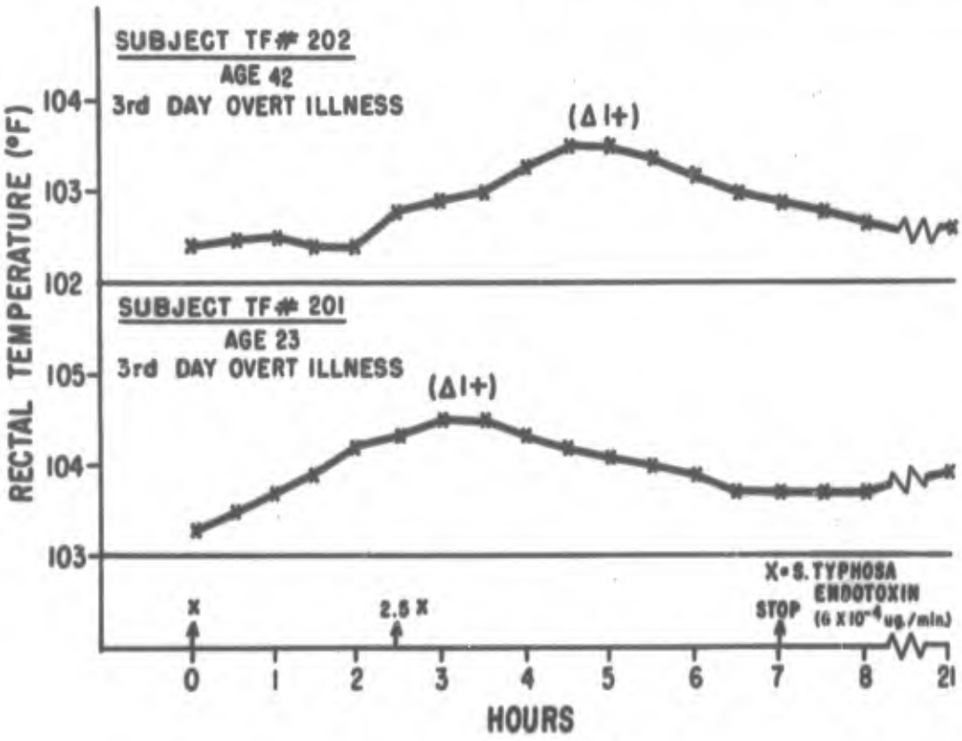

FIGURE 9.

CONTINUOUS INTRAVENOUS INFUSION OF ENDOTOXIN DURING TYPHOID FEVER

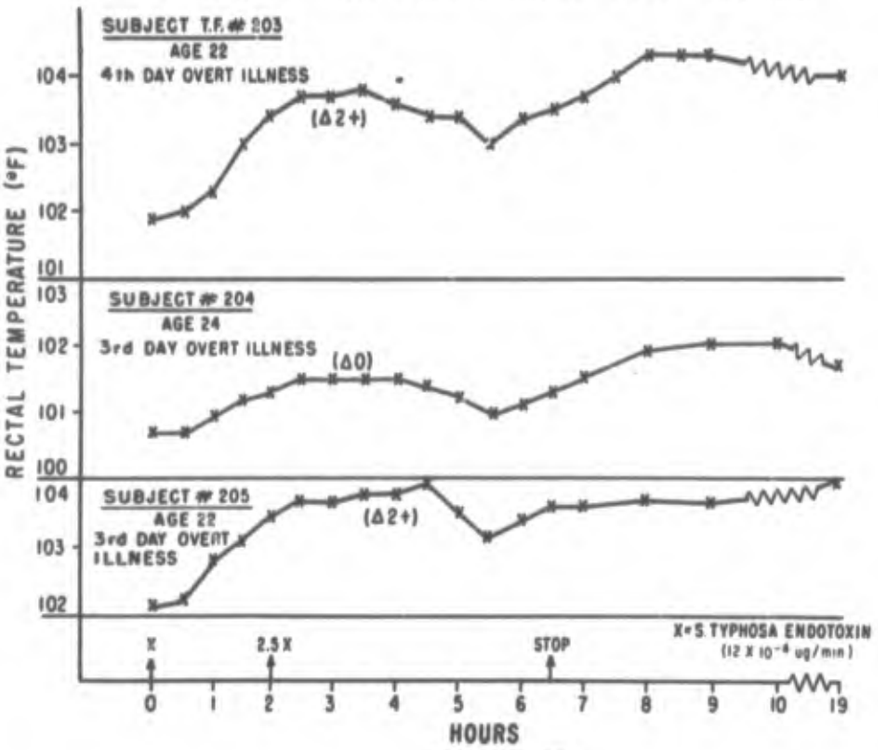

FIGURE 10.

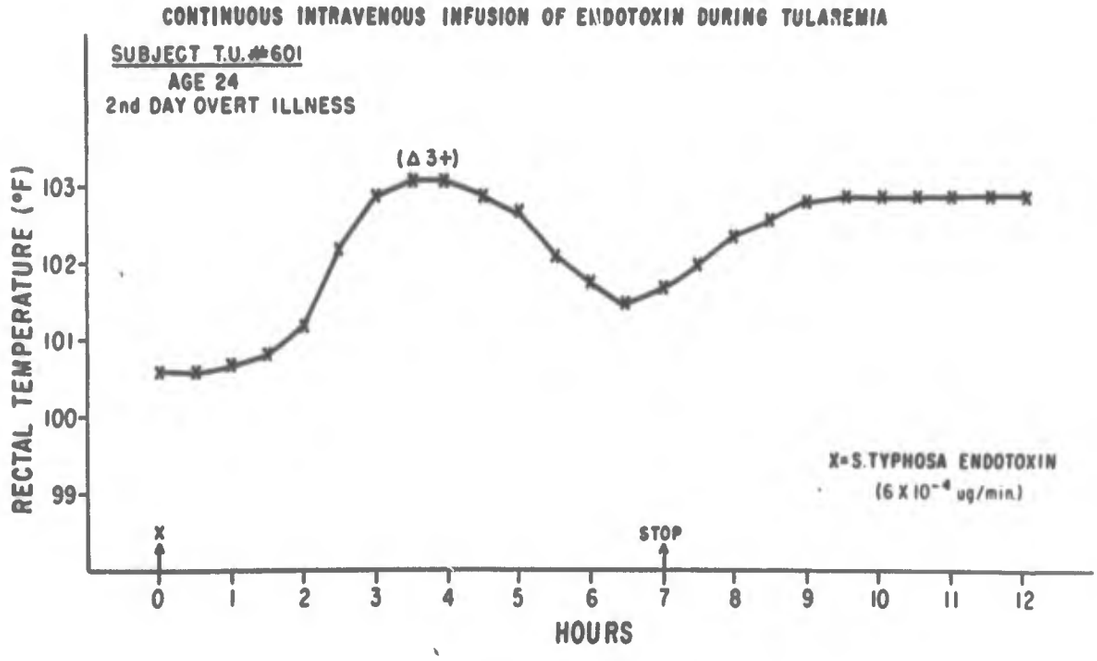
CONTINUOUS INTRAVENOUS INFUSION OF ENDOTOXIN DURING TULAREMIA
SUBJECT T.U.#601
AGE 24
2nd DAY OVERT ILLNESS
(Δ 3+)
RECTAL TEMPERATURE (°F)
103
102
101
100
99
X=S.TYPHOSA ENDOTOXIN
(6 X 10⁻⁴ ug/min.)
X
STOP
0
1
2
3
4
5
6
7
8
9
10
11
12
HOURS

FIGURE 11.

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POSSIBLE POLYMER CONFIGURATIONS OF ANTHRAX TOXIN COMPONENTS

Anne Buzzell, Ph.D.*

Work by Stanley and Smith[1,2] indicated that anthrax toxin consists of at least 3 components differing in biological activity which are demonstrable after glass filtration of anthrax culture fluids. A "Factor I," eluted from the glass filter by high pH bicarbonate buffer, was highly active in producing guinea pig skin edema when combined with filtrate which contains predominantly "Factor II," also known as the "protective antigen" of anthrax vaccine. A "Factor III" which could be found in the filtrate and also eluted from the filter, was lethal for mice or rats when recombined with Factor II. The yields of Factors I and III were low and variable, and it seemed possible that glass filtration might have altered or destroyed much of the toxin activity. Therefore, colleagues in the Bacteriology Division worked out a concentration procedure so that the components of anthrax toxin not subjected to glass filtration could be studied. The technique of concentration by ultrafiltration, introduced by Tresselt, has been worked out in detail by Gaspar and was reported to the Commission in 1963. This concentration method appears to be a very gentle one and only the yield of edema activity is lower than theoretical. Because the edema activity is relatively low in the ultrafiltrate (UFR) and because the assay for lethal activity (the measurement of the time-to-death (TTD) for Fisher rats) required such large volumes of UFR, only lethal and protective antigen (PA) activities have been studied.

Thomas Bates and the writer have been studying UFR in the ultracentrifuge and have observed a large variety of components with and without obvious biological activity. In the hope of accounting for at least some of the embarrassing plenitude of components, investigations have been conducted on the possibility of molecular aggregation, for it has been known for some time that aggregrates with lethal activity do exist.

Ultracentrifuge analyses have shown a qualitatively consistent picture of the optically visible components in all the UFR batches studied. In Figure 1 besides the major peak which consists primarily of 2 unresolved components with sedimentation constants (S) of 2 and 5 Svedberg units, there are 2 rapidly sedimenting peaks with sedimentation constants of about 14 and 19. The relative amounts of the components varied from batch to batch in a way that suggested the components 14 and 19 were aggregates of the slower pair. This batch of UFR had been concentrated 2000-fold whereas the batch shown in Figure 2 was concentrated only 480-fold and has much less of the presumed aggregates.

Figure 3 is a later photograph from the same run in which peaks of the slow components, 2 and 5, are beginning to resolve showing that these 2 components are present in roughly equal amounts, as is the usual case.

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FIGURE I. SCHLIEREN DIAGRAM FROM ULTRACENTRIFUGE ANALYSIS OF UFR. AGGREGATES PRESENT.

FIGURE 2. SCHLIEREN DIAGRAM FROM ULTRACENTRIFUGE ANALYSIS OF UFR. TWO PRINCIPLE SLOW COMPONENTS PRESENT, NOT YET RESOLVED.

FIGURE 3. LATER TIME THAN IN FIG. 2 SHOWING RESOLUTION OF TWO COMPONENTS.

Toxic activity of the presumed aggregate with a sedimentation constant 14 was suggested early in the study by use of a separation cell in the analytical ultracentrifuge. When a batch of toxin contained a large amount of component 14, activity decreased markedly in the top half of the cell after centrifugation long enough for all of this component to have sedimented out.

Earlier experiments using the separation cell had suggested that the component with sedimentation constant 2 might carry the lethal activity. Recent chromatographic data obtained by Mrs. Wilkie (Bacteriology Division), however, indicated that lethal activity might be associated with component 5 since lethal activity and PA come off a DEAE column as a single fraction.

Therefore, a complete separation of all the components of UFR, using sedimentation velocity, in a sucrose density gradient, and their biological characterization seemed called for. In the sucrose density gradient technique, one manufactures a linear gradient starting with 20% sucrose in the bottom of a swinging bucket centrifuge tube and ending with 5% at the top. One then adds a thin layer of sample on top of the sucrose. The various components appear after centrifugation as separate bands at different levels in the tube and can be retrieved by puncturing the tube at the bottom and collecting the material in a number of fractions.

The results of such an experiment both with a UFR and with a "filter factor" preparation, are shown in Table I. In column two are listed the respective fraction numbers for the 2 sucrose gradient separations. In column three are given sedimentation constants for UFR fractions calculated from the migration rate of boundaries observable with ultraviolet absorption optics in the analytical ultracentrifuge. In column four are listed the lethal activities of the UFR fractions diluted 1:10 in buffer and tested in Fisher rats by personnel of Bacteriology Division. Activities are expressed as TTD in minutes and the smallest number corresponds to the greatest activity. The results from the density gradient separation of the "filter factor" preparation are given in column five. No optically visible components were present but lethal activity could be detected by diluting the fractions 1:10 in a standard PA preparation.

The first thing to note is that the lethal activity in the "filter factor" preparation has a sedimentation constant of about 5. Also, the fractions of UFR containing the component 5 are lethal as is; that is these fractions must contain both PA and lethal factor. The fractions containing component 2 are not lethal and though it is not recorded, they have neither the antigenic components characteristic of PA nor those characteristic of "filter factor" preparations. The UFR fractions containing component 5 have both antigenic components. The function of component 2 is not fully understood at present; it is a major constituent of all the toxin preparations.

TABLE I. SEDIMENTATION IN SUCROSE GRADIENT (2 rats each)

THEORETICAL S	FRACTION	UFR		FILTER FACTOR (dil. 1:10 in PA)
		S	Avg TTD (min)	Avg TTD (min)
	1	20	--	Control = 85
	2		--	
	3	--	--	
	4	--	--	
14.6	5	15	300	
12.6	6	13	150	
	7	11	sick	
10.4	8	11	140	
	9	8.9	sick	
	10	8.6	--	--
	11	7.7	sick	sick
7.9	12	7.7	130	--
	13	5.0	60	150-180
	14	5.0	65	150-180
5.0	15	5.0	55	110
	16	4.3	60	>360
	17	4.3	80	--
	18	3.3	>360	--
	19	3.3	--	--
	20	2.0	--	--
	21	2.0		
	22	2.0		
	23	--		
	24	--		

There are a number of aggregates, sedimenting more rapidly than component 5, with full lethal activity. One can calculate (making some probably valid assumptions) the sedimentation constants to be expected for various aggregates of a monomer with sedimentation constant of 5. These are listed in the first column.

Such components are all present in the UFR. The distribution of lethal activity among them appears to be strange, however. The dimer appears to be inactive since fractions 11 and 12 which contain mainly the component 7.7 have only weak activity probably arising from diffusion spreading of monomer molecules. There is a rise in lethal activity in the region of the trimer but then a drop in activity before the next rise in the region of the pentamer. The data, therefore, seem to suggest that only odd-numbered aggregates are active. A possible reason for such a state of affairs is suggested by the fact that fraction 11, containing the dimer, has no

antigenic component corresponding to the lethal factor, only one corresponding to PA. The dimer, therefore, appears to be 2 PA particles. The timer would then be this PA dimer plus one lethal factor particle. The tetramer would be 2 dimers of PA and the pentamer, a PA tetramer plus one lethal factor particle.

SUMMARY

It is planned to test this postulate of the origin of lethal aggregates by additional experiments performed in a somewhat different fashion in order to gain better resolution of the components and to eliminate the need for theoretical corrections in the sedimentation constants occasioned by the presence of sucrose. In order to have enough fluid to test for lethal activity we have to pool samples from all 3 tubes of the swinging bucket rotor. This means that for optimal resolution of components the 3 gradients must be identical. Substantial improvements in the technique of making gradients have been made since these experiments were done. It is planned to dialyze the fractions before analyzing them in the ultracentrifuge and to determine diffusion constants as well as sedimentation constants so that the relationships among the aggregates can be specified in terms of molecular weights.

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ADRENAL FUNCTION DURING INFECTIOUS ILLNESS

William R. Beisel, Lt Colonel, MC*

At each of the last two annual meetings of this Commission, data acquired with metabolic balance techniques in volunteers were presented. Several acute infections were studied as well as a number of conditions which seemed important as major variables. In each presentation allusion was made to the fact that adrenal function was being studied concurrently. A summary is presented of the findings providing a comprehensive yet detailed evaluation of the role of the human adrenal cortex during the course of acute infectious illness.

While infection has long been recognized to induce an adrenal "stress response," details of this response have not been described heretofore. The normal cortex produces 4 major groups of steroid hormones: the mineralocorticoids, the glucocorticoids, the androgens, and the pregnanetriols. The urinary metabolites of the last 3 groups have been studied in a large number of patients.

Control period collections were obtained prior to exposure and during the course of tularemia, sandfly fever, Q fever, and also during the study of other variables including artificial fever and antibiotic administration. Urinary and plasma determinations were done in the Metabolic Department steroid laboratories of Walter Reed Army Institute of Research under the direction of Dr. Joseph Bruton and Mr. Kelly Anderson. Aldosterone determinations were performed through a special service contract. Binding of cortisol to plasma proteins was measured by an ultracentrifugation technique in the Medical Unit laboratory with the technical assistance of Specialist Gardner.

In Figure 1 change in urinary adrenal metabolites are compared to the courses of illness in 12 patients with typical acute tularemia. Arrows designate the day of exposure (above). With the onset of symptoms and fever, a progressive stepwise rise in the glucocorticoid excretion occurred. Following the institution of therapy an abrupt fall to control levels was noted. An increase in 17-ketosteroid (17-KS) excretion occurred but was less prominent; a slight rise in pregnanetriol excretion was also noted. The same data were rearranged (Figure 1, below) to align the day of maximum illness rather than the day of exposure of each of the patients.

Increased steroid excretion appeared to coincide even more exactly with early illness; days of maximum rise of each of the fractions were significantly above control values, depicted as horizontal shaded areas which represent mean ± 1 SE. Therapy was begun on the day of maximum

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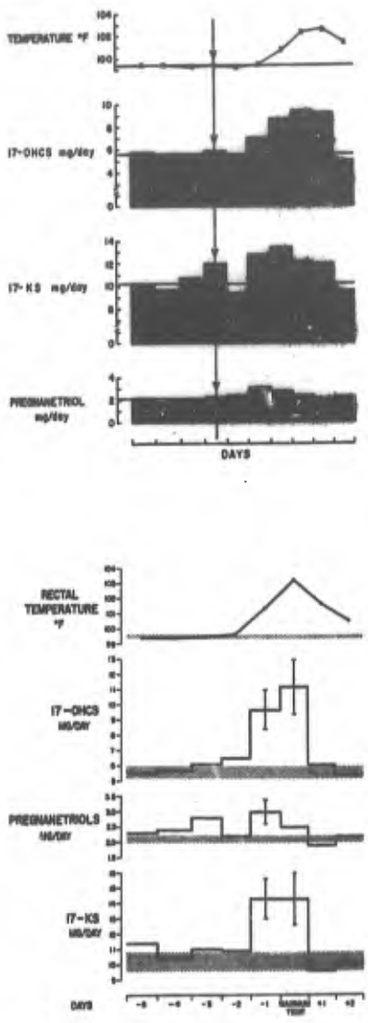

FIGURE I. URINARY CORTICOSTEROIDS IN ACUTE TULAREMIA (12 SUBJECTS).
ABOVE: BY DAY OF EXPOSURE.
BELOW: BY DAY OF MAXIMUM TEMP.

illness. Note that following therapy all steroid increases fell sharply to control values, even though fever fell slowly. It seemed possible that streptomycin might impede adrenal synthesis or interfere chemically with the analysis of steroids in urine. However, in control studies streptomycin was administered in the absence of infection, and no change in adrenal metabolite excretion was detected. The magnitude of change in adrenal response to tularemia is small, considerably less than that observed after injury or surgery. The brief duration of illness may be a factor.

Changes in urinary hydroxycorticoid excretion (Figure 2) are shown in relation to changes in nitrogen balance and excretion of urinary aldosterone. There are 2 important findings regarding a difference in timing of peak changes in steroid excretion and nitrogen metabolism. The stepwise increase in glucocorticoid excretion occurred coincident with developing fever and then fell to normal. The major changes to negative nitrogen balance did not occur until the height of illness had been reached and then persisted for almost a week thereafter. Urinary aldosterone, on the other hand, rose and fell in a stepwise fashion but lagged behind the excretion of the glucocorticoids in both peak and duration of response. This change in urinary aldosterone excretion correlated exactly in onset and duration with the timing of Na^+ and Cl^- retention by the kidneys.

Although 17-KS metabolite excretion underwent a total increase less marked than that of the glucocorticoids its composition was markedly altered. After initial isolation from urine by chemical methods changes in the major individual androgens, which comprise the 17-KS, were detected using gas column chromatography. In Figure 3 are shown the progressive changes which occurred during acute tularemia in a single subject. The individual peaks represent androsterone, etiocholanolone, dihydroepiandrosterone, and epiandrosterone (a very minor peak). On the third day following exposure the patient remained asymptomatic; urinary androgens retained the pattern seen during control days. Two days later, during the height of symptoms, there was a marked change in pattern as shown in the lower left; dihydroepiandrosterone appeared as the predominant peak, accounting for about two-thirds of the urinary ketosteroids. This androgen is produced mainly by the adrenal cortex, whereas the other androgens are mainly of testicular origin. Note that etiocholanolone (known to induce fever when produced in excess by certain individuals or when administered parenterally) played no apparent role in the fever of this infection. The total amount of etiocholanolone excreted was unaltered or actually reduced in this individual. Similar changes in the patterns of androgen excretion have been noted in a number of tularemia patients studied by gas chromatography. It is of interest that shift in the excretory pattern of the androgens could be best explained by an increase in ACTH stimulation of the adrenal cortex, without concomitant increase in testicular steroid production. Dihydroepiandrosterone, the principal adrenal ketosteroid is very weak in its androgenicity, and when administered to normal subjects produced no detectable change in nitrogen or mineral balance. Thus, infection appeared to stimulate little, if any, response by steroids (principally anabolic).

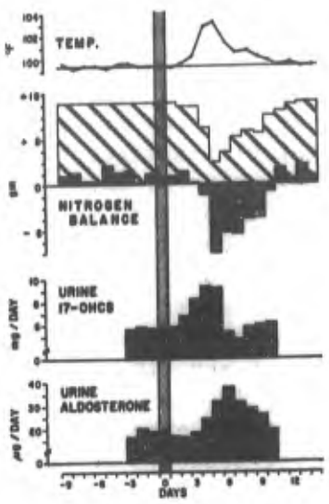

FIGURE 2. COMPARISON: URINARY CORTICOIDS TO NITROGEN BALANCE IN ACUTE TULAREMIA.

Figure 4 shows patterns of urinary steroid excretion in 8 patients who were immunized prior to exposure to tularemia. Four of these men became mildly symptomatic, experiencing a slight fever. As shown by solid lines, this group exhibited a slight, transient increase in adrenal steroid output while the 4 asymptomatic individuals shown with dashed lines had no change from control levels. Nonimmunized subjects who developed only mild symptoms following exposure exhibited only slight changes in adrenal corticoid excretion. Thus, it seemed that immunization which reduced the severity of illness also diminished the magnitude of adrenal response.

In sandfly fever the pattern of change in adrenal corticoid excretion was essentially similar to that observed in acute tularemia (Figure 5). This viral disease was studied throughout its natural course without antibiotic therapy; an abrupt fall in glucocorticoid excretion occurred once maximal severity of illness had been reached. Aldosterone determinations in sandfly fever also revealed a delay in the timing of peak excretion, a timing which was again coincident with that of urinary Na^+ and Cl^- retention.

In typical Q fever, as seen in Figure 6, changes in adrenocortical function were less clear-cut than in tularemia or sandfly fever. The rise in 17-OHCS output was only slight with a vague beginning; its peak coincided with maximal illness. Pregnanetriol rise was also gradual and slight; no discernible increase was detected in 17-KS excretion. Despite fever, the clinical impression of these subjects was that they were not as sick as those with tularemia. In Figure 7 are contrasted steroid data from 2 additional subjects exposed to Q fever; both experienced rickettsemia days 8-15. The subject at the top was the sickest of our patients; the one at the bottom developed no symptoms at any time despite his rickettsemia. He, incidentally, was the only subject in any of our studies who exhibited adrenal stimulation at the time of his exposure in a manner perhaps analogous to that reported in medical students at the time of their final examinations.

The changes in adrenalocortical output associated with the induction of artificial hyperthermia by physical means in a group of 8 men are shown in Figure 8. In these subjects the rise in excretion of the corticoid metabolites paralleled the increase in rectal temperature of the group and in so doing reversed the expected diurnal pattern of adrenal cortical hormone excretion.

Changes in the pattern of plasma steroids were investigated in these same groups of subjects. Control samples and those during fever were obtained in both the morning and late afternoon in order that any response during fever could be compared with appropriately timed plasma levels which normally fluctuate with a diurnal rhythm. This rhythm produces the highest blood corticoid levels during the early morning hours. These levels then fall to a lower concentration of blood hydroxycorticoids throughout the remainder of the 24-hr period.

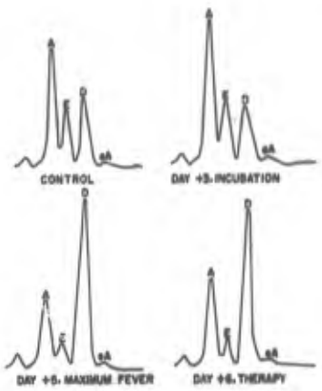

FIGURE 3. URINARY ANDROGENS IN TULAREMIA.

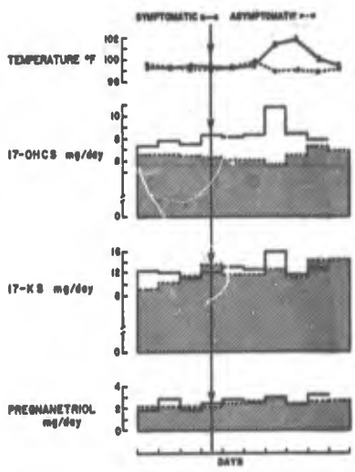

FIGURE 4. URINARY CORTICOIDS IN 2 IMMUNIZED SUBJECTS EXPOSED TO TULAREMIA.

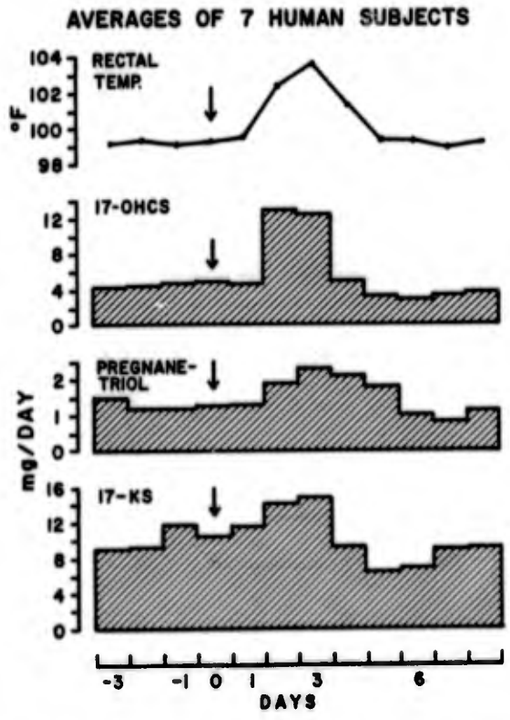

FIGURE 5. URINARY CORTICOIDS IN SANDFLY FEVER.

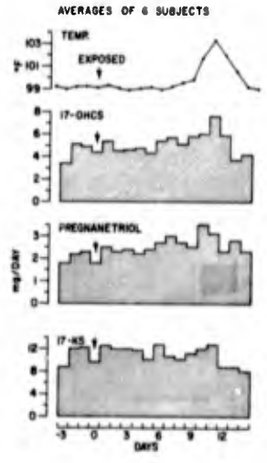

FIGURE 6. URINARY CORTICOIDS IN Q FEVER.

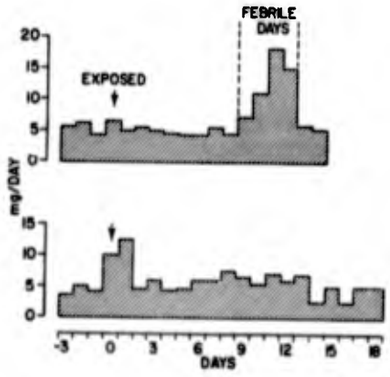

FIGURE 7. URINARY 17-OHCS IN Q FEVER.

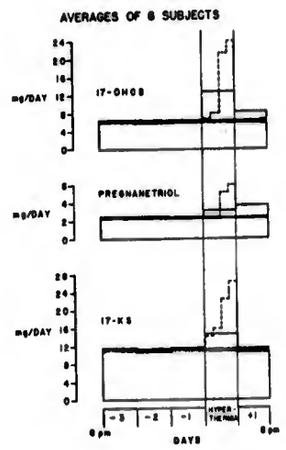

FIGURE 8. URINARY CORTICOIDS IN INDUCED HYPERTHERMIA.

Average plasma concentrations of 17-OHCS ± 1 SE are shown in Figure 9. The control morning and afternoon values for each group of subjects are shown as clear areas while the values for the same individuals during infection or fever are shown in the shaded areas. Normal diurnal fluctuation was present during the control periods. Note that in each disease studied, blood levels during the morning hours in fever were statistically indistinguishable from those obtained on control mornings. In typical acute tularemia, sandfly fever, Q fever and artificially induced hyperthermia afternoon values during fever remained approximately at the control morning concentrations. To the right of the figure are data obtained following intravenous endotoxin administration on 3 consecutive days. On these days, afternoon fever occurred but lasted only several hours: the afternoon values were consistently and significantly elevated above control levels; there was no fever during morning collections. This pattern of change, in which the normal diurnal rhythm is lost, is characteristic of patients with Cushing's syndrome. Although this pattern has been stated to be of diagnostic significance in Cushing's syndrome, we have found it to occur also in these febrile states and in acute infection. In the group of subjects with clinically mild tularemia (the second group) plasma corticoid levels retained a normal pattern and failed to remain elevated throughout the day.

In each of these plasma samples the amount of protein-bound and unbound hormone was also measured. In each group of subjects studied, during infection or fever, the binding of cortisol by plasma proteins was normal. This observation, with respect to cortisol binding, is in direct contrast to observations to be presented in greater detail by Major Shambaugh (paper follows) with respect to the plasma protein binding of thyroid hormones which is reduced during infection.

It seemed that both the extent and duration of adrenocortical stimulation were small during the short term infections studied. In these subjects the total adrenal response was far less than that reported during surgical operation. Because the adrenal response was so slight and its peak so early it seemed unlikely that changes in adrenal corticoid function alone could account for the marked changes in nitrogen excretion observed in infectious illnesses. Various authors have commented that nitrogen losses during infection could be explained entirely on the basis of adrenal overactivity. In order to gain additional information in this area of disagreement a study was devised in which a group of 4 normal control subjects were given oral cortisol in doses designed to reproduce the stepwise changes in urinary glucocorticoid excretion in the group of patients with typical tularemia.

The results of this study, shown on the right in Figure 10, are compared with data to the left with respect to the changes in nitrogen balance. As shown in the second portion of the graph the administration of corticoids leads to an excretion of glucocorticoid (17-OHCS) metabolites slightly higher each day than the amounts excreted by patients suffering acute tularemia.

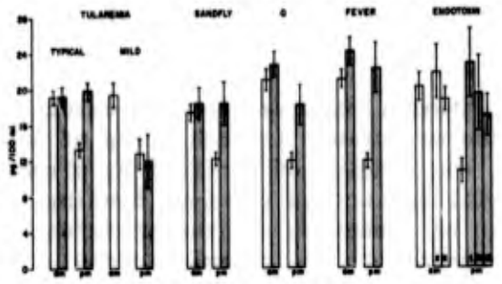

FIGURE 9. PLASMA 17-OHCS DURING INFECTION OR FEVER.

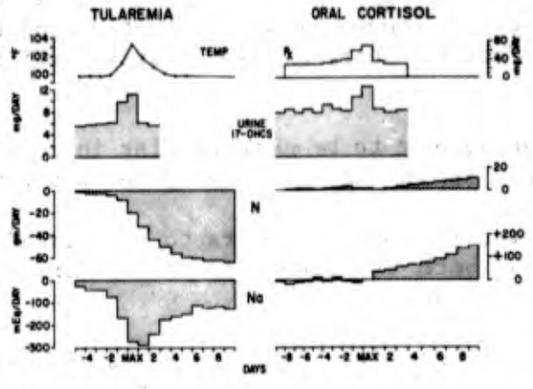

FIGURE 10. COMPARISON OF INFLUENCES OF TULAREMIA OR CORTISOL ADMINISTRATION ON NITROGEN & SODIUM BALANCE.

This dose of oral cortisol which produced urinary levels slightly in excess of those of tularemia failed to produce negative nitrogen balance. This relatively small dose of hydrocortisol was well within physiologic limits, far below the pharmacologic range.

SUMMARY

These studies for the first time permit an appreciation of the total, complex pattern of adrenal corticoid response to acute infection in man.

All functional portions of the adrenal cortex were involved in the responses observed.

These responses occurred with differences in timing, with the mineralocorticoid excretion rising after that of the glucocorticoid, and androgen, or pregnanetriol fractions. This observation is entirely compatible with the current concept that the 3 latter fractions respond primarily at ACTH stimulation, whereas aldosterone increases are stimulated primarily by a fall in circulating blood volume which activates the renin-angiotensin system. The timing of rise in aldosterone excretion could be correlated exactly with the observation, known for years, that during the height of, and following, acute infection there is also a marked fall in the urinary excretion of Cl^- and Na^+.

The adrenal response appear to be quite similar in each of the several illnesses studied as well as in the artificially induced forms of fever.

The magnitude of adrenal response appears to be related to the severity of illness; this was true both for changes in plasma corticoids as well as the excretion of urinary metabolites.

With regard to plasma concentrations the pattern noted after infection or artificial fever was typical of Cushing's syndrome with loss of the normal diurnal fall in plasma corticoids.

Changes in the plasma protein binding of cortisol were not observed in any instance studied.

From the timing and magnitude of adrenal glucocorticoid response observed, it seemed impossible that changes in body nitrogen metabolism could be related solely to the alterations induced by adrenal cortical overactivity. Despite this conclusion based on our studies of short duration illness, it is entirely possible that in long term, severe, untreated infectious disease, adrenocortical hyperfunction does contribute to negative nitrogen balance.

The increase in androgen secretion during infection failed to include those compounds with predominantly anabolic or fever-producing activity; instead, dihydroepiandrosterone of adrenal origin seemed to account in large measure for the increased excretion of 17-ketosteroids.

THYROID FUNCTION DURING GENERALIZED INFECTION

George E. Shambaugh, Major, MC*

The response of the thyroid gland in generalized infection has been the subject of only a few reports compared to the volume of literature on intrinsic diseases of the thyroid. Although diminished basal metabolic rates were reported as early as 1926 in children and adults following pneumococcal pneumonia,[1] no fundamental research into the mechanisms of thyroid alterations in generalized infection appeared until Sternberg and his co-workers demonstrated in 1955[2] a depression of thyroidal I^{131} uptake in the mouse infected with Coccidioides immitis. This report was followed by a comprehensive paper by Reichlin and Glaser[3] who observed that thyroidal release of I^{131} was delayed in rats with streptococcal pneumonia. They postulated that a reduced thyroid stimulating hormone (TSH) output associated with lowered food intake during infection might account in part for the reduction of thyroidal activity in a manner similar to the depression of TSH in rats starved for 4-8 days as reported by D'Angelo.[4] Badrick et al[5] noted a stress-induced decrease of I^{131} uptake in the thyroid of hypophysectomized rat, which appeared to result from direct suppression of the gland. In various stresses including cold, heat, fasting, formalin injection, anoxia, nephrectomy, intestinal injury, and avitaminosis, rats have responded by a decrease of thyroidal I^{131} uptake. A decrease in serum protein bound iodine (PBI) has also been reported in rats subjected to a number of different stresses.

Very little, however, is known about the alterations in thyroid physiology during a generalized infection. Because of the potential importance of the thyroid gland in determining host response to infection, an attempt was made to clarify these alterations. Since volunteer patients are not candidates, without special approval, for employment of radioisotopes even in the small doses used in thyroid diagnosis and because they could never be allowed to become severely ill from experimental infection, additional information was sought from an animal model. After conducting preliminary studies with several species, the rat was chosen as an experimental animal. A subcutaneous injection containing 5-15 organisms of a virulent type IA pneumococcus was found to produce a predictable infection after an incubation period of 40 hr. Using the rat model with a standardized dosage of pneumococcus, a large number of specific experiments were designed to survey thyroid physiology in a generalized infection. Each experiment was complete in itself with suitable controls and appropriate numbers for statistical analysis. Areas in thyroidal physiology already mentioned were covered as well as new areas not previously explored. During the course of the experiments, thyroid function was investigated using I^{131} uptake and release as parameters for the behavior of the gland itself. This was followed by studies of the response of the gland to exogenous TSH stimulation and by direct measurements of endogenous serum TSH levels. These studies were employed to gain information on thyroid-pituitary axis interrelationships.

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Serum protein binding of thyroid hormone was then studied using half disappearance times and tri-iodothyronine (T3) resin uptakes. This was followed in turn by the observations in the alteration of peripheral organ distribution of thyroxine (T4) and T3 during infection. An attempt then was made to identify the mechanisms involved and the pathogenesis of the changes observed.

Figure 1 shows thyroidal iodine uptake in infection. Following administration of 0.5 μc I^{131} given intravenously (IV) by way of the dorsal penile vein to 120 healthy and 120 rats infected 40 hr earlier; animals from each group were sacrificed at varying time periods in groups of 10 and their thyroid glands dissected free and counted.

The uptake, expressed as a percentage of the injected dose, is plotted as a function of the square root of the time in hours. The uptake in the infected rat is significantly less than in healthy animals. The ranges shown include the mean with its 95% confidence limits.

There is a fall in thyroidal iodine content in the healthy rat at 25 hr suggesting release of hormonal iodine. Thyroidal iodine in the infected rat had not fallen at this time.

To investigate the influence of infection on iodine release, 35 healthy rats were given 40 μc of I^{131}. Thyroidal radioactivity was measured 24 hr later by external counting over the neck. Counts were then made 3 times daily; the results were expressed as a percentage of the 24-hr count. Fifteen rats were then infected 72 hr after isotope injection and the results obtained on all animals were plotted on a semilogarithmic scale.

In Figure 2 the release curves expressed as the mean ± 95% confidence limits for the healthy and infected rats. The curves are parallel until 50 hr after infection. As the infected rats became sicker, their thyroidal release was progressively delayed, reaching significance at 103 hr.

To determine if the depression in thyroidal I^{131} uptake and release could be related to an inability of the gland to respond fully to TSH, specific studies were designed to compare control verus infected rats with or without administration of exogenous TSH.

The effect of exogenous TSH on thyroidal uptake was studied first (Figure 3). Thyroidal uptake of I^{131} was markedly decreased in the infected animals confirming replicated earlier experiments. When a physiologic dose of TSH was given, the uptake increased markedly in control animals as shown in clear areas to the right but in the infected animals, the stimulation of TSH induced an uptake only to normal baseline values.

TSH stimulated release of I^{131} from the thyroid of the infected rat was significantly different from the controls (Figure 4). The blood-radioactivity following TSH stimulation rose markedly in the control animals whereas the blood radioactivity in the infected animals was initially low

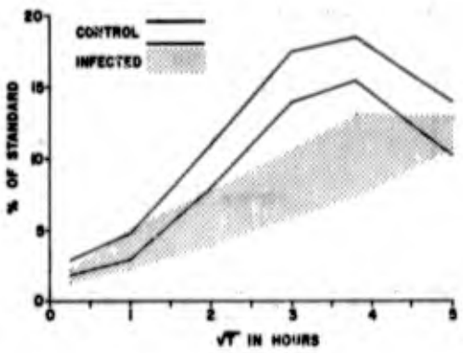

FIGURE I. I^{131} UPTAKE IN PNEUMOCOCCAL SEPTICEMIA IN RATS.

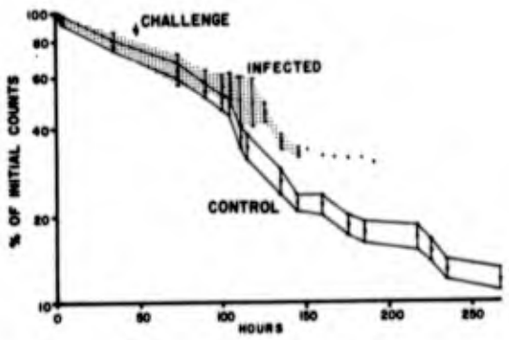

FIGURE 2. THYROIDAL I^{131} DISAPPEARANCE IN THE RAT.

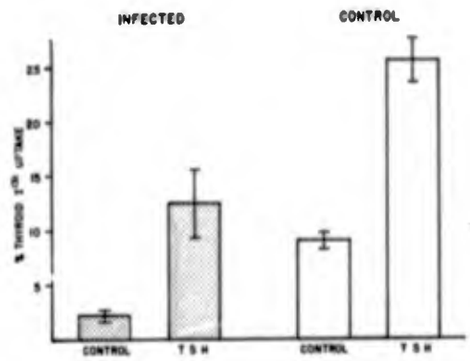

FIGURE 3. TSH-STIMULATED THYROIDAL I^{131} UPTAKE IN THE INFECTED RAT.

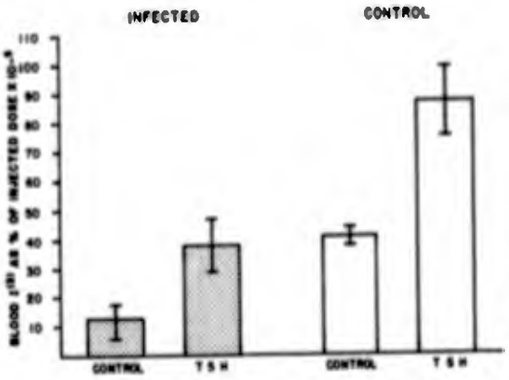

FIGURE 4. TSH-STIMULATED THYROIDAL I^{131} RELEASE IN THE INFECTED RAT.

and after stimulation rose only to the control baseline level. Furthermore, the quantity of I^{131} released by the infected animal as measured by the difference in column heights was significantly less.

To establish whether this observed difference in release might result from a lower iodine content of the thyroid glands in the infected animals, the experiment was repeated up to the point where TSH was given. The animals were then sacrificed, thyroids removed, weighed and counted. No significant difference was noted in either the weight or radioactivity of thyroid glands in infected rats. Histologic sections of the glands obtained 40 hr postinfection showed no alterations when compared to glands from healthy animals.

Thus, in the infected rat a diminished thyroidal uptake and release of radioactive iodine occurred. These changes were only partially corrected by exogenous TSH. At that stage of the investigations it became important to know whether circulating endogenous TSH concentrations had been altered by infection. Serum TSH levels were measured using a bioassay technique in the mouse as described by McKenzie.[6] Serum (0.2 ml) from infected rats was injected IV into appropriately prepared mice. The response measured by radioiodine release was compared to a dosage response curve using a TSH standard obtained from the National Institutes of Health. A mean of 0.04 milliunits/ml was similar to that obtained by McKenzie. Within the sensitivity of the assay no gross alteration of serum TSH in infection was evident, although the precision of the assay may have prevented the detection of subtle changes from normal. From these data it can be concluded that in infection, there exists an intrinsic defect in iodine metabolism in the thyroid gland with an apparent failure of the pituitary to respond appropriately. In several humans, serum TSH levels measured by the same technique failed to change during acute infection with tularemia.

To determine if infection altered the rate of disappearance of T4 from the blood, I^{131}-T4 was administered via the dorsal penile vein in a dose of 8 μc (1.2 μc/gm) to 10 control and 10 infected rats. Sequential orbital blood samples were obtained, counted, and plotted for each rat. The half disappearance times were then calculated and the mean, ± 95% confidence limits, were obtained for the infected and control groups. A second series of rats were given I^{131}-T3 and orbital blood samples obtained were counted similarly. Half disappearance times were calculated, and the infected and control groups were compared (Figure 5). The half disappearance times of T4 are significantly decreased in infection ($p > 0.01$) whereas the half disappearance times of protein precipitable T3 remain unchanged. These 2 hormones vary markedly in their responses to infection. It has long been assumed that the slower disappearance of T4 from the blood is related to its firm binding to plasma proteins in contrast to the weak binding of T3. Since the disappearance of T4 was speeded in infection, it appeared that a decrease in T4-binding proteins might have occurred during infection. The PBI in infected rats was found to be significantly depressed below normal. Using the PBI values and half disappearance times of T4, the turnover rate,

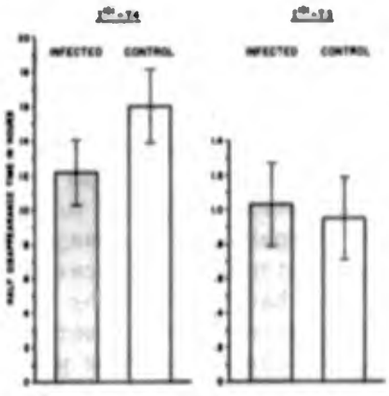

FIGURE 5. T $1/2_d$ OF THYROID HORMONES FROM THE BLOOD OF RATS.

degradation rate, extrathyroidal iodine pool and total iodine space were calculated. The increased turnover rate and decreased extrathyroidal iodine pool seen in the infected rat varied in a manner similar to those changes reported in humans with a deficiency of T4-binding proteins. To gain further information regarding binding, T3 resin uptakes were obtained on sera from infected and healthy animals. The healthy animals were divided into 2 groups, one of which was starved for 8 days (Figure 6).

The mean, ± 95% confidence limits, is plotted for infected and healthy animals. The T3 resin uptake is significantly increased in infection, but is unchanged in starvation. An increase in resin uptake is thought to be due to a decrease of the T4-binding proteins. This observation tends to strengthen the concept of a decrease in binding capacity of serum proteins in the infected rat.

Finally, changes in peripheral hormone distribution were studied. To determine if infection altered the distribution of thyroid hormones, radioactivity of various organs was measured after serial sacrifice following the administration of I^{131}-T4 or I^{131}-T3 to healthy and infected rats.

In Figure 7 the radioactivity in the thyroid, kidney, and liver at varying time periods after isotope injection is recorded. In both the T4 and T3 groups, thyroidal iodine accumulation was markedly decreased by infection. The greater uptake of radioactivity in the healthy rats given T3 is best explained by a rapid deiodination of poorly bound hormone. No change was seen in kidney distribution; in the livers of the animals given T4, a significant decrease in radioactivity, shown as a solid line, was seen during infection when compared to normal controls. This decreased quantity of hepatic radioactivity fell into a range similar to that seen in both infected and control animals given T3. Since glucuronide formation by the liver cells is not a limiting step in thyroid hormone excretion, this decrease in liver radioactivity in animals given T4 could be explained by a decrease in bound T4 in infection.

In Figure 8 are recorded the radioactivity in the stomach, small bowel and colon at varying time periods after isotope injection. No significant alteration was seen in the stomach and its contents in animals given T4. The delay in appearance of stomach radioactivity seen in infected animals given T3 suggest a delay in secretion of free iodine. In both T3 and T4 groups the passage of a bolus of radioactivity from small intestine to colon was significantly delayed by infection, and is compatible with a decrease in bowel motility.

The major alterations in thyroid physiology seen in infection in the rat have been tabulated in Table I. The depressed I^{131} uptake observed in infected rats has been recorded previously under conditions of starvation, cold and violent exercise. Curiously enough, the thyroidal I^{131} uptake in the mouse and guinea pig was unchanged by this specific infection, although it proved lethal for both these species. A delay in thyroidal I^{131} release has been described not only in pneumococcal septicemia but also other non-specific stresses including streptococcal pneumonia.

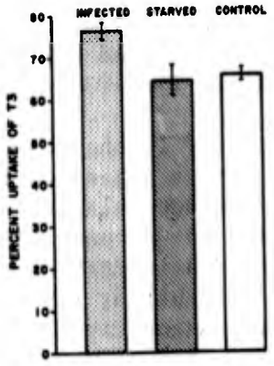

FIGURE 6. I^{131} T3 RESIN UPTAKE IN RATS.

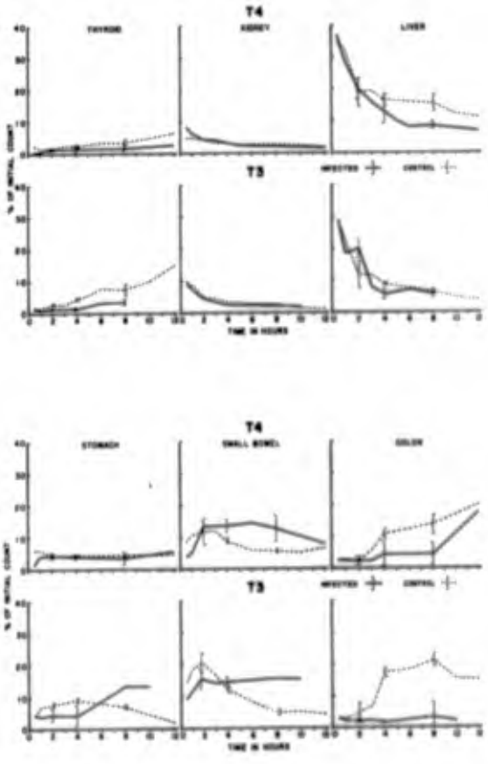

FIGURE 7. DISTRIBUTION OF RADIOACTIVITY AFTER ADMINISTRATION OF T3 &-4 TO INFECTED RATS.

TABLE I. ALTERATIONS OF THYROID PHYSIOLOGY IN GENERALIZED INFECTION

OBSERVED CHANGE	POSTULATED MECHANISM
1. Depressed thyroidal I^{131} uptake 2. Delayed thyroidal I^{131} release 3. Decreased thyroidal responsiveness to TSH 4. Decreased PBI	Direct suppression of thyroid function
5. Failure of pituitary response 6. Increased half disappearance of T4 7. Increased resin uptake 4. Decreased PBI 8. Decreased T4 concentration in liver	Diminished binding
9. Increased iodine concentration in small bowel 10. Decreased iodine concentration in colon	Delay in gut motility

The demonstration of a decreased thyroidal responsiveness to exogenous TSH, supports the concept of an intrinsic thyroidal defect. This concept was first advanced by Badrick and others,[57] who noted a further suppression of I^{131} thyroidal uptake following electric shocks in hypophysectomized rats. In the thyroid of infected rats, a metabolic defect may occur, possibly in energy metabolism which decreases both thyroid gland function and responsiveness to TSH.

The demonstrated failure of pituitary TSH response in the infected rat supports the postulations of Reichlin and Glaser[37] but the exact mechanism has not been elucidated. Since adrenocorticotropin release is increased in infection, it is difficult to postulate lack of TSH increase on the basis of a generalized pituitary suppression, although a tropin-specific depression is certainly a possibility.

In the infected rat, however, by half disappearance times and T3 resin uptakes, a fall in binding proteins has been demonstrated. This finding which has not been demonstrated previously in the rat can explain the failure of the pituitary to respond to diminished thyroid function.

When T4-binding protein diminishes there is an increase in unbound T4. Since the pituitary-hypothalamic axis responds only to circulating unbound hormone, the concentration of unbound T4 in infection may remain unchanged in spite of the depressed thyroid function and PBI. With maintenance of a normal concentration of an unbound hormone the pituitary would not be stimulated to respond. A low PBI can be accounted for by a depression of

thyroidal gland function as well as by a decrease in binding protins. Since binding proteins were unchanged in starvation and since the PBI level on pooled sera from starved rats was lower than normal it would appear that only thyroidal depression was a factor in starvation. In the infected rat, however, the PBI levels were half that of the starved animal suggesting that starvation alone could not account for a change of this magnitude. Hence, the inclusion of the decreased PBI in the altered binding category.

Although gut motility was markedly decreased in infection, this change did not appear to represent a major alteration in thyroid hormone metabolism.

SUMMARY

The eight major alterations in thyroid physiology in infection can be explained by two key changes: an intrinsic defect in the thyroid gland and a fall in T4-binding proteins.

Although the former has been well documented as a reaction to non-specific stress the fall in binding proteins in the rat has not been described previously.

Preliminary studies performed elsewhere showed a similar fall in T4-binding prealbumin in the chronically ill human, and when measured, in several instances of infection. Although T4-binding proteins in the rat behave in a similar manner pharmacologically to human T4-binding pre-albumin, thyroidal uptake in man is increased during surgical stress while uptake in the rat decreases.

In spite of these species differences, a fall in T4-binding proteins in the rat, as in man, appears to be an important but hitherto unrecognized facet in the pattern of response to the specific stress of a generalized infection. Although some insight has been gained into changes in trophic hormones and binding proteins, we continue to be ignorant of alterations in the human thyroid gland itself. Until we can define these changes in the human, as has been done with the rat, the pattern of man's response to infection will remain incomplete.

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INFLUENCE OF VIRUS INFECTION ON HOST PROTEIN AND RIBONUCLEIC ACID BIOSYNTHESIS

George Lust, Captain, MSC*

An understanding of the mechanism of action of the pathogenic effect of virus infection will have to come from research on host-cell metabolism. Much work throughout the country is being carried out on the biochemistry of viral infections, especially associated with phage work in bacterial systems, and some on various animal viruses, such as poliomyelitis virus. A great deal of the investigations in the area of animal-virus biochemistry are done using tissue culture systems, since it is easier to manipulate variables while holding other factors constant. Relatively little work has been reported employing whole animal systems. A description of some data from work involving Venezuelan equine encephalomyelitis (VEE) virus infection in mice, and in mouse L-cell tissue culture studies is presented.

A VEE virus particle consists of 6% ribonucleic acid, 24% lipid, and 70% protein. Since a virus is an obligate intracellular parasite it must obtain the materials for its synthesis from the cell. Therefore, when a virus infects a cell all of the major chemical cellular constituents may be affected. Since VEE is known to manifest its virulence through central nervous system involvement, and the virus consists of 24% lipid, host lipid metabolism, especially in the brain, may be adversely affected. Mouse brain metabolism was initially studied by Colonel Gray and later by Captain Mohrig of the Medical Unit. However, they decided to concentrate on the effects of protein biosynthesis first since proteins are basic to all biochemical reactions inasmuch as the reactions are catalyzed by enzymes and proteins are important structurally.

The first part of the present work was done with mice using both uninfected controls and Trinidad strain VEE-infected animals. The usual inoculum was 3,000 median lethal doses (LD_{50}) given intraperitoneally (IP). These animals died within 6-7 days.

In vivo protein synthesis was measured. The results obtained with liver are presented in Figure 1. In this experiment, C^{14}-leucine was injected via the tail vein into groups of 4 mice each day. The subcellular constituents of the liver were isolated and the radioactivity was measured as described in the previous presentation. Results are expressed as counts per minute (cpm)/mg protein. A reproducible decrease from control in C^{14}-leucine incorporation occurred after 20 hr of infection, particularly in the microsomes and mitochondria. Subsequently, an increase in synthesis rate was observed on days 3 and 4. As expected, the microsomal protein contained the greater quantity of radioactivity followed by the mitochondria.

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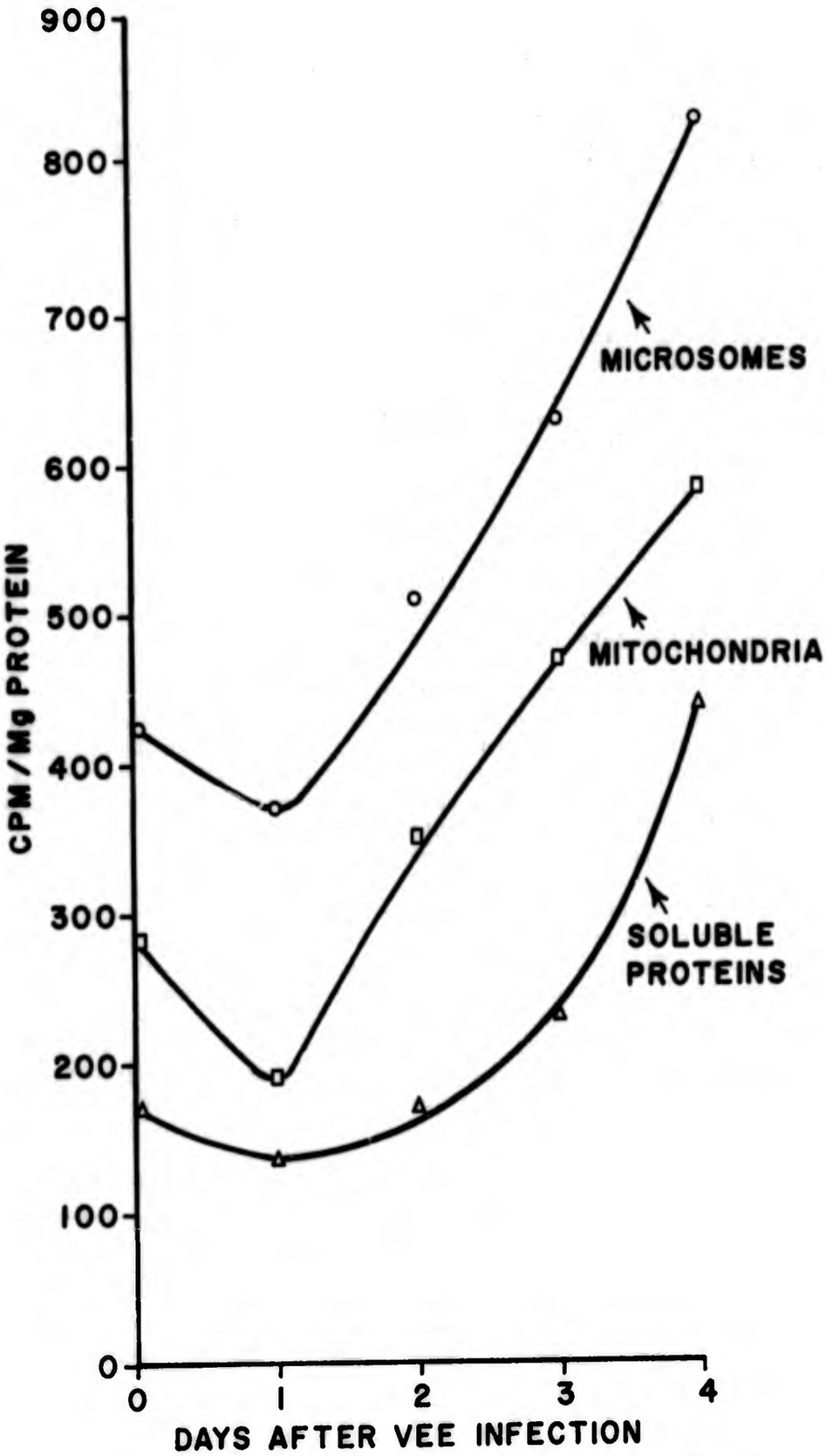

FIGURE 1. IN VIVO C^{14}-LEUCINE INCORPORATION INTO CELLULAR FRACTIONS OF MOUSE LIVER.

In the same mice we also investigated protein synthesis in the brain. The pattern obtained is shown in Figure 2. In this case the amount of radioactivity incorporated was less and the changes were similarly less pronounced. An interesting finding here was that the soluble protein of the brain homogenate contained the highest radioactivity, where as in the liver it was lowest. In the liver and even here in the brain, the increased C^{14} uptake as the infection progressed may be due both to some increased new protein synthesis as well as incorporation of C^{14}-leucine into virus-coat protein. We did not distinguish between them in this experiment.

In vitro microsomal protein synthesis experiments were then conducted with the same tissues. The isolated microsomes were incubated with soluble cytoplasmic enzymes, ATP, GPT, Mg^{++}, and an amino acid mixture, of which leucine was radioactive. The data obtained are presented in Figure 3. In the liver, a decrease occurred which reached a minimum at day 2. The rate of protein synthesis then increased to the normal control level. In the brain no apparent alteration was observed. The trends observed in the in vivo experiments were essentially reproduced in the in vitro studies. The effect of VEE infection on the rate of C^{14}-leucine incorporation into brain protein was relatively small in both the in vivo and in vitro experiments. In the liver, however, a marked decrease occurred followed by an apparent recovery to the control rate of synthesis.

It appears that the greatly enhanced incorporation observed in the in vivo liver study is probably due to incorporation of C^{14}-leucine into virus protein. A host stress response to the infection such as increased cortisol in the circulation and a rise in temperature may also be exerting an influence on protein synthesis in the whole animal which may be negated in a test tube. This increase over control values was not observed in the microsome experiment; in this case the rate returned to control values.

Before going on to the work on mouse L-cells in tissue culture, a brief orientation is necessary on the general scheme of protein synthesis as it applies here. The general outline is depicted in Figure 4. Information as to the kind of protein to be synthesized is stored in DNA in the nucleus. DNA then directs specific RNA synthesis which in turn is directly responsible for protein synthesis. An RNA virus, such as VEE, has no DNA, therefore its genetic information is stored in RNA, which directs the kind of protein that is to be made. There are new proteins induced by the virus after it is in the cell. The drug Actinomycin D inhibits the DNA-directed RNA synthesis. It does not affect RNA-directed RNA synthesis which may occur during viral infections. Thus, when Actinomycin D is added to infected cells and RNA and protein synthesis continue, or increase, it must be directed by viral RNA.

The data for protein synthesis in L-cells is presented in Figure 5. In this experiment, C^{14}-leucine was incubated with L-cells. One group of cells was infected with VEE and another served as control. Protein was

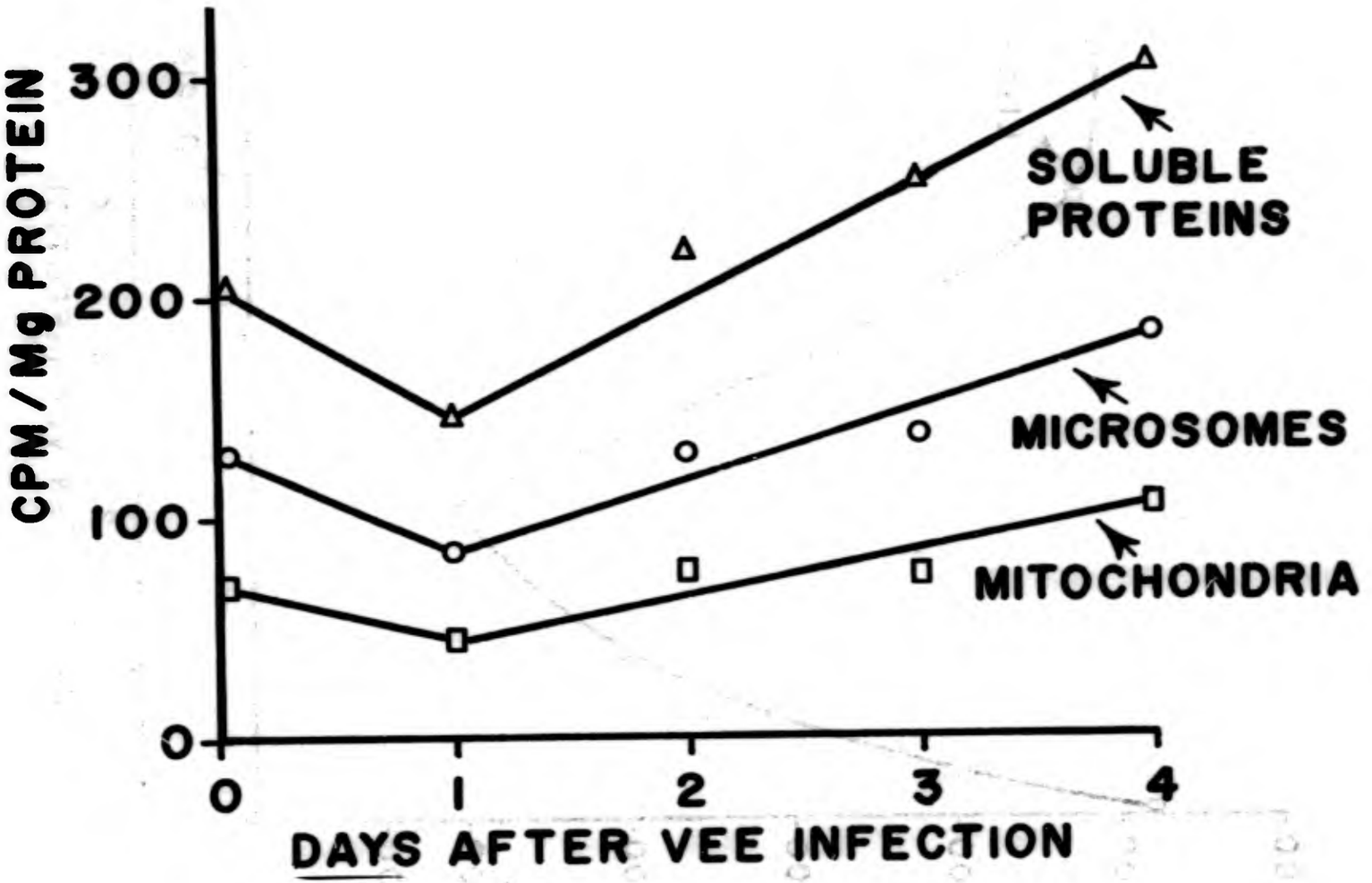

FIGURE 2. IN VIVO C^{14}-LEUCINE INCORPORATION INTO CELLULAR FRACTIONS OF MOUSE BRAIN.

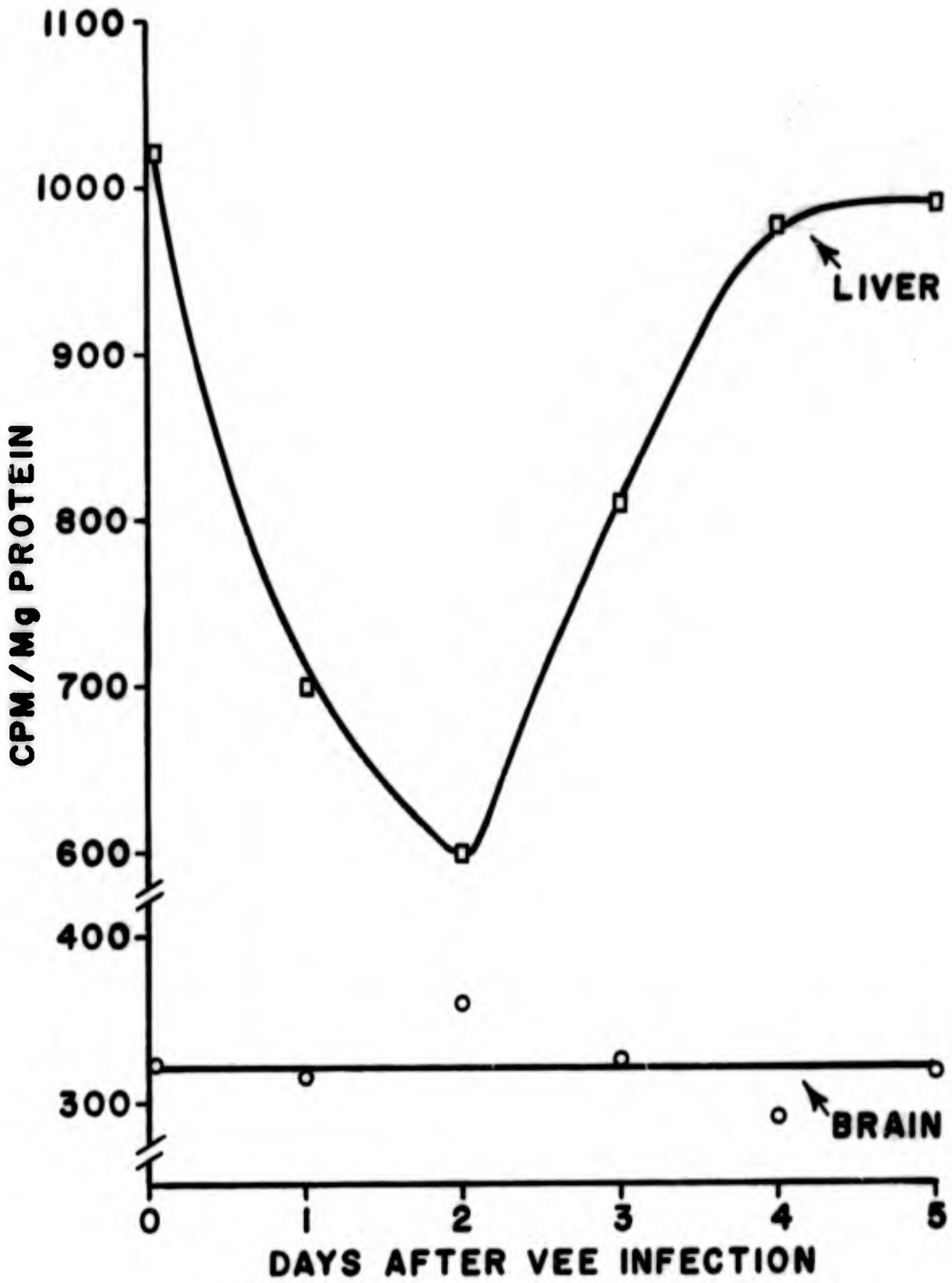

FIGURE 3. EFFECT OF VEE INFECTION ON MOUSE IN VITRO MICROSOMAL PROTEIN SYNTHESIS.

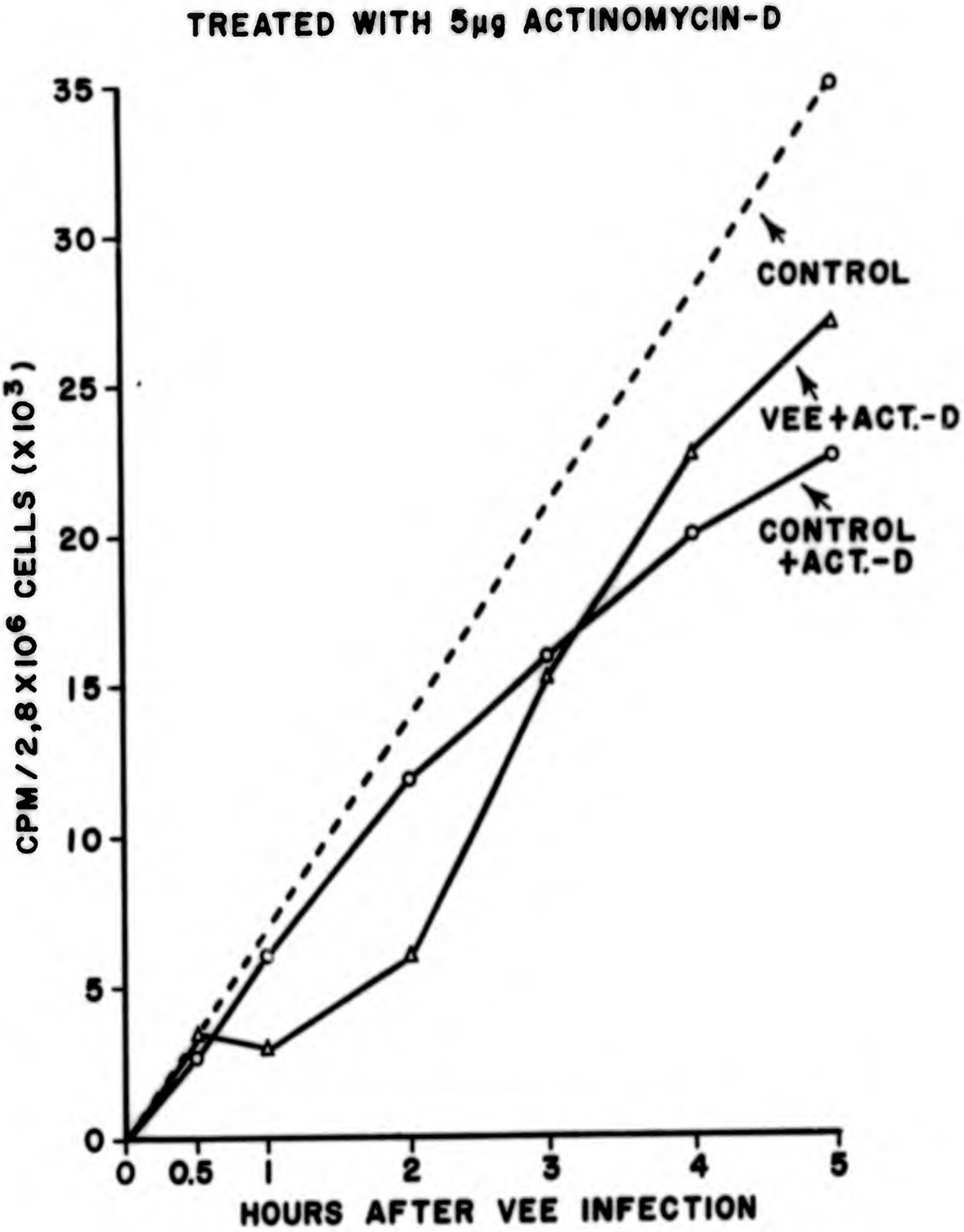

FIGURE 5. LEUCINE-ωC^{14} INCORPORATION INTO PROTEINS OF L-CELLS INFECTED WITH VEE.

precipitated with trichloroacetic acid (TCA), washed and counted as described earlier. Actinomycin D depresses protein synthesis 30% at 5 hr. It is not completely inhibited because the RNA present when the drug is added, continues to function, even though no new RNA is made. In the VEE-infected cells a definite decrease in protein synthesis was seen at 1 and 2 hours. The rate of protein synthesis then increases and is greater than the uninfected cells at 4 hr.

The L-cells were infected by adding 50-150 VEE particles per L-cell, in phosphate buffered saline. This insured that all cells were infected. The virus was allowed to attach 30 minutes, the excess virus was then removed by pipetting, and Medium 199 was added. "Time after infection" starts at this point.

In Figure 6 the pattern of RNA and protein synthesis is illustrated by "pulse" measurements. Thirty-minute "pulse" experiments were performed with whole L-cells using C^{14}-leucine to measure protein biosynthesis and uridine-2-C^{14} to measure RNA synthesis. All these cells were treated with Actinomycin D at a concentration of 1 μg/ml of medium. At each time indicated, the C^{14} precursor was added to the incubation mixture for 30 minutes. Both protein and RNA synthesis decreased after infection and both started to rise after 2 hr. A minimum apparently was reached between 1 and 2 hr, because after 3 hr the rate of synthesis increased and reached a maximum at 4 hr. Cellular RNA synthesis of uninfected cells was inhibited after addition of Actinomycin D. When no drug was added to the uninfected control cells both RNA and protein appeared as a straight, horizontal line. The VEE virus infection depressed rapidly both protein and RNA synthesis, irrespective of whether drug was present. In infected cells, 1 hr after infection both cellular RNA and protein synthesis were drastically reduced. The reason for the increase after 3 hr was that by that time new, virus-induced protein appeared and the new virus RNA began to appear. When no drug was added to the uninfected control cells both RNA and protein appeared as a straight, horizontal line. Since cellular RNA synthesis was inhibited by Actinomycin D the increased RNA production after the depression was probably viral RNA. The increased protein formation may be both early enzymes and virus-coat protein.

It was of interest to look closer at some of the specific changes in protein synthesis that occurred in the cell after infection. The term "early protein" or "early enzyme" has been used several times. This means proteins, specifically enzymes, which are not present in the cell prior to the introduction of virus and may include any enzyme required by the virus in its initial stage of replication. If the virus needs a specific compound for replication which is not normally in the cell, it may call forth an enzyme to make the compound. Thus when mammalian cells are infected with a RNA virus a new RNA polymerase is synthesized in the cytoplasm of the cell which is responsible for the synthesis of new viral RNA. This had been reported for Mengo virus by Baltimore and Franklin[1] and for polio virus by Baltimore,[2] Holland et al.[3] In part,

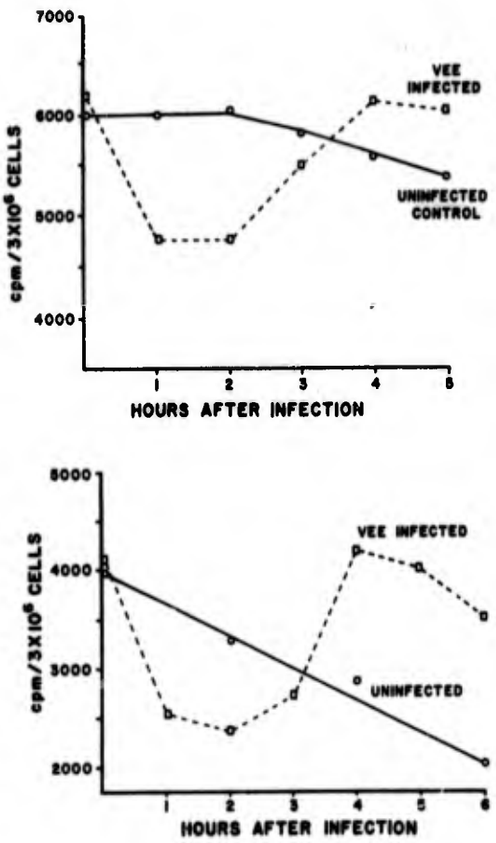

FIGURE 6. RNA & PROTEIN SYNTHESIS IN VEE-INFECTED L-CELLS.

we modeled our experiments after some of this work in an attempt to demonstrate a new RNA-directed RNA-polymerase during VEE infection. Cells infected for 4 hr, and uninfected L-cells, were homogenized and the nuclear and large microsomal fractions were isolated and used as the source of enzyme. The reaction mixture used to demonstrate the RNA-polymerase contained in 0.7 ml final volume, 20 µg pyruvic kinase, 10 µmole phosphoenolpyruvate, 100 µmole Tris buffer (pH 8.1), 10 µmole magnesium acetate, 0.1 µmole-UTP-2-C^{14}, and 40 µg each of ATP, CTP, and GTP. After incubating at 37 C for 15 min the enzymatic reaction was stopped by adding rapidly sodium pyrophosphate and 2 ml of 5% TCA. The precipitate was washed, dissolved in KOH and the radioactivity was measured as described earlier. RNA synthesis in normal cells occurred in the nucleus of the cell, and was DNA-dependent. However, upon virus infection this mechanism was somehow shut off and cytoplasmic RNA synthesis occurred. This latter is viral RNA.

When L-cells were infected with VEE for 4 hr a cytoplasmic RNA polymerase was demonstrated which was absent in normal cells (Table I). It may be seen

TABLE I. COMPARISON OF C^{14}-UTP INCORPORATION BY FRACTIONS OF UNINFECTED AND VEE-INFECTED L CELLS

INCUBATION MIXTURE	UNINFECTED		VEE-INFECTED	
	Nuclear (cpm/mg protein)	Microsomal	Nuclear (cpm/mg protein)	Microsomal
Regular	1470	630	685	2200
+ 15 µg DNase	125	100	80	2350
+ 5 µg Actinomycin D	185	130	100	2050

Cells were homogenized in 0.25 M sucrose plus 10^{-3} M $MgCl_2$ at 0-5 C. The nuclear fraction was centrifuged at 600 x g for 10 min and prepared as described by Baltimore and Franklin.[47] The mitochondria were removed at 8000 x g for 10 min. The large microsomal particles were obtained by centrifuging at 105,000 x g for 20 min. The reaction mixture was incubated with 2-3 mg protein. Protein was determined by the method of Lowry et al.[57] Unincubated blanks were subtracted from the counting rate.

in the table that microsomal polymerase was insensitive to Actinomycin D and DNase, in contrast to the nuclear RNA-polymerase which was present in normal cells. Nuclear enzyme was decreased 50% in VEE-infected cells and was sensitive to Actinomycin D + DNase. Both polio virus and mouse encephalomyocarditis virus replication had also been shown to occur in association with relatively large microsomal particles in the cytoplasm.

Some of the properties we found for the VEE-induced cytoplasmic RNA-directed, RNA-polymerase are shown in Table II. For optimal activity

TABLE II. PROPERTIES OF THE CYTOPLASMIC RNA POLYMERASE OF VEE-INFECTED L-CELLS

INCUBATION MIXTURE	UTP-2-C^{14} INCORPORATION (cpm/mg protein)
1. Complete	2150
- Mg^{++}	380
- PK, -PEP	1350
2. Complete	1950
- GTP, -CTP	650
- GTP, -ATP	680
- ATP, -CTP, -GTP	550
3. Complete	1560
+ 5 µg Actinomycin D	1430
+ 10 µg DNase	1600
+ 100 µg RNase	85

The large microsomal particles were isolated and incubated as before.

the enzyme required all 4 nucleotide triphosphates, Mg^{++}, and RNA. Since the TCA-precipitated product became TCA-soluble after hydrolysis with NaOH it appeared to be nucleic acid. The data presented were obtained using UTP-2-C^{14}. GTP-8-C^{14} and ATP-8-C^{14} were similarly utilized. All attempts to purify the induced enzyme further, have been unsuccessful to date. The enzyme was very unstable and became inactivated by freezing overnight at -20 C, or by standing at 5 C for 40 min. Sonication, sodium deoxycholate and protamine sulfate, procedures which separate RNA from proteins, also inactivated the microsomal preparation, and the enzyme activity could not be restored by the addition of exogenous RNA.

SUMMARY

It was found that in vivo liver protein synthesis is depressed early in VEE virus infection and subsequently increases above uninfected control levels. The same trend was found in liver microsomal protein synthesis experiments; the increase returning only to the control value. The effects in the brain were less pronounced.

In mouse L-cells in tissue culture studies it was possible to see the effects again, but under more controlled conditions. By inhibiting host RNA synthesis with Actinomycin D we could show clearly the appearance of

virus-induced protein and virus RNA. Specifically we demonstrated the appearance of a new RNA polymerase in the cytoplasm of the cell which was probably involved in the synthesis of virus RNA. This enzyme proved to be very difficult to work with, since it was apparently highly labile.

Presently, experiments are in progress to purify the enzyme further and to attempt to demonstrate the presence of the new polymerase in VEE-infected mouse liver and brain.

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PROTEIN SYNTHESIS BY LIVER CELL COMPONENTS OF MICE INFECTED WITH DIPLOCOCCUS PNEUMONIAE

David G. Van Ormer, 1st Lieutenant, MSC*

Protein biosynthesis occurs in at least 3 areas of the cell: the nucleus, the mitochondria, and in the ergastoplasm of the microsomal fraction. The mechanism of microsomal protein synthesis, subsequent to the genetic stages, has been recognized for several years[1,2] to be as shown in the following formulae:

1. $AA + ATP + sRNA \underset{}{\overset{\text{amino acyl synthetase}}{\rightleftharpoons}} \text{amino acyl-sRNA} + AMP + PP_i$

2. $\text{amino acyl-sRNA} + \text{polyribosome} \underset{}{\overset{\text{transfer enzyme, GTP}}{\rightleftharpoons}} \text{amino acid-ribosome} + sRNA$

The second reaction, the ribosomal reaction, is illustrated diagrammatically in Figure 1. We see that the messenger RNA (mRNA) serves as a kind of track on which the ribosome, the large sphere, "rolls" along at a speed (in the reticulocyte) of about 0.004 inches/hr.[3] The illustration shows the same ribosome in 2 positions. At each of these positions a particular amino acyl-sRNA complex attaches itself to both ribosome and mRNA. A sequence of 3 bases on the mRNA determines which amino acyl-sRNA attaches at that position of the ribosome. The amino acid is added to the peptide chain, the sRNA leaves to pick up another amino acid, and the ribosome moves along to another position where another amino acyl-sRNA can attach. A ribosome making a hemoglobin strand requires about one minute to traverse a strand of mRNA. In reticulocytes there are at any one time about 5 ribosomes per "strand" or per polyribosome.

At the Commission meeting in 1964 Lt Colonel Beisel expressed the hope that studies of biochemical changes within host organs might lead to a more detailed description of the host response to an infectious agent. In this connection it has been possible to confirm earlier reports that viral[4,5] and, particularly, bacterial[6,7] infections, cause increases in protein synthesis in some of the viscera. The studies presented here also show that this increased activity occurs in both mitochondrial and microsomal liver-cell fractions of mice infected with Diplococcus pneumoniae.

Male, white mice (CD-1 strain, 25-35 gm) were maintained on ordinary laboratory chow at least a week before use. Under the dorsal skin of each mouse were injected 3-4 organisms of D. pneumoniae type I (strain A_5)

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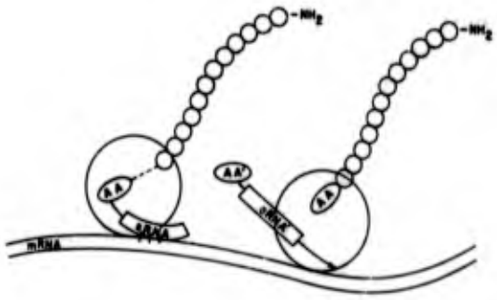

FIGURE I. DIAGRAM OF MICROSOMAL PROTEIN SYNTHESIS.

contained in 0.5 ml of tryptose-phosphate broth (pH 7.8). The mice then were fasted but were allowed water. Eighteen, 42 and 66 hr later 4 infected and 4 control mice were injected via the tail vein with 1.0 μc of L-leucine-C^{14} in 0.1 ml of physiological saline. The specific activity of the leucine was 5.0 μc/μmole. The tissue was minced in ice-cold 0.25 M sucrose and homogenized as 10% (w/v) suspension by using 6 passes of the Teflon pestle of a Potter-Elvehjem apparatus. Differential centrifugation of the resulting homogenate with the No. 40 rotor of the Spinco Model-L ultracentrifuge yielded the cellular fractions. The mitochondria were removed at 15,000 x g for 15 min and the microsomes at 100,000 x g for 90 min. To remove glycogen, the pellets were rinsed with water. Stirring the pellets with 2 ml of ice-cold, 5% trichloroacetic acid (TCA) precipitated the mitochondrial and microsomal protein, while the supernatant or soluble fractions and the total homogenate samples were precipitated with equal volumes of 10% TCA. The protein precipitates were washed by stirring with 2 ml of ice-cold ethanol-ether (2:1, v/v). The dried protein samples dissolved in 3 ml of 1 N KOH to provide convenient aliquots for biuret-protein determinations and for liquid scintillation counting in Bray's solution.[8]

Figure 2 shows the C^{14}-leucine incorporation as per cent of the corresponding control value versus the time of liver excision. (The mice rarely lived longer than 72 hr after injection.) During the first 42 hr after D. pneumoniae injection, leucine incorporation increased in all fractions. At 66 hr incorporation was greatest in the mitochondrial fraction, followed by the total homogenate, soluble fraction, and microsomal fraction. The points on the graph represent averages for 2 separate infected groups.

Infected mice which were not deprived of food exhibited feeding rates differing appreciably from control mice. An attempt to remove this nutritional effect utilized mice pair-fed with their controls. Possibly due to erratic feeding characteristics of mice, the data from this attempt allowed no definite conclusions concerning hepatic protein synthesis during semistarvation.

There is no direct explanation for the observed increased liver activity. One rather obvious possibility is that there is an increase in mitochondrial-generated ATP.[9] The catabolic hormones, thyroxine and cortisone, and the stimulators of ACTH, such as adrenalin and insulin, also accelerate liver protein synthesis.[10] Growth hormone increases liver protein. A study of regenerating liver, which presents a pattern of protein synthesis resembling the effect of thyroid stimulation, may help explain liver protein synthesis during infection.[11] In the case of thyroxine, in vitro experiments indicate that acceleration of protein synthesis occurs at the step involving transfer of sRNA-bound amino acids to microsomal protein.[12] In the case of hydrocortisone, on the other hand, acceleration of protein synthesis is preceded by stimulation of RNA synthesis.[13]

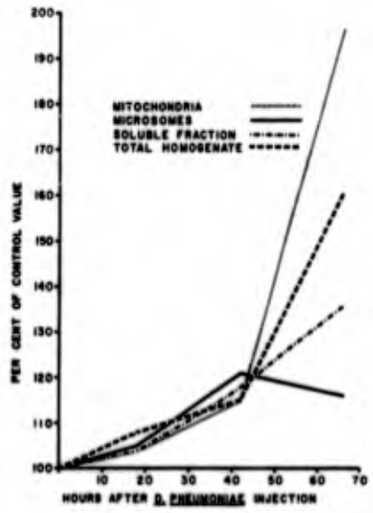

FIGURE 2. IN VIVO INCORPORATION OF L-UC^{14} LEUCINE IN LIVER-CELL COMPONENTS OF MICE INFECTED WITH D. PNEUMONIAE.

SUMMARY

The findings show increased liver mitochondrial and microsomal protein synthesis as a result of D. pneumoniae infection.

ACKNOWLEDGMENTS

This work has involved the efforts of several individuals at all levels. Acknowledgment is given particularly to 1st Lt James W. Higbee, MSC, for providing the D. pneumoniae organisms and Mrs. Lorene L. Neville for performing much of the technical work.

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ALTERATIONS IN TISSUE ALKALINE PHOSPHATASE ACTIVITY DURING ACUTE INFECTION

George Lust, Captain, MSC*

The intracellular function of alkaline phosphatase is presently not understood. The enzyme is found in high concentrations in the small intestine, bone, and kidney; other tissues have appreciably less, with brain and muscle having very low activity. Changes in serum activity of alkaline phosphatase have, of course, had extensive diagnostic application in medicine, since the serum activities are elevated in diseases affecting the skeletal system and the hepatobiliary system.

It has been known for approximately 20 years that the alkaline phosphatase content of neutrophilic leukocytes will vary in certain disease states. The existing literature implies that various conditions associated with a neutrophilic leukocytosis are characterized by increased alkaline phosphatase activity within the neutrophils. In patients with leukemia the leukocytic alkaline phosphatase activity appears to be more variable, being markedly depressed in some cases and elevated in others. Generally, in these studies there was no correlation between the serum and the leukocytic alkaline phosphatase activity.

Changes of tissues as well as serum alkaline phosphatase levels during acute bacterial and viral infection have received relatively little attention. Because virtually nothing was known concerning the timing of the change of leukocytic alkaline phosphatase with respect to the timing of onset of symptoms and fever during acute infectious disease, serial studies of leukocyte alkaline phosphatase activity were initiated in volunteer subjects during studies of infection, reported by Beisel in the 1964 report to the Commission. The changing content of alkaline phosphatase can be quantitated either chemically or histochemically. A histochemical technique was used in which all serial blood smears from a given patient were stained simultaneously after completion of hospitalization. A modification of a standard Gomori staining technique was used.

Changes can be seen in association with different amounts of alkaline phosphatase in the neutrophils. With this stain the enzyme appears as dark discrete granules. Leukocytes with no alkaline phosphatase activity fail to show dark granules. Increasing activity can be graded on a 1 to 4 scale in individual cells, with a 4 exhibiting intense cytoplasmic blackening. If 100 white blood cells (WBC) are counted and given a rating the alkaline phosphatase index for a given blood smear is obtained; this could range from 0-400.

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In Figure 1 the hematologic response is shown compared to the febrile response in a large group of subjects during acute tularemia. In this group of men the day of maximum fever was aligned for all subjects and the data plotted as changes which occurred prior to, and following the day of maximum fever. Note at the bottom that with the onset of symptoms and fever there developed an increased number of adult neutrophils, although the average total WBC count did not exceed the normal range. Development of fever was also accompanied by an increased appearance of band forms which reached a maximum several days following insitution of therapy. In the center it can be seen that the alkaline phosphatase index showed a progressive increase with the onset of acute illness. Significant elevations of alkaline phophatase over controls are shown.

Figure 2 shows a similar pattern in subjects exposed to sandfly fever virus. Here, a neutrophilia occurred, although there was a shift to the left and a very early abrupt appearance of increased alkaline phosphatase activity, which occurred despite the neutrophilic leukopenia. This was associated with an increased number of band forms. Following recovery, restitution of the WBC count to its normal limits was associated with a second appearance of band forms which did not occur in association with an increase in the alkaline phosphatase index.

Figure 3 shows the alkaline phosphatase index as it developed in a group of subjects accidentally exposed to aerosolized staphylococcal enterotoxin B. This group experienced considerable leukocytosis with a shift to the left and a simultaneous prolonged increase in alkaline phosphatase activity.

In Figure 4 it may be seen that leukocytosis and a shift to the left did not necessarily involve an increase in alkaline phosphatase activity when volunteers were subjected to artificial hyperthermia. The alkaline phosphatase content of the leukocytes in this case did not show any changes.

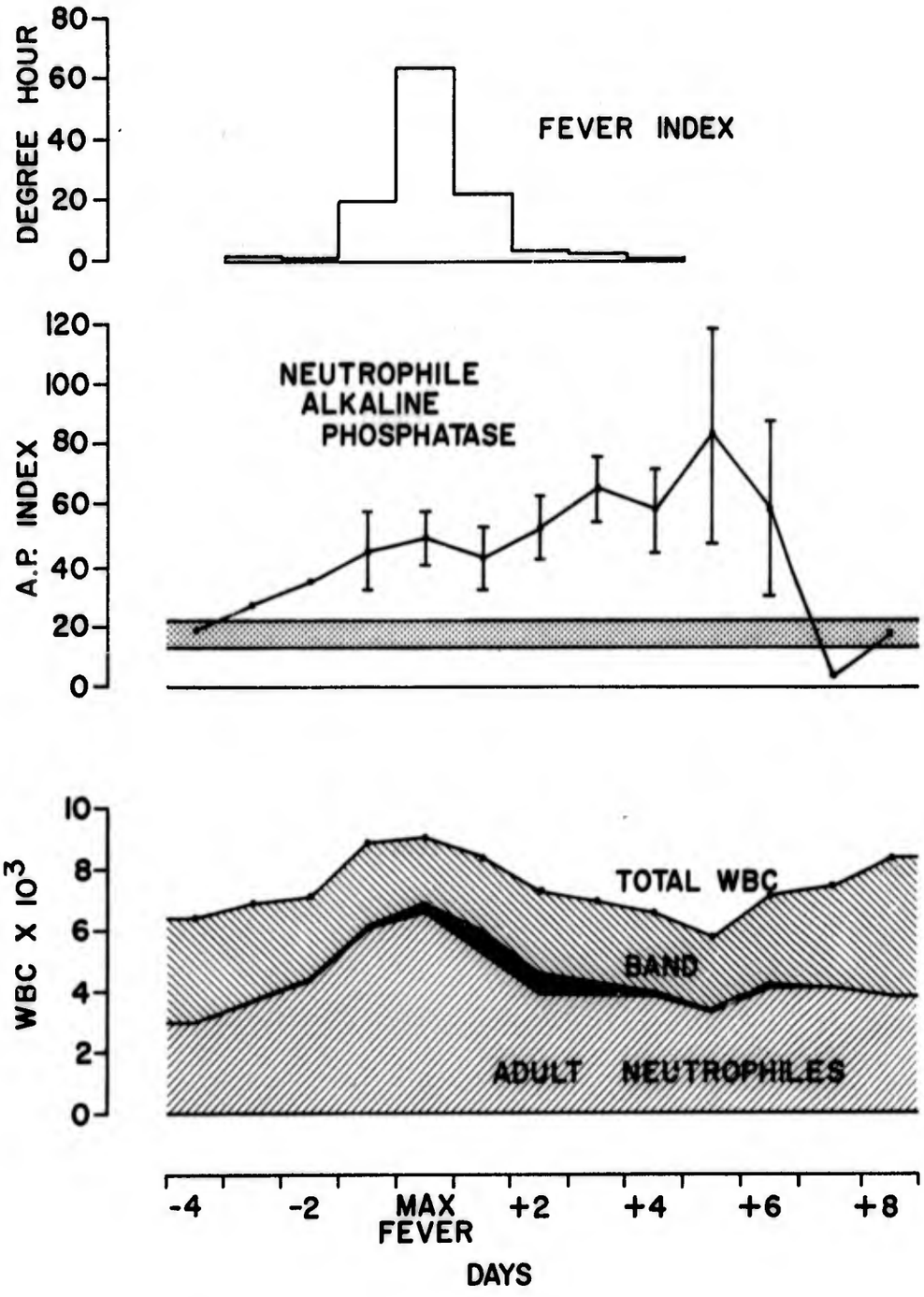

FIGURE 1. NEUTROPHILE ALKALINE PHOPHATASE RESPONSE.
TYPICAL ACUTE TULAREMIA.

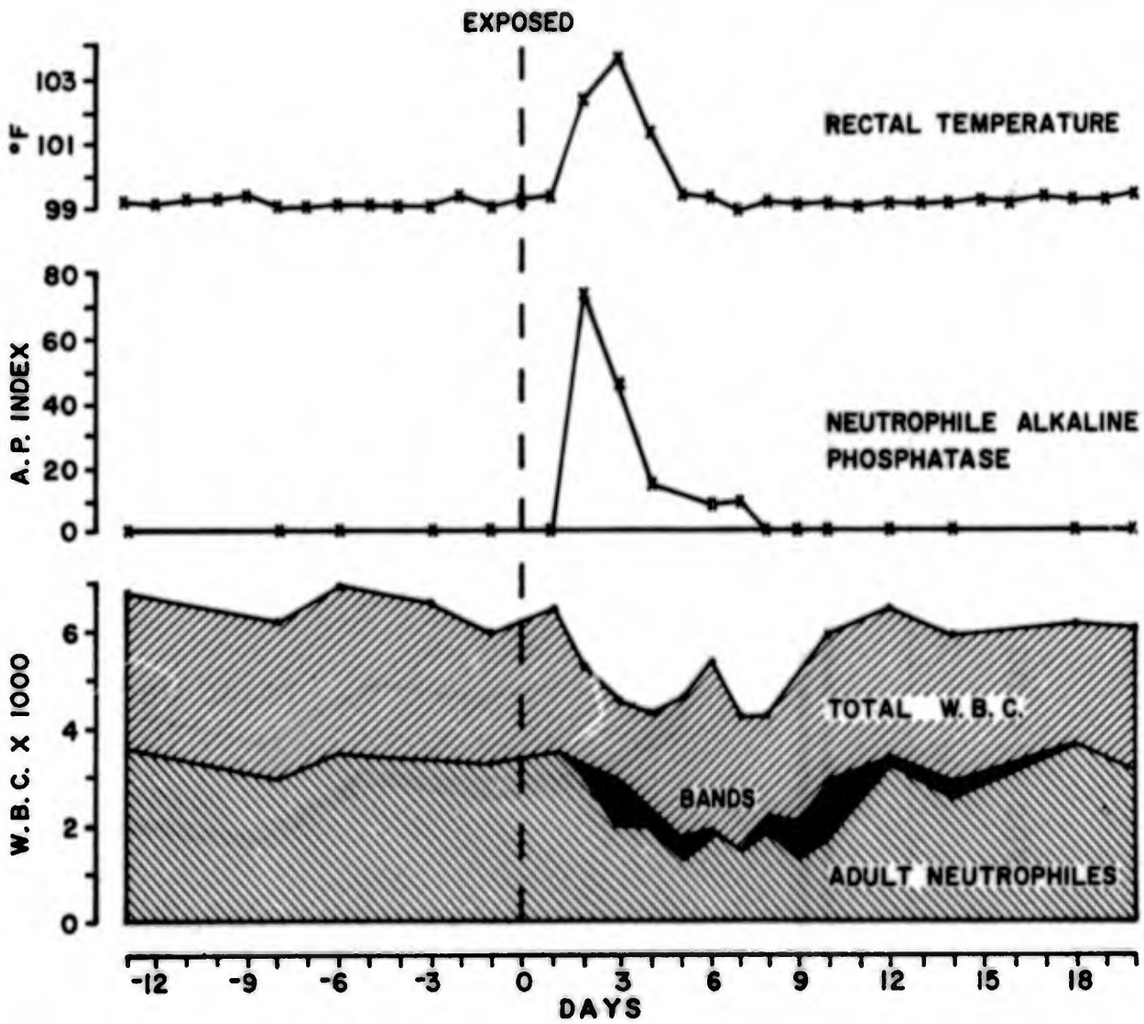

FIGURE 2. NEUTROPHILE ALKALINE PHOSPHATASE RESPONSE. SANDFLY FEVER.

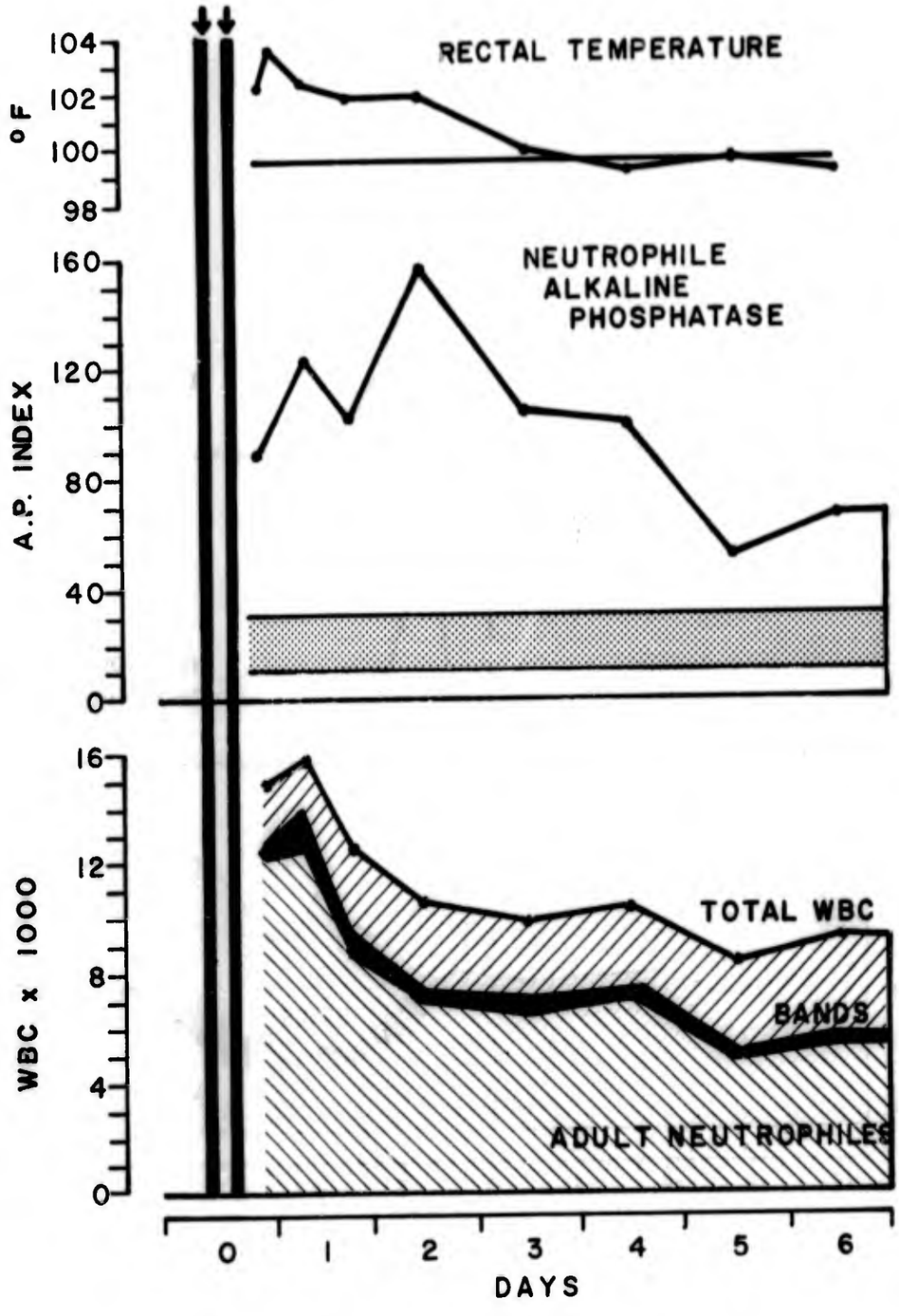

FIGURE 3. NEUTROPHILE ALKALINE PHOSPHATASE RESPONSE. ACCIDENTALLY AEROSOLIZED SEB IN 9 SUBJECTS.

With these clinical observations on leukocyte alkaline phosphatase as a background, investigations were carried out more specifically on the mechanism of action involved which results in an increase in alkline phosphatase activity in leukocytes as well as in other tissues. Mice and guinea pigs were used. Studies are now in progress to help explain the increase in alkaline phosphatase activity in WBC and to relate these changes to those occurring in other tissues. Data are presented on alterations of alkaline phosphatase activities in mouse tissues and serum during an acute bacterial infection and two viral diseases.

Groups of white mice were injected subcutaneously in the back with 5-12 organisms of Diplococcus pneumoniae (Type I, Strain A_5). This infectious dose caused death within 72 hr. At 16, 40, and 64 hr, groups of 10 mice were killed and the necessary tissues excised. These tissues were homogenized in 0.25 M sucrose and then centrifuged at 12,000 x g for 15 min to remove cell debris and mitochondria. The supernatant liquid contained only microsomes and was used as the enzyme solution. Virtually all the alkaline phosphatase within the cell was associated with the small particulate fraction.

The enzyme assay was patterned after the method of Shinowara et al[1/] with beta glycerol phosphate as the substrate in Veronal buffer at pH 9.5. Inorganic phosphate was determined in each case.

Figure 5 depicts the reactions that are catalyzed by alkaline phosphatase. Reaction I is a general reaction. Reactions II and III were used to assay for enzyme. Inorganic phosphate may be determined in each case. In Reaction III the para-nitrophenolate ion can be measured directly at 410-420 mμ since it is yellow.

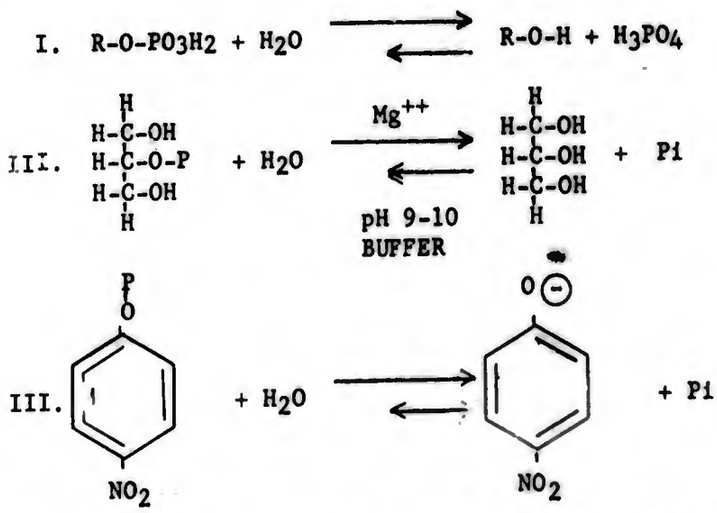

FIGURE 5. REACTIONS CATALYZED BY ALKALINE PHOSPHATASE

In Table I are presented the changes of alkaline phosphatase in various mouse tissues and serum after infection with D. pneumoniae. The tissues are arranged according to decreasing enzymatic activity. The results are

TABLE I. ALKALINE PHOSPHATASE ACTIVITY IN TISSUES OF MICE INFECTED WITH D. PNEUMONIAE

TISSUES	ACTIVITY[a] BY HR ($\times 10^{-2}$)			
	Control	16	40	64
Small intestine	52.2	66.5	74.3	89.0
Femur	7.32	8.83	7.70	8.17
Sternum	6.45	6.35	6.63	5.87
Kidney	3.47	3.89	3.50	3.45
Liver	0.752	0.645	0.580	0.477
Lung	0.302	0.280	0.307	0.274
Spleen	0.300	0.313	0.282	0.277
Serum	0.110	0.115	0.129	0.155

a. µmole Pi/min per mg protein.

expressed in international enzyme units, µmoles inorganic phosphate liberated/min per mg protein. Under the experimental conditions used no change in alkaline phosphatase was found in the femur, sternum, kidney, lung, and spleen. However, alterations were found in the small intestine, liver and serum (Figure 6).

A 40% decrease from the control level occurred in the liver at 64 hr. In the serum the enzyme level increased 30%. The greatest change, however, was found in the small intestine where a 70% increase in activity was found. The control value obtained from the uninfected animals during each experiment was arbitrarily assigned a value of 100. The changes are thus expressed as per cent of control value.

In an attempt to ascertain the origin of the serum enzyme some organ-specific inhibition studies were done. The effect of various inhibitors on alkaline phosphatase activities of the mouse liver, small intestine, and serum are presented in Table II. In particular, the following compounds should be emphasized: Zn^{++} inhibited liver, but not intestinal enzyme activity.

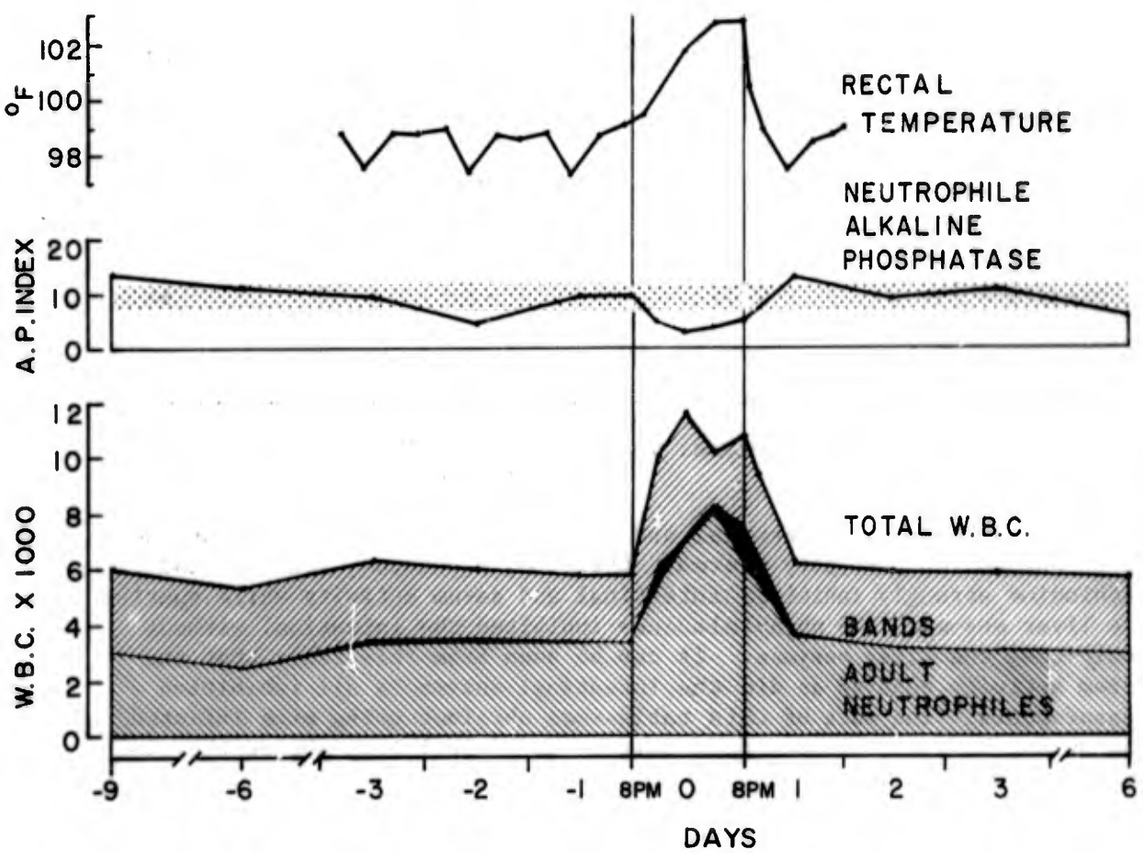

FIGURE 4. NEUTROPHILE ALKALINE PHOSPHATASE RESPONSE. PHYSICALLY-INDUCED HYPERTHERMIA IN 8 NORMAL SUBJECTS.

TABLE II. EFFECT OF VARIOUS INHIBITORS ON ALKALINE PHOSPHATASE ACTIVITIES OF 2 MOUSE TISSUES AND SERUM

INHIBITOR	CONCENTRATION M	% INHIBITION[a] BY		
		Liver	Serum	Intestine
$AlCl_3$	0.01	96	94	96
NaF	0.01	6	7	3
NaCN	0.005	37	98	98
$ZnCl_2$	0.01	88	6	9
L-cysteine	0.005	43	88	93
L-histidine	0.01	53	89	90
L-leucine	0.01	49	86	89
L-methionine	0.01	55	90	90
DL-phenylalanine	0.01	8	58	64
DL-fluorophenylalanine	0.01	10	62	65

a. % inhibition is expressed as: $100 = \frac{(IU + inhibitor)}{(IU - normal)} \times 100.$

Cyanide inhibited the intestinal and serum activities strongly, but the liver enzyme was partially spared. Cysteine, histidine, leucine, and methionine strongly inhibited intestinal and serum activity while sparing the liver enzyme. The phenylalanines inhibited the intestinal without inhibiting the liver enzymes. It can be seen from this table that the serum activity behaved as did the intestinal enzyme in all inhibition measurements. The data of this table were obtained using mice infected for 60 hr; however, tissues from uninfected control mice showed the same patterns of inhibition.

The influence of infection upon in vivo intestinal protein synthesis is shown in Figure 7. Here, C^{14}-leucine was injected into the veins of 4 mice and the intestinal microsomes were isolated. The results are expressed as counts per minute per milligram microsomal protein (cpm/mg). It can be seen that the rate of C^{14}-leucine incorporation into proteins is greater in infected mice. The rate decreases before death, but still remains elevated over the control values as the infection becomes terminal.

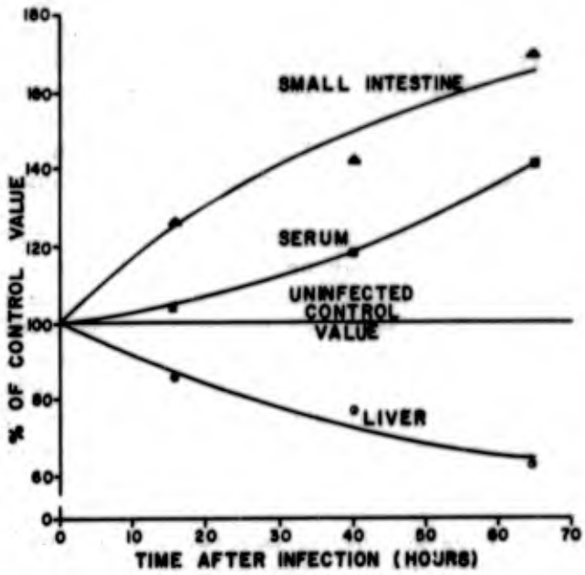

FIGURE 6. ALTERATION OF ALKALINE PHOSPHOMONOESTERASE ACTIVITY IN D. PNEUMONIAE-INFECTED MOUSE TISSUES AND SERUM.

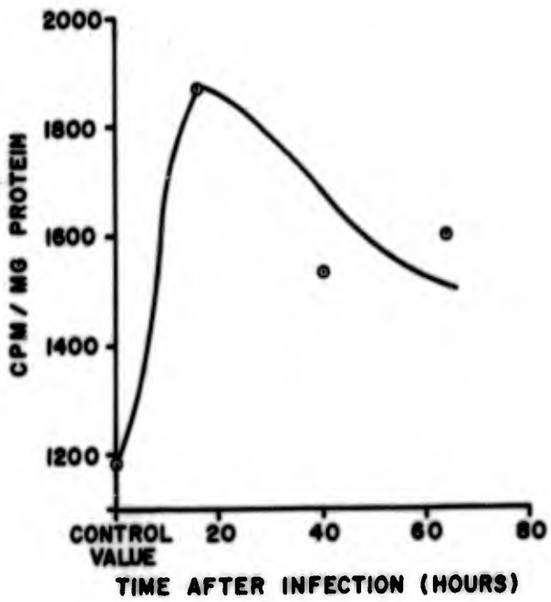

FIGURE 7. IN VIVO INCORPORATION OF C^{14}-LEUCINE INTO INTESTINAL MICROSOMAL PROTEINS AFTER D. PNEUMONIAE INFECTION.

To review the effect of this bacterial infection the major change was an increase in intestinal alkaline phosphatase. This seemed not to be the result of excretion of alkaline phosphatase in bile, for despite an apparent increase in bile production no increase in bile alkaline phosphatase could be detected. Furthermore, the enzymatic activity decreased in the liver as the infection progressed. On the basis of the inhibition studies it appeared that the slight increase in serum enzyme represented alkaline phosphatase arising in the gut mucosa. The increased quantity of bile may have been partially dissolving the intestinal enzyme in greater quantity. In support of this concept it had previously been shown in rats by Fishman et al[27] that serum alkaline phosphatase of intestinal origin is markedly decreased in the serum following bile duct ligation.

It is known that the intestinal mucosal cells have a turnover time second only to the leukocytes. It may be that the observed increase in the intestinal activity is related in some manner to a more rapid cell turnover during infection. This apparent pattern may be similar to the elevated leukocyte alkaline phosphatase associated with increased WBC production in infection. The rate of intestinal mucosal cell turnover was not measured directly; by another approach it was shown that the rate of C^{14}-leucine incorporation into proteins by intestinal mucosal cells was greater during infection.

It was of interest to know whether a general trend could be seen with respect to type of disease and host in the enzymatic alterations. An investigation was carried out on the effect of an acute virus infection on mouse tissue alkaline phosphatase. Mice were infected intraperitoneally with 3,000 median lethal doses (LD_{50}) of Venezuelan equine encephalomyelitis virus, and the liver, small intestine, and serum were obtained as before. The results of this experiment are presented in Figure 8. During Venezuelan equine encephalomyelitis infection the liver activity decreased after an initial increase. Again, the serum and intestinal enzyme followed a similar pattern although in the opposite direction from that of the bacterial disease.

In Figure 9 data are presented which show the trends obtained for alkaline phosphatase in the same tissues of chickens infected with Newcastle disease virus. This experiment was performed in conjunction with Dr. Robert Squibb from Rutgers University (Contract No. DA-49-193-MD-2694). The most drastic changes were observed here. The serum and intestinal levels again were parallel but were more depressed during the infection. It may be noted that the effect of the 2 viral diseases, although in different hosts, was to decrease the intestinal and serum activities. In the bacterial infection the trends observed were the reverse.

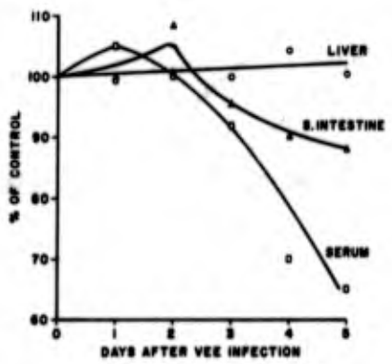

FIGURE 8. CHANGES IN ALKALINE PHOSPHATASE IN VEE-INFECTED MOUSE TISSUES AND SERUM.

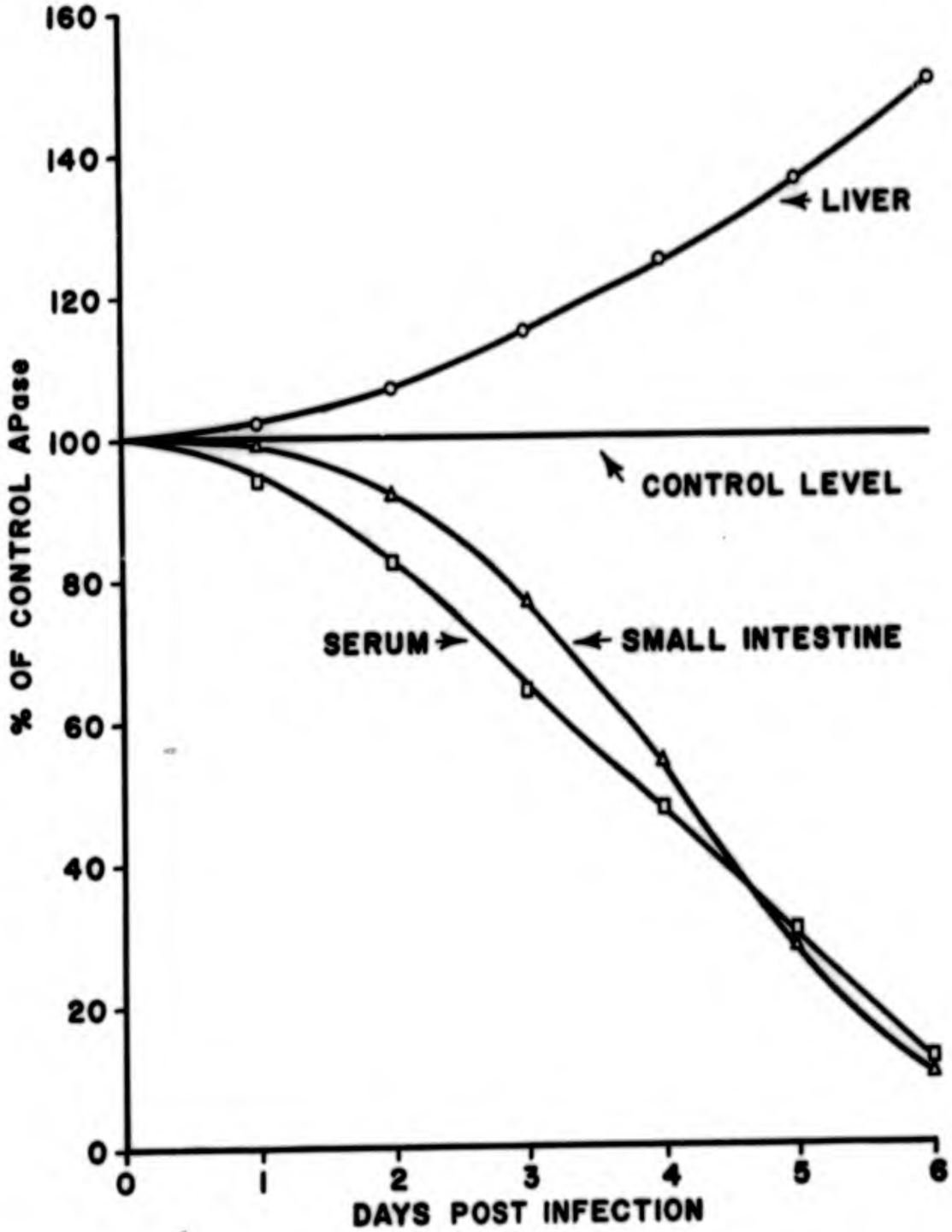

FIGURE 9. ALTERATION OF ALKALINE PHOSPHATASE IN NEWCASTLE DISEASE-INFECTED CHICKEN TISSUES AND SERUM.

SUMMARY

While these studies still leave the specific intracellular function of alkaline phosphatase unanswered, they nevertheless provide additional information that alkaline phosphatase alterations do occur in tissues during acute bacterial and viral infection. The exact mechanism responsible for the changes must await additional investigations.

ACKNOWLEDGEMENTS

The very able technical assistance of Sp4 Arthur Kanna is gratefully acknowledged.

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DETECTION OF PASTEURELLA PSEUDOTUBERCULOSIS ANTIBODIES BY MICROHEMAGGLUTINATION

John D. Marshall, Jr., Major, MSC, and Julius A. Currie*

The widespread distribution of Pasteurella pseudotuberculosis in nature was recognized by Klein in 1899,[1] who demonstrated its presence in the soiled water of two rivers. Since that time, natural epizootics have occurred in rodents,[2-6] fowl,[7,8] and primates.[9] An epidemic in humans was reported by Mollaret and Berthon.[10] Several investigators [11-13] have protected animals against virulent Pasteurella pestis infection by immunization with avirulent P. pseudotuberculosis. This is good evidence of the close immunogenic relationship of these species. A corollary may be inferred in nature by the work of Deoras,[14] who found that plague-resistant Bombay rats had antibody against P. pseudotuberculosis, while the susceptible country rats did not. Simultaneous isolation has occurred of P. pseudotuberculosis and P. pestis from plague foci. This causes one to ponder what role, if any, this organism may have in producing a plague-resistant rodent group in what normally would be expected to be a uniformly susceptible population.

The complex antigenic interrelationships that exist between these 2 organisms would not complicate diagnostic differentiation if the disease syndromes were different, or if the physiological differentiation of the causative organisms were mutually exclusive. The fulminating acute septicemia of P. pseudotuberculosis in guinaepigs is pathologically indistinguishable from septicemic P. pestis infections in the same animal. Chronic P. pseudotuberculosis infection in the susceptible animal and in man is characterized by a transient, gastric enteritis, complicated by a mesenteric lymphadenitis, resembling acute appendicitis[15] or Salmonella infection.[16]

Identification of P. pseudotuberculosis and P. pestis is based on an antigenic analysis of no less than 20 antigens. The majority of strains of both species share at least 18 of these factors.[17-20]

An attempt to type P. pseudotuberculosis based on their somatic antigens was proposed.[21-23] The designations were based on the heat stable antigens and were referred to as lipopolysaccharides (LPS).

Thal's scheme[22] for the antigenic typing of P. pseudotuberculosis was divided into 5 groups with numerical listings of type specific antigens 1-10, the various types having different combinations of these antigens.

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Davies[24] and Crumpton et al[25] showed that there is a close relationship if not identity, between antigen 1 of P. pseudotuberculosis and antigen LPD of the plague bacillus. Antigen 1 or LPS is described as a rough somatic factor. Common factor 1 is not to be confused with fraction 1, which is the major virulence and protective antigen of many strains of P. pestis, and of which P. pseudotuberculosis is devoid.

Table I is a review of the somatic factors involved in the various types of P. pseudotuberculosis. There are several shared factors, plus factor 1, which is common to all types and the plague bacillus. Common factor 1 has been the cause of much trouble in typing P. pseudotuberculosis. Antigen 1 is the so-called common antigen of Davies and perhaps should be parenthetically enclosed to denote its possible absence or masking in smooth strains of P. pseudotuberculosis. In preparing a typing kit, it is desirable that smooth strains rich in type-specific factors be employed.

TABLE I. ANTIGENIC CONSTITUTION OF P. PSEUDOTUBERCULOSIS[23]

TYPE	SUBTYPE	SOMATIC ANTIGEN	FLAGELLAR ANTIGEN
1	1A	1, 2, 3	A
	1B	1, 2, 4	A
2	2A	1, 5, 6	A
	2B	1, 5, 7	A
3		1, 8	A
4		1, 9	B
5		1, 10	A
P. pestis LPS		1	

The LPS preparations of the different types of P. pseudotuberculosis were prepared from smooth colonies. It was determined very early in the investigations that this was a very important step in obtaining type-specific results. The cells for preparing type-specific antigens were grown on suitable media, harvested, and autoclaved for 15 min, centrifuged and the supernatant fluid precipitated with ethyl alcohol.

Reference antisera were prepared by injecting 20 μg of antigen intravenously for 5 consecutive days. On the 8th day, animals were trial bled and tested for antibody titers by the hemagglutination (HA) technique.

This rapid immunization schedule, usually produced high titered specific antiserum in 3 days, or 8 days after beginning immunization. Individual rabbit responses resulted in titers ranging from 1:1024 to 1:8192.

The amount of sensitizing antigen necessary for maximum sensitization ranged from 5-37 μg and differed with each type of LPS preparation and each lot.

PERFORMANCE OF THE HA TEST

Standard procedures were followed for the sensitization of erythrocytes; they were then diluted with buffered saline to give a final concentration of 1% sensitized erythrocytes/ml of saline.

The microtiter technique was employed throughout these investigations. Antisera for testing were diluted 2-fold in buffered saline in parallel rows. Sensitized erythrocytes (0.025 ml) were added to each dilution; the same amount of nonsensitized erythrocytes were added to each dilution of the control row. Other necessary controls were included. All were incubated at 37 C in a sealed moist chamber for 30 min. Patterns of readings were based on agglutinated erythrocytes. Table II shows the crossing and

TABLE II. COMPARISON OF ANTISERA PRODUCED AGAINST LPS DERIVED FROM ANTIGENICALLY SMOOTH, ROUGH, AND MIXED CULTURES OF *P. PSEUDOTUBERCULOSIS*, TYPES 3 AND 4

ANTISERUM	TYPE	ABSORBED WITH	RECIPROCAL TITER Sensitized LPS of:				
			P. psuedotuberculosis[a]				
			3R	3S	4R	4S	*P. pestis*
P. pseudotuberculosis	3SR	-	512	2048	512	128	64
	3SR	*P. pestis*	—[b]	2048	-	-	-
	3S	-	-	2048	-	-	-
	4R	-	512	128	4096	256	128
	4R	*P. pestis*	-	-	32	-	-
	4S	-	-	-	-	4096	-
P. pestis			64	-	64	-	512

a. R = Common antigen predominates.
SR = Mixed common and specific antigen.
S = Specific antigen predominates.

b. Negative at 1:16.

sharing of common antigens of P. pseudotuberculosis types 3 and 4 and of the plague bacillus. It can be seen that when one absorbed antiserum containing common antigen "R" antibody with P. pestis cells, the titer of 3SR antiserum dropped from a 1:512 to negative. The same was seen with 4R antiserum when a specific homologous titer of 1:4096 dropped to 1:32 when absorbed with P. pestis. It can be seen that 4S antiserum vs. 4S antigen gives a 1:4096 titer, yet is negative against 4R and P. pestis antigens. This lends further emphasis to the necessity for selecting smooth strains of P. pseudotuberculosis for production of type-specific antisera.

Table III shows the antigenic relationship of P. pseudotuberculosis types, P. pestis, and the shared somatic factors of Salmonella Groups B and D.

TABLE III. ANTIGENIC RELATIONSHIPS OF P. PSEUDOTUBERCULOSIS AS DETERMINED BY HA OF LPS-SENSITIZED SHEEP RBC

		RECIPROCAL TITER									
		Sheep RBC sensitized with LPS of:									
ANTISERUM TYPE		P. pseudotuberculosis type							P. pestis	S. typhimurium	S. typhosa
		1A	1B	2A	2B	3	4	5			
P. pseudotuberculosis	1A	4096	1024	-[a]	-	-	-	-	-	-	-
	1B	512	2048	-	-	-	-	-	-	-	-
	2A	-	-	8192	512	-	-	-	64	64	-
	2B	-	32	2048	4096	-	-	-	64	32	-
	3	-	-	-	32	2048	-	-	-	-	-
	4	-	-	-	-	-	4096	-	64	-	128
	5	-	-	-	-	-	--	8192	32	-	-
P. pestis		-	-	-	-	-	-	-	512	-	-
S. typhimurium		-	-	512	128	-	-	-	-	512	-
S. typhosa		-	-	-	32	-	512	-	-	-	2048

a. Negative at 1:16.

It became apparent early in our investigations that there was a desperate need for a polyvalent HA test. With the use of such a test it would be possible to screen antisera with a one-step procedure; any suspicious sera could then be tested individually. Such a procedure has been worked out; it consists

primarily of preparing a polyvalent antigen by taking equal quantities of all 7 types of P. pseudotuberculosis, rich in antigenic factors 2-10, mixing and using to sensitize erythrocytes. The polyvalent HA test looks promising.

Table IV shows the results obtained with such a test, utilizing type-specific antiserum as a control.

TABLE IV. EVALUATION OF A POLYVALENT SENSITIZED RBC FOR SCREENING TYPE-SPECIFIC ANTISERA

ANTISERUM	TYPE	RECIPROCAL TITER	
		Specific[a]	Polyvalent[b]
P. pseudotuberculosis	1A	4096	1024
	1B	2048	1024
	2A	8192	8192
	2B	4096	8192
	3	2048	4096
	4	4096	4096
	5	8192	8192
P. pestis		512	16

a. Titer against RBC sensitized with homologous antigen.

b. Titer against RBC simultaneously sensitized with all 10 P. pseudotuberculosis specific antigens.

A group of 513 sera from humans and animals were tested for antibody to P. pseudotuberculosis and the plague bacillus (Table V). Of the 97 human sera, 47 were from individuals living in Madagascar during the plague outbreak of 1952. In this series, 27 individuals had factor 1 antibody titers against P. pestis ranging from 1:128 to 1:20,480 and were negative when tested against P. pseudotuberculosis polyvalent sensitized sheep RBC. In 4 cases, antibodies were found against both organisms and 16 were negative. In a second group of 50 sera collected from patients in the Washington, D. C., area with symptoms of abdominal disease, 4 sera were positive for P. pseudotuberculosis, but not the plague bacillus, while 46 were negative. It must be pointed out here that no follow-up on these sera were made; neither is an implication made that the positive sera were from suspected cases of P. pseudotuberculosis.

TABLE V. SURVEY OF 513 SERA FOR THE PRESENCE OF HA ANTIBODY AGAINST *P. PSEUDOTUBERCULOSIS* AND *P. PESTIS*

SERUM SOURCE	NO. OF SERA TESTED	NUMBER POSITIVE		
		P. pseudotuberculosis only	Both	*P. pestis* only
Humans	97	4	4	27
Dogs	77	19	0	0
Chimpanzees	59	11	0	0
Gorillas	7	2	0	0
Orangutans	6	4	0	0
Baboons	7	0	0	0
Rats[a/]	200	70	10	52
Guinea pigs[a/]	60	40	0	10

a. Experimentally immunized or infected animals.

Rats were experimentally inoculated with either avirulent *P. pestis*, one of the types of *P. pseudotuberculosis*, or were control animals. The 10 animals showing titer to both species had received immunization with a mixture of *P. pestis* and all *P. pseudotuberculosis* types. In no instance did an animal have antibody for a type with which he had not been immunized. Serological titers from chimpanzees, gorillas, orangutans, and baboons were usually low, 1:64 to 1:256, when tested in the polyvalent HA test. These animals were considered to be "conditioned" inasmuch as they are from well established laboratory colonies at Walter Reed Army Institute of Research or Yerkes Laboratories.

Gel diffusion testing of our antigens and antisera revealed a single precipitin line. In the cases where subtypes existed, lines of identity occurred. When the purified antigen preparations were tested by disc electrophoresis, each gave a single protein staining band.

The use of the polyvalent HA test as a screening procedure reduced the workload to half of that necessary when each serotype had to be tested individually. Serum found positive in a screening test was subjected to the various type-specific tests to determine which serotype was involved. When either type 2 or 4 was involved, the positive serum was subjected to Group B and D *Salmonella*-sensitized cells. The difference in titers between the homologous and heterologous reactions were sufficiently different to resolve the problem.

SUMMARY

The HA procedure differentiated the various types of P. pseudotuberculosis; the use of such a test eliminated the cross agglutination expected with the plague bacillus. The polyvalent HA test permitted the screening of large numbers of sera in a short time. The small quantity of serum needed in the microhemagglutination test made possible the screening of very small wild rodents.

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REVIEW OF VIROLOGY DIVISION PROGRAM

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Work has continued with the attenuated Venezuelan equine encephalomyelitis (VEE) vaccine. Approval for use of vaccine produced in our laboratories was received from the Army Investigational Drug Review Board in April 1965. At the 1964 meeting results were reported of 6-month stability assays of a single lot of freeze-dried vaccine. Assays of this material showed no detectable loss in titer following 18 months storage at -20 C. The 2-year assays are scheduled for November.

The question of whether persons administered the attenuated virus develop sufficient levels of viremia to serve as a source virus for mosquitoes is still unresolved. It was planned to perform these studies in collaboration with Dr. Barnett of the University of Maryland. However, because of certain administrative and logistical problems this approach was abandoned and plans were developed to perform these studies within the Medical Unit.

To date 3 persons have been tested for viremia following administration of attenuated virus. Blood samples were collected every 12 hr beginning 24 hr after inoculation for a total of 7 days; they were diluted 10^{-1} to 10^{-5}; each of 6 guinea pigs was inoculated per dilution. Fourteen days later the animals were challenged with virulent virus. All 3 persons developed significant levels of antibody. In no case was viremia demonstrated.

These studies were suspended during the summer because of the lack of adequately screened facilities for quartering study subjects. It is planned to resume them this fall when mosquitoes cease to be a problem.

Because viremia was not demonstrated the protocol was modified to the extent that only the 10^{-1} dilution of blood would be initially tested by inoculation of 2.0 ml into each of 4 guinea pigs. The balance of each sample will be stored frozen for further testing as required. This procedure will permit testing of a larger number of persons within the same period of time.

Suggestions or comments from the Commission concerning this problem and the proposed approaches would be appreciated.

As part of the studies with the attenuated virus vaccine we have reevaluated the procedure for performing serum neutralization (SN) tests in an attempt to develop a procedure which would yield reproducible results.

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The initial experiments were concerned with evaluation of several diluents and VEE virus preparations. The 4 diluents evaluated were 2% beef heart infusion broth, phosphate buffered saline (PBS) pH 7.4, PBS with 1% human serum albumin, and PBS with 1% inactivated normal rabbit serum. These were used for diluting the 2 virus preparations, a 20% suspension of infected suckling mouse brain and a whole egg slurry.

Consistently reproducible virus titers were obtained with the 20% infected suckling mouse brain diluted with PBS containing 1% inactivated normal rabbit serum; this did not alter the problem of variation between tests. Because of the inconsistent patterns and time to animal deaths in tests incubated at 37 C for 1 hr, the conditions of incubation were examined. Two conditions of test incubation were evaluated: 1 hr at 37 C or 4 C overnight. Although virus titers in the presence of normal serum did not differ significantly; results obtained with tests incubated at 4 C indicated that neutralization was incomplete. In the light of Morgan's[1/] suggestion that complement was involved in the neutralization of Western equine encephalitis (WEE) the tests were repeated with 0.2 ml of a 1:30 dilution of complement added to tests incubated under the 2 conditions. Results of the tests were in agreement; in repeated tests of a single pool of immune serum, with incubation at 4 C, and the addition of complement, results were essentially identical with those obtained with incubation at 37 C and with less variation between tests.

While the foregoing studies were performed with 21-day old mice, other studies indicated that the age and sex of mice employed in neutralization tests were responsible for variation in results. This parameter was examined and the initial experiments showed that comparable results could be obtained using 3-, 4- or 5-week old females and 3- or 5-week old males whereas SN titers obtained with 4-week old males were consistently lower, and varied significantly between tests.

These studies showed that consistent results could be obtained when: 3-week old male or female mice were used and the tests were incubated at 4 C overnight in the presence of complement. This procedure offered a technical advantage in that a larger number of tests could be performed within a given time frame. These studies are being expanded to include Group A arboviruses under investigation in other studies.

In addition to the studies dealing with the SN test in mice, work is in progress on the development of metabolic inhibition tests as well as a plaque reduction test for measurement of antibody to the Group A arboviruses.

A procedure has been developed for performance of a metabolic inhibition test which yields reproducible results with animal sera. However, tests performed with human sera have not given consistent results. Irregular patterns occur when human immune sera are used. Treatment of sera with trypsin or Kaolin or the addition of fresh serum does not influence

this variation. The impression is that time of serum collection following vaccination may in part account for the variations. Studies to examine this question are in progress.

Work on the plaque reduction assay has recently been initiated. Limited results indicate reasonably close correlation between results and those obtained in intracerebral neutralization tests in mice.

Last year's report mentioned that studies had been initiated on development of an inactivated multivalent Group A arbovirus vaccine.

During the past year a major portion of the work in this program involved development of a method for obtaining materials of suitable antigenic activity. Chick embryo cell cultures have been used for propagation of the viruses of VEE and WEE. Neutral formalin has been used for inactivation. Although both viruses grow well in the chick embryo cell, the antibody responses following administration of multiple doses of inactivated virus were disappointing.

Several methods for increasing the virus yield were tried. The most promising of these involves transfer of supernatant fluids through 3 consecutive cultures. The procedure is outlined in Table I. With this procedure significant differences in antigenic activity were obtained among the 3 harvest fluids and the activity increased with each transfer.

TABLE I. OUTLINE OF PROCEDURE FOR PREPARATION OF INACTIVATED VIRUS

CULTURE 1.	Incubate at 35 C for 24 hr. Harvest supernate.
CULTURE 2.	Infect in same manner as Culture No. 1. Hold 2 hr at 35 C - decant excess seed. Add supernate from Culture No. 1. Incubate at 35 C for 24 hr. Harvest supernate.
CULTURE 3.	Repeat procedure used for Culture No. 2. Harvest supernate. Add formalin to 0.1-0.2% final concentration. Incubate at 37 C for 24 hr. Filter - HA pad. Hold at 4 C for 7-10 days.

VEE virus propagated by this method and inactivated with 0.1% formalin was assayed in guinea pigs, mice and hamsters. The median effective dose (ED_{50}) for each species was 0.003 to 0.004 ml when 3 doses of the vaccine were administered at 7-day intervals and the animals were challenged 7 days after the last dose with 1,000 median lethal doses (LD_{50}) of virulent virus. Storage stability studies with this vaccine are in progress.

The same procedure was followed for preparation of WEE vaccine except that 0.2% formalin was required for inactivation. Vaccines were prepared with 2 strains of virus. The vaccine prepared with the California strain had an ED_{50} of 0.1 ml. Because this value was high, a vaccine was prepared with a strain of virus obtained from Dr. Gorelick, U. S. Army Biological Laboratories. This was compared with the chick embryo vaccine currently in use for immunization in man.

The animals were administered 3 doses of 0.5 ml each at 7-day intervals and were challenged 21 days after the last dose. The results are presented in Table II. As may be seen the experimental vaccine appears slightly more effective than the product in current use. However, this difference may be due in part to strain differences and the challenge virus employed.

TABLE II. COMPARISON OF CURRENT HUMAN AND AN EXPERIMENTAL WEE VIRUS VACCINES

VACCINE	SURVIVORS/10	
	Current	Experimental
Undiluted	10	10
1:5	7	10
1:25	1	7
1:125	2	1
1:625	0	0
None	0	0

Preparation of vaccine with Eastern equine encephalitis (EEE) virus is in progress, after completion, Sindbis virus vaccine will be prepared.

On completion of the preparation of the 4 vaccines they will be tested for their effectiveness to confer protection to homologous and heterologous challenge either individually or in combination. The possible effect of the order of administration will also be examined.

As a corollary to the vaccine studies, experiments have been conducted with attenuated VEE virus vaccines to determine the earliest time post-vaccination that protection against homologous challenge occurs and secondly to determine whether animals vaccinated with attenuated VEE virus develop resistance to heterologous challenge.

In initial experiments it was found that adult mice vaccinated with attenuated VEE virus developed hemagglutinating inhibiting (HI) antibody to the homologous virus as well as to EEE, WEE and Mayaro viruses.

The relative insusceptibility of older mice to challenge with the available strains of EEE, WEE and Mayaro viruses made it necessary to select the hamster for cross-protection studies.

The first phase of these studies involved the inoculation of a group of hamsters with approximately 10^3 guinea pig intraperitoneal immunizing doses ($GPIPID_{50}$) of attenuated VEE virus.

The animals were divided randomly into 3 groups. On days 1-10 five animals were selected from each group and were bled for viremia and serum, and challenged with either VEE, EEE or WEE viruses. The results are presented in Table III.

TABLE III. VEE ANTIBODY DEVELOPMENT IN HAMSTERS VACCINATED WITH ATTENUATED VEE VIRUS AND RESPONSE TO CHALLENGE WITH VEE, EEE, WEE VIRUS

DAY POST-VACCINATION	VEE ANTIBODY TITER (HI) (Reciprocal)	VIREMIA FROM ATTENUATED VEE VIRUS	SURVIVORS/5		
			VEE	EEE	WEE
1	<10	+	5	5	4
2	<10	+	4	5	5
3	<10	+	5	4	5
4	<10	+	5	3	3
5	10	-	4	5	3
6	20	-	5	3	4
7	20	-	5	2	5
8	40	-	5	2	3
9	160	-	5	1	5
10	80	-	5	0	5

Hamsters had demonstrable viremia for 4 days following inoculation of attenuated VEE virus, with antibody detectable on day 5. A significant number of animals were refractory to both homologous and heterologous challenge during the period of viremia as well as following the appearance of antibody. It is recognized that these are short term studies; the

results suggest that the attenuated VEE virus vaccine could serve as the primary immunizing antigen in the induction of immunity to other Group A arboviruses.

These studies will be extended in an attempt to determine: (1) to what degree interference is protective, i.e., can it be overcome by large doses of challenge virus; (2) the duration of heterologous protection; and (3) if primary immunization with attenuated VEE virus augments the antibody response to other Group A arbovirus antigens.

A brief discussion follows of the results of studies on the use of immune serum in the prophylaxis and treatment of VEE infection in the guinea pig.

Sixty guinea pigs were each inoculated with 4 ml of homologous VEE immune serum by the intraperitoneal route. This serum had a HI antibody titer of 1:1280 and a serum neutralization index (SNI) of 7.0 logs.

Beginning 24 hr after administration of serum and at weekly intervals thereafter 6 animals were selected at random from the group, bled for serum and challenged with approximately 1,000 mouse LD_{50} of virulent VEE virus.

The results, presented in Table IV, show that protection conferred by passively administered antibody persisted for > 63 days. Antibody titers

TABLE IV. DURATION OF PROTECTION AFFORDED BY PASSIVELY ADMINISTERED HOMOLOGOUS VEE IMMUNE SERUM IN THE GUINEA PIG

DAYS AFTER SERUM ADMINISTRATION	MEAN RECIPROCAL HI TITER	NO. SURVIVORS/6[a]	SURVIVORS[a] SHOWING INCREASE IN TITER OVER SERUM CONTROL
1	76	5	1/5
7	53	6	0/3
15	28	6	1/5
22	13	4	2/4
29	8	6	5/5
36	8	6	4/5
42	5	4	4/4
50	5	4	4/4
57	5	2/5	2/2
63	5	3/4	3/3

a. No. of survivors disagree because of nonspecific deaths.

of animals challenged within 15 days of administration of serum usually decreased at the same rate as the controls. In contrast, antibody

titers of animals challenged 30-60 days after administration rose to high levels. These increases were taken as evidence that infection had occurred.

In experiments concerned with treatment of VEE infection, guinea pigs were administered 4 ml of homologous immune serum at 0, 4, 8, 24, 48 and 72 hr after inoculation of virulent virus. Results presented in Table V show that animals were protected against the lethal effects of infection when immune serum was administered within 24 hr after infection.

TABLE V. EFFECT OF PASSIVE ANTIBODY AS TREATMENT IN VEE INFECTION IN GUINEA PIGS

TIME SERUM ADMINISTERED AFTER VIRUS INOCULATION (hr)	NO. SURVIVORS/6[a]	SURVIVORS[a] SHOWING INCREASE IN TITER
0	6	0/4
4	5	3/5
8	5	2/4
24	6	6/6
48	0	-
72	0	-
No serum	0	-

a. No. of survivors disagree because of nonspecific deaths.

HI tests performed on sera obtained in serial bleedings of these animals subsequent to the administration of serum showed that the rate and magnitude of antibody response varied as a function of the time between inoculation of virus and serum respectively (Figure 1). Although not shown the antibody titers of animals administered serum immediately after inoculation of virus decreased at the same rate as those of animals which received serum alone. Approximately 50% of the animals in the 4- and 8-hr groups responded with increases in antibody; however, the rate and magnitude were less than that seen in animals administered serum at 24 hr. The "no response" curve represents the 8 animals in the 0-, 4- and 8-hr groups which did not develop an increase in HI antibody.

Subsequent experiments have shown that 0.5 ml of the homologous immune serum administered 24 hr after infection was sufficient to prevent death in 4 of 6 animals. This result is of interest since the level of viremia in control animals averaged 5.8 logs/0.3 ml of blood at the time serum was administered.

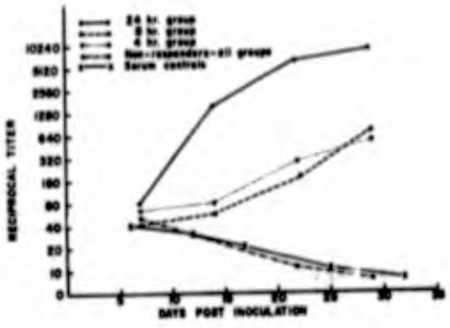

FIGURE I. HAI TITERS IN ANIMALS ADMINISTERED IMMUNE SERUM AFTER INOCULATION OF VIRULENT VEE VIRUS.

It appeared that the effectiveness of antibody therapy was related to clearing of the viremia and consequent prevention of virus entry into the central nervous system. This supposition was tested in recent experiments in which immune serum was administered intrathecally to animals immediately and 24 hr after inoculation of VEE virus. Two volumes of sera were used; 0.1 and 0.5 ml.

All animals given serum immediately survived. In the groups administered serum at 24 hr, 3 of 6 given 0.1 ml and 4 of 6 given 0.5 ml survived. It is of interest that in both groups the times to death were prolonged. While not conclusive, the results suggest that prevention of death of animals infected with VEE virus is the result of excluding or neutralizing virus in the brain.

SUMMARY

Progress on investigations on VEE, EEE, and WEE within the Virology Division are presented. Major effort continued on VEE in the following categories:

1. Storage of freeze-dried living vaccine for 18 months resulted in no loss in titer.

2. Testing in vaccinees for viremia sufficiently high for arthropod transmission was delayed.

3. Procedures for serum neutralization tests for detection of antibodies following immunization were revised and continued under investigation.

Other tests for use with Group A arboviruses were studied; a metabolic inhibition test showed promise for use with animal sera, but has been disappointing when used with human sera. Further study is required.

Development of an inactivated multivalent Group A arbovirus vaccine was begun; VEE, WEE, EEE and Sindbis viruses are under consideration for this combination.

Preliminary studies were conducted on the use of immune serum for prophylaxis and treatment of VEE in guinea pigs.

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COMPARATIVE PATHOLOGY OF CUTANEOUS ANTHRAX IN THE IMMUNIZED AND UNIMMUNIZED GUINEA PIG

Alexander DePaoli, Captain, VC, Martha K. Ward, Captain, USPHS, and Kenneth R. Dirks, Lt Colonel, MC*

One year ago Dr. Ward reported to this Commission that immunization with combined anthrax protective antigen and live attenuated tularemia vaccine enhanced guinea pig survival after subcutaneous challenge with Bacillus anthracis spores to a greater extent than did immunization with anthrax protective antigen alone. On the other hand, the protection afforded guinea pigs by the live attenuated tularemia vaccine against tularemia challenge was unaffected by this combination. The present study was undertaken to identify, if possible, the morphological basis for the observed improved protection against anthrax provided by the vaccine combination. This work was performed by Captain Alexander DePaoli in collaboration with Dr. Martha Ward.

Eighty Hartley strain guinea pigs were immunized subcutaneously with anthrax protective antigen (APA) and 80, with a combination of anthrax protective antigen and live attenuated tularemia (APA-LVS) vaccine. Thirty days later each of these and 14 nonimmune guinea pigs were challenged intradermally (ID) with 1,000 B. anthracis spores, strain NH-6. Animals of both immunized groups were sacrificed at 4-hr intervals for 24 hr, and then every 12 hr through 144 hr postchallenge. All survivors were sacrificed at 14 days.

In a previous similar study no changes were observed in the skin of nonimmune animals sacrificed prior to 20 hr, and in a parallel study no animals survived beyond 60 hr. For these reasons control animals were sacrificed at 20, 24, 36, and 48 hr.

Following sacrifice the cutaneous challenge sites were examined grossly, excised, and fixed in phosphate-buffered 10% formalin for histopathological examination.

Typically the skin lesions in control animals at 20 hr were light to dark red, slightly raised, 0.3-0.5 cm in diameter, and 1.5-2.0 cm in diameter at 48 hr. Moderate congestion of the underlying subcutaneous tissue and mild edema were present by 20 hr; these changes progressed, to become severe and accompanied by focal hemorrhage by 36 hr. They persisted in control animals dying or sacrificed at later times.

In both immunized groups of animals the cutaneous lesions were, like the controls, light to dark red. However, their rate of development was accelerated so that by 20 hr they were twice the size recorded in the

* Presenter, U. S. Army Medical Unit, Fort Detrick, Maryland.

controls. Subcutaneous congestion and edema, on the other hand, were less marked at all parallel sacrifice times. The peak reaction in the immunized groups was reached by 72 hr and noticeable regression was evident by 120 hr. Only a small, 0.4-0.7 cm, discoid area of epilation identified the challenge site 2 weeks postchallenge.

Histopathological differences among the 3 groups were observed in the neutrophilic leukocyte, mononuclear cell, and eosinophilic leukocyte responses, the numbers of bacilli in the lesions, and the appearance of phagocytosis.

While the neutrophilic response was only mild in controls, it was moderate to marked in the vast majority of the immunized animals. Among immunized it was more intense and more rapid in development in the APA-LVS group than in the group receiving APA alone. Figure 1 depicts this comparison graphically. While a 3+ intensity of the neutrophilic response was achieved by 20 hr postchallenge in the combined vaccine group, a comparable intensity of response was not reached in the group immunized with APA alone until 16 hr later, and such an intensity was never reached in the control animals.

The numbers of bacilli in the lesions were catalogued as "few" or "many," 150 organisms per microscopic section being chosen as the upper limit for the "few" category. All lesions from the control animals contained many bacilli (Table I). In contrast sections from less than half of the APA and less than one-fourth of the APA-LVS immunized animals contained comparable numbers of organisms. Sections from only 56% of the APA and 34% of the APA-LVS groups exhibited any organisms whatsoever. Furthermore, only 3 of the latter group sacrificed later than 24 hr displayed recognizable bacilli.

TABLE I. INCIDENCE OF ANTHRAX BACILLI IN SKIN LESIONS OF GUINEA PIGS

ORGANISMS/SECTION	NO. OF GUINEA PIGS		
	Controls	Immunized	
		APA	APA-LVS
None	0	34	53
< 150	0	11	9
> 150	14	32	19
TOTAL	14	77	80

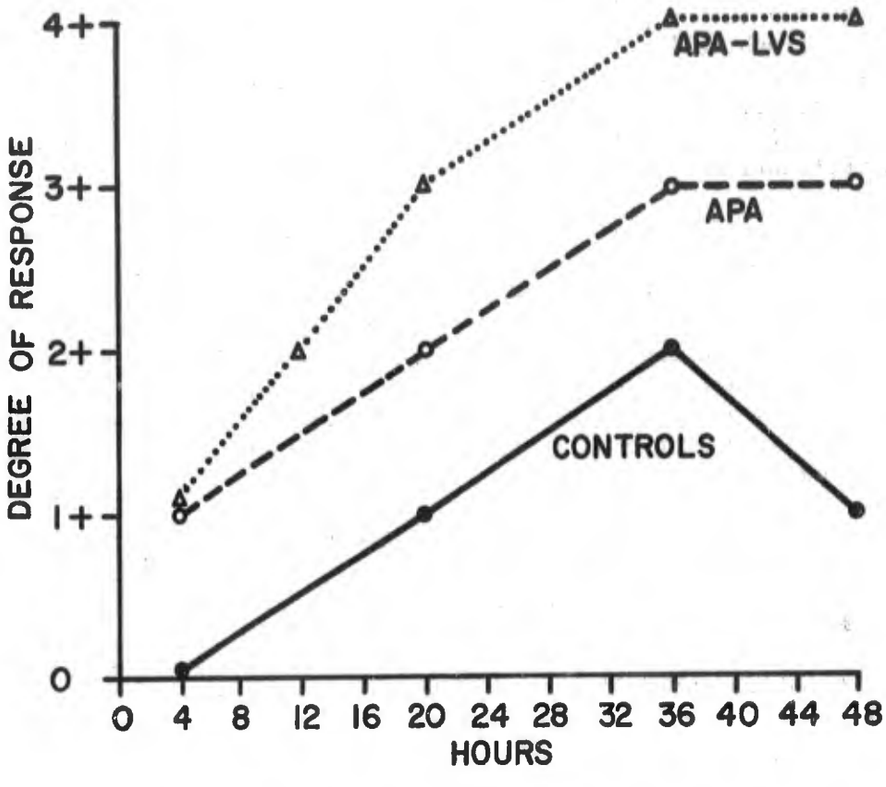

FIGURE 1. NEUTROPHILIC RESPONSE TO ID CHALLENGE WITH 10^3 B. ANTHRACIS SPORES, STRAIN NH-6, IN GUINEA PIGS. (APA = animals immunized with anthrax protective antigen; APA-LVS = animals immunized with anthrax protective antigen combined with live attenuated tularemia vaccine.)

As a corollary to the foregoing, phagocytosis of bacilli (Table II) was conspicuous in approximately half of the animals of both immunized groups. It was entirely absent in all sections from the control animals.

TABLE II. INCIDENCE OF PHAGOCYTOSIS OF ANTHRAX BACILLI IN GUINEA PIGS

PHAGOCYTOSIS	NO. OF GUINEA PIGS		
	Controls	Immunized	
		APA	APA-LVS
Absent	14	42	40
Present	0	35	40
TOTAL	14	77	80

A significant mononuclear cell response was observed in the majority of animals of both immunized groups (Table IIIO. It was slightly more intense in the combined vaccine than in the APA group. This response was entirely absent in controls.

TABLE III. INCIDENCE OF MONONUCLEAR RESPONSE IN GUINEA PIGS

DEGREE	NO. OF GUINEA PIGS		
	Controls	Immunized	
		APA	APA-LVS
None	14	25	29
Mild	0	37	29
Moderate	0	13	12
Marked	0	2	10
TOTAL	14	77	80

Sixty-one of 77 APA-immunized and 50 of 80 APA-LVS-immunized animals displayed a notable eosinophilic response at the challenge site (Table IV).

TABLE IV. INCIDENCE OF EOSINOPHILIC RESPONSE IN GUINEA PIGS

DEGREE	NO. OF GUINEA PIGS		
	Controls	Immunized	
		APA	APA-LVS
None	10	16	30
Mild	4	16	32
Moderate	0	18	9
Marked	0	27	9
TOTAL	14	77	80

Variable degrees of epidermal, dermal, and hair follicular necrosis, epidermal ulceration, congestion, edema, hemorrhage, vascular degeneration, and vascular thrombosis were observed. These changes were least conspicuous in the combined vaccine group and most prominent in the unimmunized animals. Table V summarizes the frequency of local vascular thrombosis in the various groups. This phenomenon was least common in the combined vaccine group.

TABLE V. INCIDENCE OF VASCULAR THROMBOSIS IN GUINEA PIGS

VASCULAR THROMBOSIS	NO. OF GUINEA PIGS		
	Controls	Immunized	
		APA	APA-LVS
Absent	8	40	57
Present	6	37	23
TOTAL	14	77	80

The following figures illustrate the principal histopathologic features just described. Figure 2A is a section of the ID inoculation site of a non-immune animal 20 hr postchallenge. Note the large numbers of bacilli superficial to the cutaneous muscle layer. A higher magnification (Figure 2B) reveals that these organisms have excited only a minimal neutrophilic leukocytic inflammatory response. Phagocytosis is not evident.

Figure 3 shows a section of the skin lesion of an APA-immunized guinea pig sacrificed at 20 hr. A more intense neutrophilic response is readily apparent. Bacilli are fewer in number and may appear fragmented. Phagocytosis is not seen, however. A few mononuclear cells are present.

Figure 4 is a section from the challenge site of a combined vaccine immunized guinea pig sacrificed at 20 hr. It demonstrates still greater neutrophilic leukocytic aggregation, smaller numbers of bacilli, and the presence of bacterial phagocytosis. Moderate numbers of mononuclear cells are present.

At 36 hr (Figure 5) numerous bacilli are still visible in this lesion from a control animal; only a few neutrophils have migrated into the area; phagocytosis is absent; and no mononuclear cells can be seen.

In contrast (Figure 6) numerous neutrophils infiltrate the challenge site of the APA-immunized animal at this time. Bacilli of normal staining reaction are difficult, if not impossible, to discern; moderate number of mononuclear cells are present; and phagocytosis is active.

Figure 7 from the challenge site of a combined vaccine protected animal demonstrates an even greater neutrophilic response and sharper localization of the inflammatory process. Normal staining bacilli are not recognizable, mononuclear cells are visible, and phagocytosis is present.

Reflecting the absence of bactericidal effects and phagocytosis in the unimmunized animal, Figure 8 demonstrates the normal staining reaction on Wolbach-Giemsa stain of B. anthracis in the cutaneous lesion of a control guinea pig sacrificed at 48 hr.

Eosinophilic leukocytic infiltration of variable degree are apparent in occasional control animals and in immunized animals from 16 hr post-challenge until the end of the 2-week observation period. Figure 9 shows marked eosinophilic response in an APA-immunized animal which died 54 hr postchallenge. This unfavorable outcome and that of 6 other animals with similar responses suggests that local tissue eosinophilia in this circumstance, as in other better known entities, may be more closely related to hypersensitivity than to protection.

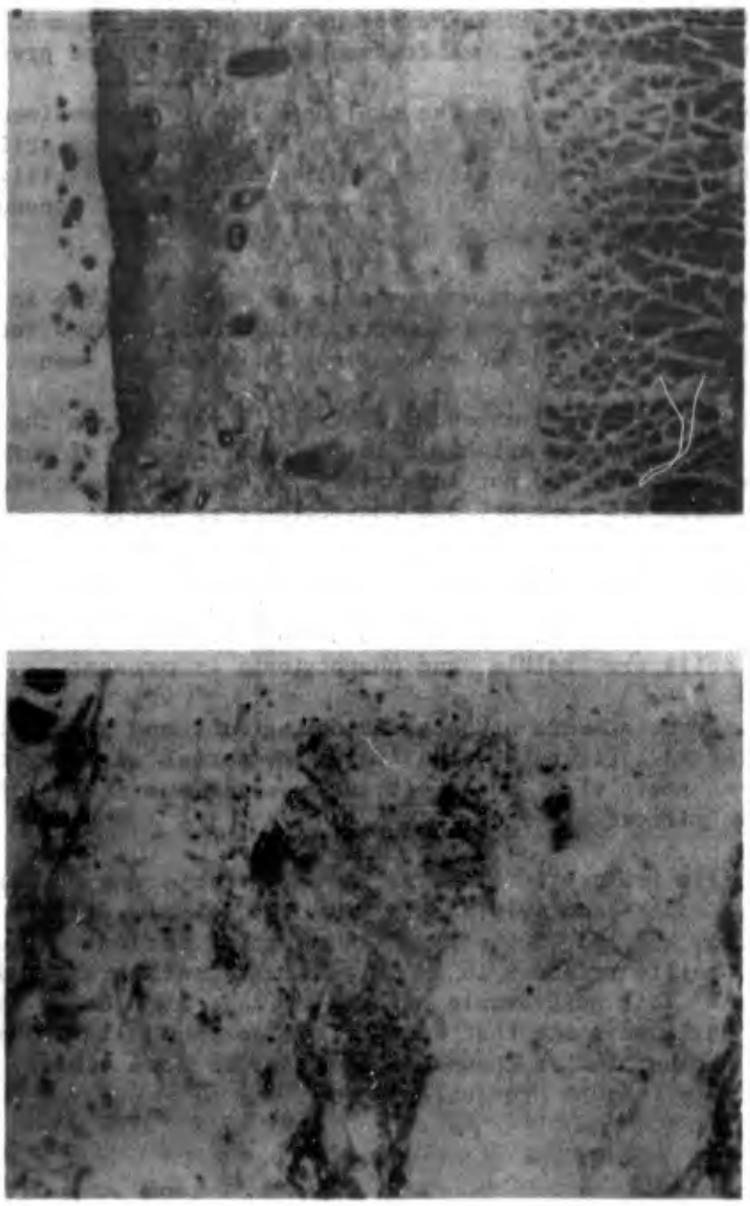

FIGURE 2. ID INOCULATION SITE OF NONIMMUNE ANIMAL AT 20 HR. (A) APPROX. 35X. (B) APPROX. 100X.

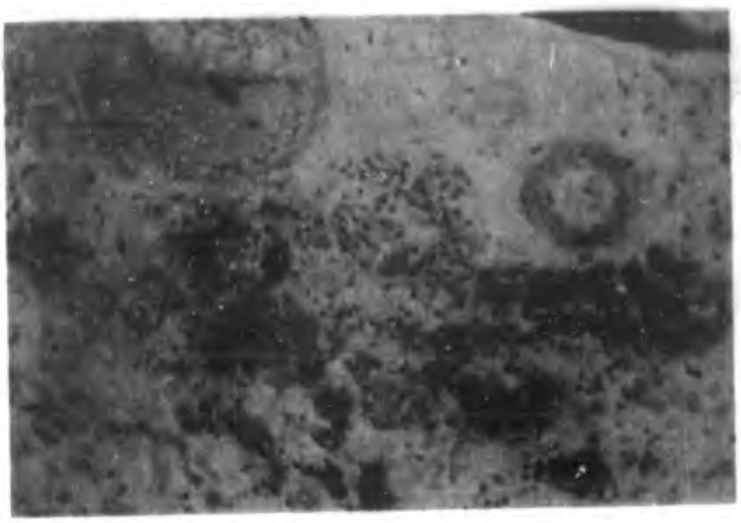

FIGURE 3. SKIN LESION OF APA-IMMUNIZED GUINEA PIG SACRIFICED AT 20 HR. (APPROX. 100X)

FIGURE 4. CHALLENGE SITE OF APA-LVS-IMMUNIZED GUINEA PIG SACRIFICED AT 20 HR. (APPROX. 100X)

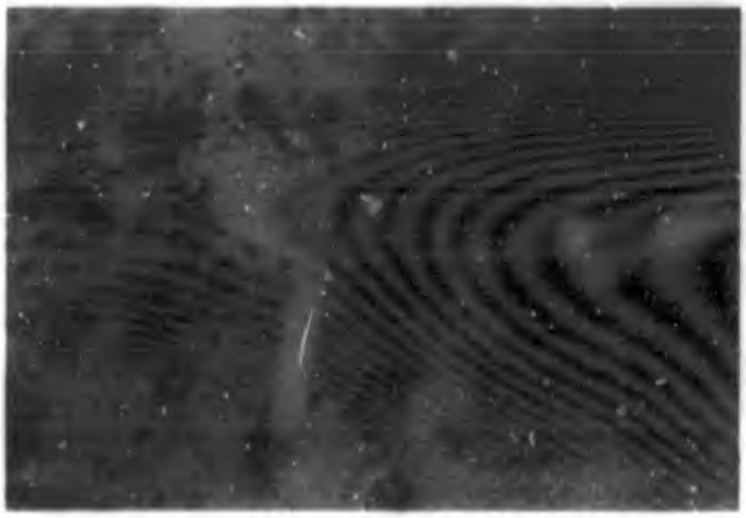

FIGURE 5. SKIN LESION FROM CONTROL ANIMAL AT 36 HR. (APPROX. 100X)

FIGURE 6. CHALLENGE SITE OF APA-IMMUNIZED ANIMAL AT 36 HR. (APPROX. 100X)

FIGURE 7. CHALLENGE SITE OF APA-LVS-VACCINE PROTECTED ANIMAL. (APPROX. 100X)

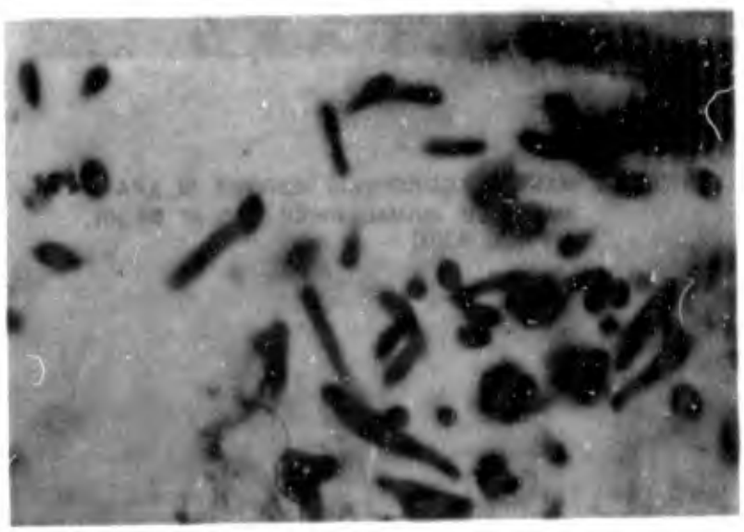

FIGURE 8. NORMAL STAINING REACTION ON WOLBACH-GIEMSA STAIN OF B. ANTHRACIS IN CUTANEOUS LESION OF A CONTROL GUINEA PIG SACRIFICED AT 48 HR. (APPROX. 970X)

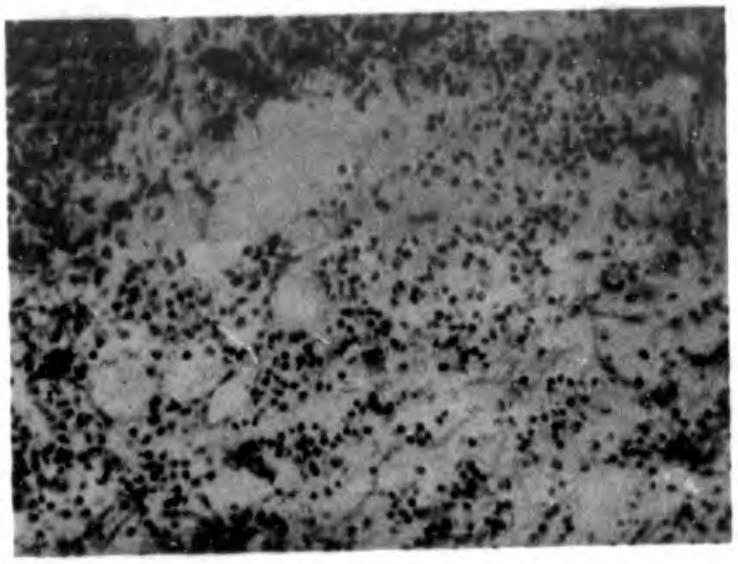

FIGURE 9. MARKED EOSINOPHILIC RESPONSE IN APA-IMMUNIZED ANIMAL WHICH DIED AT 54 HR. (APPROX. 430X)

Culture of the skin lesions (Table VI) in all of the control animals yielded anthrax bacilli. Nearly three-fourth of the lesions in the APA-immunized group and nearly half of those in the combined vaccine group yielded positive cultures. Very few organisms were observed in animals of the immunized group sacrificed more than 36 hr postchallenge.

TABLE VI. POSTMORTEM BACTERIOLOGY OF SKIN LESIONS OF GUINEA PIGS

SKIN CULTURE	NO. OF GUINEA PIGS		
	Controls	Immunized	
		APA	APA-LVS
Not done	0	4	8
Negative	0	20	35
Positive	14	53 (4-206 hr)	37 (4-144 hr)
TOTAL	14	77	80

It is worth noting that no animals of the combined vaccine group died of anthrax during the experiment, while 12 (16%) of the APA group died of this disease prior to their scheduled sacrifice times.

SUMMARY

It would appear from these observations that the morphologic counterpart of the acquired increased resistance to ID B. anthracis, strain NH-6, spore challenge conferred on the guinea pig by immunization with APA has 4 recognizable components. These seem to have as their objective localization of the infection and consist of: (1) rapid mobilization of neutrophilic leukocytes to the site of challenge; (2) inhibition of replication of B. anthracis at the challenge site; (3) mobilization of mononuclear cells to the challenge site; and (4) phagocytosis of anthrax bacilli by neutrophilic leukocytes and mononuclear cells.

The enhanced protection against such challenge afforded guinea pigs by the combination of APA with LVS vaccine is reflected in acceleration and intensification of these 4 responses, with resultant increased efficiency in localization of the infectious process.

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FISCAL YEAR 1965

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034 450

UNCLASSIFIED
Security Classification

DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) Commission on Epidemiological Survey of the Armed Forces Epidemiological Board and U. S. Army Medical Unit, Fort Detrick, Frederick, Maryland 21701	2a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED 2b. GROUP NA

3. REPORT TITLE

ANNUAL REPORT TO THE ARMED FORCES EPIDEMIOLOGICAL BOARD

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)
Annual Report, Fiscal Year 1965

5. AUTHOR(S) (Last name, first name, initial)
DAWKINS, A. T., JR., MacNAIR, D. S., JAEGER, R. F., BUZZELL, A., BLEMLY, N. R., HORNICK, R. B., WOODWARD, T. E., BEISEL, W. R., SHAMBAUGH, G. E., LUST, G., Van ORMER, D. G., MARSHALL, J. D., JR., CURRIE, J. A., McKINNEY, R. W., DePAOLI, A., WARD, M. K., DIRKS, K. R.

6. REPORT DATE February 1966	7a. TOTAL NO. OF PAGES 140	7b. NO OF REFS 62
8a. CONTRACT OR GRANT NO. b. PROJECT NO. 1C622401A096 c. d.	9a. ORIGINATOR'S REPORT NUMBER(S) 9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)	

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY U. S. Army Medical Research and Development Command, Office of The Surgeon General, Department of the Army, Washington, D. C.

13. ABSTRACT

Progress is reported in selected areas of research of the U. S. Army Medical Unit. A separate report covers research on staphylococcal enterotoxin B under Project No. 1B533001D164.

DD FORM 1 JAN 64 1473

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14 KEY WORDS	LINK A ROLE	LINK A WT	LINK B ROLE	LINK B WT	LINK C ROLE	LINK C WT
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