WALTER REED ARMY INSTITUTE OF RESEARCH

WALTER REED ARMY MEDICAL CENTER

WASHINGTON, D.C. 20012

ANNUAL PROGRESS REPORT

Reports Control Symbol MEDDH-288

1 July 1963 - 30 June 1964

Volume I

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WALTER REED ARMY INSTITUTE OF RESEARCH
Walter Reed Army Medical Center
Washington, D. C. 20012

ANNUAL PROGRESS REPORT

1 July 1963 -- 30 June 1964

Volume I

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

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ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36153

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8020104

1. REQUESTING AGENCY
Army Medical Service
Office of The Surgeon General
Washington, D.C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
A Walter Reed Army Inst of Rsch
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723-1000, Ext 3552

49

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576-5129 or Interdepartmental Code 98, Ext 5129 See Continuation Sheet

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6. TITLE OF: PROJECT ☐
TASK ☐
SUBTASK ☒ Acute renal injury and failure (U)

7. DATE OF REPORT | DAY 30 | MONTH Jun | YEAR 1964

8. RESUME' (U) Renal hemodynamic and functional alterations have been studied in anuric and oliguric hemorrhagic hypotension in the dog.

Several agents, including urea, 20% mannitol, low molecular weight dextran (Rheomacrodex), and phenoxybenzamine (Dibenzyline) have been compared in terms of directly measured renal blood flow alterations and other renal functional changes during hemorrhagic hypotension. Related observations on the hemodynamic effects of mannitol plasma volume expansion upon simultaneously measured cerebral, cardiac, and renal blood flows have been made in the dog, sheep, goat, baboon, and monkey. The effect of chronic partial renal arterial constriction on renal morphology and function has also been determined and studied with particular emphasis on alterations during osmotic diuresis in directly measured renal blood flow, and the renal extractions of PAH, creatinine, and inulin.

On monkeys behaviorally conditioned to perform tasks requiring sustained vigilance and attention, various experimental uremic states have been produced and related to quantitated behavioral deficits. Differences in effects on behavior between urea infusion, bilateral nephrectomy, bilateral ureteral ligation, and continuous urine reinfusion have been determined.

9. KEY WORDS

Renal function, acute renal failure, osmotic diuresis, hemorrhagic hypotension, Mannitol, urea.

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

ACCESSION NO. 36153	ARMY RESEARCH TASK REPORT
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ANNUAL PROGRESS REPORT

Project No. 3A012501A802 COMBAT SURGERY

Task No. 01 Combat Trauma

Subtask No. 04 Acute renal injury and failure

Description:

These interrelated studies concern (1) measurement of alteration of hemodynamic factors (renal blood flow and renal function as related to ischemic hemorrhagic hypotension) in the prevention and pathogenesis of ARF, (2) the development of a model to determine the renal effects of various bacterial toxins as exemplified by the alpha toxin of Clostridium perfringens, and (3) observations in behavior-conditioned primates including the nature of uremic toxemia, ARF, and renal transplantation.

Progress:

I. Renal alterations in hemorrhagic hypotension (with and without treatment including osmotic diuretics and other agents):

a. The rise in renal blood flow noted during osmotic diuresis with mannitol or urea in normotension and hypotension has been demonstrated to be due to several interrelated factors which are individually inseparable. These factors include plasma volume expansion, decreased blood viscosity (hemodilution) and increased cardiac output. Similar increases in renal blood flow when these agents are directly infused into the renal artery have not been demonstrated. Comparable increases in cardiac, renal, and in cerebral blood flow during mannitol osmotic diuresis were noted in the monkey, baboon, sheep and goat.

b. In 11 dogs in controlled states of normotension and hypotension, intravenous infusion of 10% Rheomacrodex (dextran) induced directly measured increases in cardiac output, stroke volume, and renal blood flow without augmenting the urinary flow rate. Comparison with previously studied hypotensive dogs given mannitol revealed that the increases in cardiac and renal blood flow persisted longer when similar volumes of Rheomacrodex were administered. Some of these increases are related to the viscosity altering the effect on blood flow but not to differences in urinary excretion.

Infusion of Rheomacrodex resulted in decreases in the extractions of inulin, creatinine, and PAH that were independent of the urinary flow rate or hypotension. No increase in GFR was noted. These results are consistent with a hypothesized intrarenal redirection of flow. These are apparently nonspecific effects since similar patterns

have been observed during infusion of 20% dextrose, 20% mannitol, or during plasma and red cell exchange in the dog.

c. The use of a modified saline solution derived from sea-weed algae (glyco-alginate) was evaluated for its possible beneficial effects in the treatment of hemorrhagic hypotension. No striking improvement in renal blood flow, persistent plasma volume expansion, urinary flow rate, or catecholamine responses followed infusions of this agent; the results were comparable to equivolume normal saline replacement therapy.

d. In another series of experiments it was determined that treatment with phenoxybenzamine (Dibenzyline) (0.43 mgm/kg.) increased renal blood flow in hemorrhagic hypotension. This improvement correlated with a measured fall in renal vascular resistance. An increase in filtration fraction (C_{CR}/C_{PAH}) was noted in the drug-treated normotensive dogs. Generally, there was seen a consistent rise in glomerular filtration rate, urinary flow rate, renal plasma flow, and the maintenance of the renal extraction ratios of PAH, creatinine, and inulin.

e. Unilateral constriction of the left renal artery was performed in 17 dogs. Severe decrease in renal blood flow and GFR resulted. Studies were performed for periods of up to 54 days following the occlusive procedure. A dilute urine was excreted with an increase in sodium concentration during urea-saline-ADH diuresis. This affirms that the nephron population, while functioning relatively well in terms of distal function (T^cH_2O/per 100 ml GFR x 100), was damaged in the more proximal tubular areas. The kidneys in these states of chronic reduction in renal blood flow did not demonstrate the phenomena of an increase in water reabsorption previously noted by Berliner et al during acute reduction of GFR and renal blood flow. We also noted that significant levels of renal blood flow (up to 40 ml/min) can be measured in such a chronically damaged kidney, in the presence of no urine flow and no effective extraction of inulin, PAH, or creatinine during maximal stimulations by infusions of urea, dextrose, or mannitol. These results indicate that in the presence of chronic renal arterial occlusions in the dog, significant collateral blood flow from the renal pelvis and ureter can occur without any benefit to glomerular filtration or renal secretory tissue.

f. Studies were performed to assess the ability of the Hg-197 neohydrin renograms and scintiscans to reflect acute alterations of renal blood flow in the dog during hypotension, normotension, renal arterial occlusion or osmotic diuresis. It was possible under these conditions to alter the steady state relationship between RBF and urine flow rate. The Hg-197 scintiscans and renograms failed to reflect the induced blood flow alterations, but often did reflect urine flow rate changes.

II. Studies in a model to determine the renal effects of alpha toxin of Cl. perfringens, believed to induce acute renal failure:

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a. A feasible and practical *in vivo* isolation technique for the perfusion of the dog kidney by bacterial toxins was devised, by diverting the left renal vein blood, and replacing this amount of blood loss by a carefully graduated jugular vein infusion. A steady state was attained for the period of the experiment. *Clostridium perfringens* alpha toxin was infused through a small needle inserted in the left renal artery. A prompt decrease in renal blood flow with overt cortical ischemia was followed by a rise in blood flow, a fall in renal vascular resistance, an increase in urinary flow rate, and gross renal vascular engorgement. Red blood cell fragility and hemolysis were noted in the absence of detectable circulating toxin as tested by the L-V test, which was apparently insensitive to the dilutions of toxin which produced the foregoing functional and morphological alterations. Acute renal failure was not produced. The toxic effects of the toxin-induced hemolysis were shown to be distinct from its lethality producing effects.

In current studies, the sites of localization of the toxin in the renal tissue are being determined by I-131 and I-125 isotope tagging and by autoradiography.

III. Studies in experimental primates on the nature of uremic toxemia, ARF, and renal transplantation:

a. Studies on the previously reported model featuring behavioral testing in the monkey during urine reinfusion, were extended to include other uremic states including bilateral ureteral ligation, bilateral nephrectomy and urea infusion. Several other behavioral tests were added, including conditioned-avoidance and paced-avoidance scheduling. Fifteen experiments have been completed.

Alterations in behavior were comparable in the presence of bilateral ureteral ligation, nephrectomy, continuous urine reinfusion, or urea infusion. Performance decrements, however, occurred at different times in relation to the stressors and the levels of plasma urea nitrogen concentration. Urine reinfusion performance failures were noted at levels of 59 mgm% compared to values of 183 to 257 mgm% noted in the other experimental uremic states. This difference was not related to abnormalities in plasma potassium, sodium concentration, osmolarity, blood pH, the mechanics of infusion, or bacterial contamination. Similar quantitations of injected fractions of human uremic material are projected.

b. In a series of six pilot experiments, male Rhesus monkeys were determined (by Dr. Huser, Department of Hematology, WRAIR) to be human blood group B with anti-human A material in their sera. Following infusion of AB human blood these animals exhibited transient elevations of plasma urea nitrogen. Additional studies are currently in progress to further document the response to repeated mistransfusion insults as well as the concomitant roles of hydration and hypotension. The ultimate goal is an experimental primate model of ARF.

c. In line with the current attempts at human transplantation for chronic renal insufficiency and uremia, a conjoint effort with the Department of Surgical Metabolism and Pathology was instituted. Eighteen renal autotransplants and four hemotransplants were performed in male and female monkeys, using an American Stapler Device for arterial and venous anastomosis. This device was successful on renal arterial vessels as small as 1.1 mm in diameter. Animals were sacrificed at periods of up to 3 weeks following transplantation. The complete assessment of the technical features, alterations in renal function, and morphology is being prepared.

Summary and Conclusions:

I. Renal Hemodynamics: Alterations in renal function have been tabulated in reference to measured changes in hemorrhagic hypotension and following treatment with a wide variety of agents. Evidence is presented on the comparative responses to such agents as mannitol, urea, or Dibenzyline. In other studies radioisotope techniques have been tested to determine their potentialities in reflecting changes in renal blood flow in various states including hypotension. Such agents as Hg-197 neohydrin were found to yield valid data, but with serious limitations in states of changing urine flows.

II. Renal Effects of Toxins: An alternate view to the failure of production of ARF in the dog by hypotension has been the suggestion that bacterial toxins could induce such a state. Studies herein reported on the alpha toxin of Cl. perfringens indicate that it does not induce ARF. An experimental model for the study of other agents has been devised.

III. Studies in Primates on Uremia: Behavioral testing in the monkey has successfully pointed out significant differences in uremic states induced by different means. These differences will be exploited in an attempt to assay the effect upon behavior of the injections of human uremic material into behaviorally trained monkeys. An experimental demonstration of a possible toxic uremic factor appears plausible on the basis of the results currently obtained.

The possibility of obtaining a primate model of ARF has been explored and pilot study results presented. Other experimental manipulative procedures in primates (monkeys) have included the successful application of a stapler device to perform renal transplantation.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER 36154

PROJECT, TASK, OR SUBTASK NO. 3A012501A8020105

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY

NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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6. TITLE OF: PROJECT ☐
TASK ☐
SUBTASK ☒ Metabolic and nutritional problems associated with injury (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME' (U) The Department of Surgical Metabolism and Pathology has initiated, expanded, or continued investigations in the areas of: (1) The factors responsible for lethality in generalized peritonitis. Studies include development of model systems for the identification of causal microflora, and the understanding of pathophysiological changes and metabolic pathway affected by these microorganisms and/or their toxins. (2) The use of adhesive polymers for restoring tissue continuity following injury and the development of histologic and biochemical procedures for evaluating their biological behavior. (3) The mechanisms of wound healing which include (a) comparison of the healing strength of skin incisions orientated longitudinally and transversely, (b) the effects of microflora and antibiotics on the healing of colonic anastomoses, and (c) evaluation of a vascular stapler. (4) Bilirubin metabolism attending cholecysto-ileostomy in the dog and common duct obstruction in primates. (5) The antimetabolic effects of sulfonamide therapy on the thyroid gland as shown by histologic and biochemical alterations. Also, the antagonistic effects of compounds structurally related to sulfa drugs. (6) The sanitization and sterilization of patient isolators while in use.

9. KEY WORDS
Infection, adhesive, healing, injury, cholecystostomy, antimetabolite, isolator.

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36154	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	3A012501A802 / 01 / 05 / /
14. DATE OF REPORT (30-33)	0664
15. SECURITY OF WORK (34)	4
16. TYPE OF REPORT	3 / 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	011000 /
18. OSD CLASSIFICATION (65-66)	AR
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Card "D"

20. CONTRACT NUMBER	DA
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23. PRIORITY (11-14) 24. PROGRAM ELEMENT (15-26)	1 / 6.11.25.01.1
25. CMR&D CODES	N/A
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	1412 / a / 6

Card "F"

27. FUNDING

a. Est. Total Cost (11-15)	
b. % Spent Intern. (16-18)	1
" " Extern. (19-21)	2
c. Total Obligation (22-26)	
d. Progmd. Cur. FY (27-33)	
e. " " " +1 (34-40)	
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ANNUAL PROGRESS REPORT

Project No. 3A012501A802 COMBAT SURGERY

Task No. 01 Combat Trauma

Subtask No. 05 Metabolic and nutritional problems associated with injury

Description:

The Department of Surgical Metabolism and Pathology has continued a multi-disciplinary approach to the metabolic and nutritional problems associated with injury. Studies were conducted in the areas of (1) host factors associated with lethality in peritonitis attributable to the pharmacologic and physiologic effects of bacterial toxins and metabolites. These included the development of model systems for (a) characterizing the role of the microflora, (b) identifying the complex toxic substances, and (c) elaborating the metabolic pathways affected, (2) the use of adhesive polymers for restoring tissue continuity following injury, and procedures have been established for evaluating the tissue toxicity of the polymers, (3) the basic processes in wound healing with special regard to the effects of microflora and antibiotics on the healing of colonic anastomosis; and the effects of orientation on the healing of incisional skin wounds, (4) the metabolic and excretory products of bilirubin after cholecysto-ileostomy and common bile duct obstruction, (5) the metabolic effects of chronic sulfonamides therapy with special reference to inhibition of normal thyroid function, and (6) the sanitization of patient isolators while in use which includes the evaluation of germicidal agents for cleansing and the relative antibacterial efficiencies of ultraviolet germicidal lamps for pass-through locks.

Progress:

I. Studies on the factors responsible for lethality in generalized peritonitis:

Host factors associated with lethality in peritonitis appear attributable to the various pharmacologic and physiologic effects of bacterial toxins and metabolites. Studies have been initiated to: (a) characterize the role of the microflora, (b) identify the complex of toxic substances, and (c) elaborate the metabolic pathways affected. Two experimental models have been developed for this research. The first system emphasizes the role of complex fecal microflora, and their relative significance in peritonitis. The second system was established to simulate abdominal injury, and to yield a standardized model for metabolic studies. Experiments were designed to (1) demonstrate the feasibility of the model systems for the study of peritonitis, (2) provide evidence of bacterial population densities in the lethality of peritonitis, (3) study the properties of toxic metabolites, (4) devise assay techniques for endotoxins, and (5) explore effects of the toxins on metabolic pathways.

(1) Development of experimental models for the study of bacterial peritonitis: Study of the host response to bacterial peritoneal infection requires a standardized, reproducible system. Therefore, experiments were designed to demonstrate the feasibility of an animal based model, and to provide evidence as to which microorganisms are implicated in lethal peritoneal infections. Mice were injected with feces contained in various suspending agents, and in different combinations and dilutions. This provided a means for in vivo enrichment of peritoneal, pathogenic microorganisms, and also allowed the examination of the effects of peritoneal injections of known pure cultures of bacteria.

Adult albino mice were used. Feces were obtained from the ceca of albino mice, pooled, and diluted to a final concentration of 20% (wet wt./vol.) in normal saline. Inocula were prepared in suspensions of 1.0% and 0.25% agar, 10% gelatin, 10% cornstarch, 10% mineral oil, and normal saline; the fecal concentration was 10%, 1.0% or 0.1%, with saline as a control. In each experiment, 5 to 10 mice were injected intraperitoneally with 0.25 ml. of the inocula. Data were obtained on time, gross peritoneal pathology, and bacterial flora of the peritoneal cavity at death. Exudates were obtained from all the mice that died. These were cultivated under selective conditions in order to isolate and identify the microorganisms. Plating procedures were designed to provide both presumptive identifications and quantitative estimations of the various bacteria present.

The data obtained from the culture procedures are summarized as follows: (1) No gram-positive anaerobic forms (Clostridia) were isolated with consistency or in significant numbers from any of the exudates. (2) All peritoneal exudates yielded Escherichia coli, Aerobacter aerogenes and one or more species of Proteus; these species were present in the exudates in numbers in excess of 10^6 per ml. (3) Several exudates yielded the following organisms in possibly significant numbers (10^5 per ml or greater): Streptococcus faecalis, E. freundi, Pseudomonas aeruginosa, Staphylococcus epidermidis and typable strains of Klebsiella pneumoniae. This latter organism, differentiated from A. aerogenes on the basis of capsule formation and typing reactions, was found to predominate in exudates obtained from mice dying after infections of 12 to 24 hours' duration.

The results further indicated that (1) the injection of feces alone in concentrations of 10 per cent induced lethal peritonitis. (2) The injection of feces mixed with agents which could undergo enzymatic degradation or be cleared by the RES (gelatin, mineral oil) could also induce peritonitis but, as with suspensions of feces in saline, only in high concentrations. (3) The injection of feces suspended in agents which were not susceptible to enzymatic degradation or which could act as blocking agents in the RES (starch, agar) could induce lethal peritonitis at lower concentrations (1.0 per cent). Further, the infections were of relatively longer duration (18-24 hr.); this latter aspect was preferable to short-term infections (6-12 hr), in that, the various physiologic parameters

associated with shock, and attributed to such infections, could be better monitored. (4) Data obtained from these experiments revealed the following: In the model employed, such anaerobic organisms as Clostridia appeared to play no role in the induced peritoneal infections; in contrast, but in agreement with the literature concerning peritoneal infections in man (excluding infections restricted to the lumina of various portions of the gut), the predominant microorganisms isolated from such infections were members of the gram-negative enteric group. The significance of these results bears directly on the pos ible role of bacterial endotoxins in shock syndromes associated with massive peritoneal infections,

(2) Studies on the agents responsible for the experimental peritonitis: Further studies were conducted to isolate all possible agents causing peritonitis. In these experiments, exudates from lethal peritoneal infections were used as the inocula. Exudates from the different experimental groups were pooled, diluted to 4 ml and separated into two unequal volumes 2.5 ml and 1.5 ml. Albino mice, 10 per group, were injected with 0.25 ml of one pool, and an additional group of 5 mice were injected with 0.25 ml from the second volume which had been heated to 80°C. for 15 minutes. The latter heat-treated exudate group served (1) as controls for the unheated exudate group, (2) as a method of enrichment for the isolation of clostridia, and (3) as a screen for the detection of endotoxin present in the original exudates. The parameters studied were the same as in previous experiments.

Although isolation procedures were specifically designed to assure detection of any Clostridia present, none were found in significant numbers. One isolation of an unidentified species of Clostridium was made from an isolated lesion found in the liver of a 24-hour survivor. Several animals in experiments similar to that described above, however, yielded exudates containing relatively high numbers of Streptococcus faecalis.

The principal objection to the use of fecal suspensions is the lack of microfloral uniformity; therefore, the effects of injections of pure cultures of bacteria were determined. In addition, reports in the literature indicated that erythrocytes or hemoglobin had the ability to enhance virulence of otherwise nonpathogenic bacteria. Experiments were designed in which pure cultures of several species of enteric bacteria were injected with hemoglobin and other suspending vehicles.

The results of these experiments are as follows: (1) Injection of pure cultures in saline produces lethal peritoneal infection only if the challenge inocula are massive (10^7 or greater). (2) Injection of pure cultures combined with various suspending agents produces lethal infection only if, first, a heavy inoculum is used and, second, the suspending agent cannot be effectively cleared without producing a blocking effect of the RES (10^6 or greater). (3) Use of hemoglobin as a suspending agent will produce a lethal peritoneal infection in nearly all combinations of per cent agent and assayed microorganisms and at bacterial

counts of inocula below those required with other agents or vehicles (10^5 or greater). (4) A successful peritoneal infection model can be developed through the use of either pure cultures or pooled cultures in such suspending agents as hemoglobin, agar and starch.

(3) The establishment of the Escherichia coli-erythrocyte system for the study of peritonitis: Bacteria, nonlethal when injected intraperitoneally, can become lethal if certain adjuvants are added. Previous studies (Davis and Yull, J. Trauma, 2:291, 1962) have shown that a combination of red blood cells and E. coli is lethal to rats and dogs when inoculated intraperitoneally. Since this combination is not unusual in abdominal trauma, studies were undertaken to characterize this mixture for peritonitis studies.

Male rats, 250-260 g, were used and were fasted, but allowed water ad libitum, 12 hours before challenge. Escherichia coli, 0111:B 4, were grown on Bacto nutrient broth (.8%) with added washed RBC. The final hemoglobin concentration was adjusted to 4 grams per cent by the cyano-methemoglobin method. The E. coli-erythrocyte mixture was incubated 18 hours at 38°C, thoroughly mixed and injected intraperitoneally. The volume of inoculum was 5 ml/kg body weight, contained 10^8 organisms/ml and hemoglobin concentration of 4 grams per cent. Control animals were given nutrient broth-RBC mixtures, or E. coli in nutrient broth containing no RBC. The animals were observed for moribundity, diarrhea, and general physical condition. In addition, the animals were weighed, then anesthetized with ether and a blood sample was drawn from the inferior vena cava. Peritoneal exudate, when present, was removed aseptically and transferred to sterile tubes. If organ weights were desired, the organ was excised, wet weight taken immediately, and the organs dried at 65°C for 48 hours in a vacuum oven.

The combination of E. coli (10^8) and hemoglobin (4 g%) was uniformly lethal when injected intraperitoneally, death occurring between 20 and 24 hours. In the experimental animals that died, large quantities of peritoneal exudate were present. Mortality decreased proportionately when the number of organisms was decreased, or when the hemoglobin concentration was less than 1 gram per cent. No deaths were observed in controls although the animal receiving E. coli (10^8) in nutrient broth appeared ill for 24 to 48 hours following injection. It was found that RBC from outdated human blood were nontoxic, and therefore all subsequent experiments were performed using the RBC from fresh rat blood.

(4) Results of initial studies using the Escherichia coli-erythrocyte system: The experimental animals were subjected to gross observations and some physiochemical measurements were performed. The organs of the animals were altered in both weight and appearance. The spleen and kidneys were enlarged and distended with blood. The gastrointestinal tract was hemorrhagic with the vessels dilated. The liver was enveloped in a granular film or membrane, which was easily removed, leaving a normal appearing surface.

The animals were difficult to bleed, 18-24 hours after injection, because of rapid clotting and vein collapse. Therefore, only minimal amounts, 1 to 2 ml of blood could be withdrawn; this is in contrast with the 5 to 10 ml that can be withdrawn from the control animals. The hemoglobin content of the blood increased with time after injection until death. There was also a gradual decrease in blood pH and lowering of the oxygen saturation. Serum potassium levels showed slight elevation but the serum sodium levels were normal.

The fact that comparatively large quantities (4 to 5 ml) of peritoneal exudate were present, contrasted with the few ml of blood available, motivated a shift of interest to this fluid. The original E. coli-hemoglobin infusion is absorbed in several hours. By the 12th hour after injection, a light brown fluid is found in the peritoneal cavity, which increases in volume with time. The pH of this fluid ranges from 6.9 to 7.3. The lethality of this exudate was tested in several experiments. In all instances, the exudate was more lethal volume for volume than the injected combination of E. coli and RBC. The bacterial content of the exudate is now being investigated. The lethality of the exudate is not affected by freezing, heating at 60°C for one hour, or lyophilization, but can be nullified by agents which precipitate protein. Further studies are planned on the physiological and biochemical response of the animal to the combination of E. coli and RBC and to the exudate. Also, studies will be undertaken to isolate and identify its toxic components.

(5) Examination of the role of bacterial endotoxins in shock associated with or induced by bacterial peritonitis: The demonstration of a toxin in the plasma, exudate and other fluids or organs is essential to the examination of the pathologic or pharmacologic role of bacterial endotoxins in shock. Accordingly, emphasis was placed on the development of an endotoxin assay. Due to the limitation of physical and environmental requirements, the chick embryo assay method was selected. Experimentation is now being undertaken to quantitate this bioassay procedure. Two phases of the investigation are now underway: (a) determination of the age of embryo most appropriate for bioassay, and (b) standardization of the assay by determination of the LD_{50} of known endotoxins.

The effects of age on the lethality of endotoxins was determined using White Leghorn embryonated eggs and purified endotoxin. The embryonic eggs were from 7 to 13 days old. The endotoxins concentrations ranged from 0.01 ug/0.05 to 100 ug/0.05 ml. The eggs were prepared as follows: After candling, chorioallantoic veins were identified, and the location marked on the shell of the egg. Windows, ½ inch square, were then cut in the shell over the vein, and the exposed shell membrane cleared with sterile mineral oil and painted with tincture of iodine. Using a 27-gauge needle and tuberculin syringe, 0.05 ml of the saline-endotoxin solution or saline control was injected into the vein. Eggs in which marked bleeding immediately after injection occurred were discarded. Following inoculation, the windows were sealed with melted paraffin and

the eggs returned to the incubator. The observations included (1) time of death of embryo, (2) gross appearance of the embryo at death, (3) appearance of the brain at death; and (4) the appearance of selected vital organs.

Technical problems associated with intravenous inoculation of the 7- to 9-day embryo made interpretation of results difficult. However, even in embryos succumbing to experimental artifacts (hematomas, etc.), the 7- to 9-day-old group exhibited little or no indication of endotoxin effects. Sufficient data were obtained for the 11- and 13-day-old embryos to permit approximation of LD_{50} values.

The same experimental procedure was used in expanded determinations of LD_{50} values. These tests were performed on the 10-to 11-day and the 12-to 13-day embryos. Poor correlation was found when these results were compared with the results from previous experiments. Check with supply agents revealed that the embryos, in fact, were 14 days old. The embryo of this age has been found refractory to both lethal and toxic effects of endotoxin.

The results of experiments, using embryonated chicken egg, in the development of a bioassay for endotoxin may be summarized as follows: (1) The lethal effects of endotoxin in the chick embryo are interdependent upon embryonic age and dosage. (2) The optimal age for the bioassay appears to be 10 - 11 days. At the 13th day and beyond of embryonic development, the chicken embryo manifests a marked increase in resistance to both the lethal and the toxic effects of endotoxin. (3) Approximate LD_{50} determinations for E. coli 0111:B4 endotoxin in embryonated chicken eggs for application in bioassay are as follows:

10-11 day embryos, ca. 0.01 gamma
11-12 day embryos, ca. 0.05 gamma
12-13 day embryos, ca. 0.1 gamma
13+ day embryos, in excess of 1 gamma

II. The use of adhesive polymers for supplanting or implementing suture techniques:

The management of wounds of violence in large numbers could be facilitated tremendously by a procedure for the rapid repair of vascular and organ injuries. The self-polymerizing plastics, especially the alkyl-2-cyanoacrylates, have shown promise as tissue adhesives. Methyl-2-cyanoacrylate has been used in restoring vascular continuity and nonsurgical suture of other tissues but is highly necrotoxic. The Department has continued and expanded studies on ester homologues of 2-cyanoacrylates polymers. Investigation has included their general properties, bonding strength, histotoxicity and uses as skin and tissue adhesives. The polymers were synthesized by the U. S. Army Medical Biomechanical Research Laboratory.

(1) Studies comparing the properties and histotoxicity of cyanoacrylate tissue adhesives: The potential value of a biologically innocuous tissue adhesive prompted extended studies of the biophysical and biological properties of the alkyl-2-cyanoacrylates. The hypothesis was examined that lengthening of the alkyl side chain would result in a more flexible and biologically acceptable adhesive. Areas of study were heats of polymerization, bond strengths, and histotoxicity.

The heats of polymerization were performed on the mesentery of an anesthetized rat. A thermistor probe and a capillary tube containing 0.01 cc. of the monomer were placed on either side of the moist mesentery. The methyl-2-cyanoacrylate caused a 4°C temperature rise in less than 10 sec., the hexyl and decyl produced only a 2°C rise in 20 and 80 sec. respectively. It was concluded that the heat generated by polymerization on surfaces such as the mesentery probably represents a minor factor in the histotoxicity of these materials.

The bond strengths of a series of ester homologues of cyanoacrylate were compared. The dermis was chosen as representative of adhesive strength in tissue. The epithelial surfaces of split-thickness skin grafts were fastened to 1 cm^2 metal blocks with an alkyl-2-cyanoacrylate, 0.01 cc. of the monomer forming a cyanoacrylate-dermis-cyanoacrylate bond between the metal blocks. Bond strength measurements were made on an Instron test device advanced at the rate of 0.05 in./min. The results of 10 determinations per compound are summarized in Table 1. E-910 is a commercial preparation of methyl-2-cyanoacrylate containing stabilizers and plasticizers.

Table 1. Comparison of bond strength of the ester homologues of 2-cyanoacrylate.

Ester homologue	Bond strength ($gm/cm^2 \pm S.C.$)
methyl-2-cyanoacrylate	1072 ± 35
E-910	1212 ± 75
ethyl	1347 ± 88
butyl	1533 ± 69
hexyl	1178 ± 87
octyl	1464 ± 55
decyl	920 ± 40

It was concluded that all the cyanoacrylates tested possessed bond strengths sufficient enough for biological adhesiveness.

Histological studies were performed using adult male rats. The inflammatory responses to methyl, hexyl, and decyl-2-cyanoacrylate were examined from 6 hr. through 63 days at subcutaneous, hepatic, and humoral marrow cavity implant sites. The methyl homologue was found to be intensely and persistently necrotizing and pyogenic, while hexyl and decyl homologues elicited a foreign-body granuloma response preceded by

transient inflammation. In vitro polymerization of the compounds only minimally modified these effects.

In an attempt to provide quantitative estimates of histotoxicity of cyanoacrylates for fibroblasts, which are extremely hardy cells, and to remove the variable of in vivo polymer fragmentation, standard size and weight polyvinyl alcohol sponge discs were implanted subcutaneously in rats. Two control sponges were dipped in saline; paired experimental sponges contained weighed amounts of methyl-, hexyl-, and decyl-2-cyanoacrylate monomer, or Eastman 910. All sponges were permeable and wet weights of control and experimental sponges were statistically alike. Hydroxyproline content and histological characterization of both control and experimental sponge pairs were obtained at 14 days. Methyl- (or E-910) inhibited hydroxyproline elaboration and fibroblastic proliferation within sponges. Hexyl- and decyl- sponges were strikingly different from methyl-sponges in that neither hydroxyproline content nor fibroblastic proliferation were depressed.

Thus, it has been shown that the extreme histotoxicity associated with methyl-2-cyanoacrylate does not characterize the higher homologous hexyl- and decyl-2-cyanoacrylate. Continuing investigation of the biological properties of the cyanoacrylates may ultimately yield acceptable tissue adhesive.

(2) The use of methyl-2-cyanoacrylate for adhesive closure of skin incisions: The effectiveness of methyl-2-cyanoacrylate as an adhesive closure for skin incisions was compared to standard suture techniques. Standard 5-cm dorsal skin incisions in paired rats (4 groups of 10 each) were closed alternately with five #36 stainless steel wire sutures or adhesive painted over the apposed skin edges. Closure by chemical adhesion was much more rapid, and resulted in a more stable accurately apposed wound. The paired animals were sacrificed at 7 and 14 days postoperatively, while the sutures were removed from the control group 6 days postoperatively. Wound separation did not occur in either group. Wounds were examined grossly, histologically, and by tensile strength techniques.

Incisions closed with methyl-2-cyanoacrylate appeared significantly stronger at 7 days than incisions closed by stainless steel wire sutures. Increased strength was noted also at 14 days, but the results were not statistically significant at the 5% confidence level. The adhesive-closed incisions, in addition, were cosmetically more acceptable because of the absence of cross-hatching scars by sutures. Histologic differences between the adhesive and stainless steel sutured tissues were not significant. The increased strength of the adhesive closed incisions could be explained by either better approximation of skin edges and splinting of the wound, or a nonspecific inflammatory reaction in the wound to the plastic. It has been observed by others that wounds irritated by ground glass and other substances show increased tensile strength. The initial strength of adhesive closures are considerably weaker than

incisions closed by standard suture techniques (28 g/linear mm compared to 1700 g/linear mm), therefore an adequate subcutaneous closure is necessary to avoid tension at the suture line. In all animals, approximation of the panniculus carnosus was necessary for adequate closure. It can be concluded that adhesives have a possible application for the closure of skin lacerations, particularly where cosmetic considerations are important and for mass casualty situations.

(3) The use of methyl-2-cyanoacrylate for sealing pancreatic wounds: Methyl-2-cyanoacrylate was evaluated in respect to its potentialities as a surface sealing agent in wounds of the pancreas, produced in cats. In both the control and the experimental animals, a one centimeter portion of pancreas was resected from the greater pancreatic lobe. The open ends of the control animals were left untreated, while the ends of the treated group were sealed with methyl-2-cyanoacrylate. The animals were sacrificed at one, seven, and thirty days, and examined grossly and histologically. The control animals showed evidence of pancreatic juice leakage as demonstrated by intraperitoneal fat necrosis, pseudocysts, and cutaneous fistulas. The treated animals sacrificed at the same time intervals, developed no pseudocysts or cutaneous fistulas, and showed only minimal and infrequent areas of intraperitoneal fat necrosis. Histologic sections showed surface necrosis of the pancreatic tissue adjacent to the plastic adhesive. Thus, methyl-2-cyanoacrylate proved an effective surface sealing agent for pancreatic wounds, even though some surface necrosis resulted.

III. Studies on the mechanisms of wound healing:

The Department has continued a diversified approach to better understanding of the basic processes of wound healing. Previous progress reports have detailed the standard technique employed, and given extensive data on the strength and histologic appearance of healing incisions. The establishment of criteria for normal wound healing has allowed a comprehensive approach to factors which influence the healing of an incision. The obvious importance of bacteria in colon healing has initiated a study of the effect of antibiotic controlled microflora on healing. The healing of skin incisions has been studied in respect to time after operation, age of the animal, and thickness of the skin, and now another factor, orientation of the incision has been examined. In addition, evaluation of the American Vascular Stapler for anastomosing small blood vessels is under investigation.

(1) The effects of microflora and antibiotics on colon wound healing:

a. As reported initially neomycin at an oral dose level of 160 mg/kg/day had no beneficial effect on healing of the anastomosed colon in the rat. Groups of rats with colonic resections were then administered two and four times the above dosage of neomycin. The animals were sacrificed at 4 and 11 days postoperatively. Fecal cultures showed only minimally increased bacterial suppression when compared with the previous dosage, and this was limited primarily to the gram-positive enterococcus

group of enteric bacteria. Biophysical and morphologic studies on the healing colonic wounds after massive doses of oral neomycin revealed significantly weaker anastomoses, as well as a histologic picture of marked submucosal edema and round cell infiltration.

b. The effects of other agents and conditions upon colonic healing were also investigated. Experiments were performed using 10 treated and 10 control rats each to study the effects of (a) oral neomycin (160 mg/kg/day), (b) oral bacitracin (20,000 u/kg/day), (c) combined oral neomycin and bacitracin (same dosages), (d) colonic healing in the germfree rat, and (e) preliminary fecal stream diversion (colostomy). All animals were sacrificed on the 7th postoperative day. Based on prior experience, this seemed the most optimal time for both biophysical and morphological studies. Quantitative bacteriologic studies were routinely performed on fresh stool specimens to monitor the effects of intra-enteric antibiotics. Stool antibiotic assays also were performed. Germfree animals were likewise monitored to detect unexpected bacterial contamination. In the fecal diversion-group, a preliminary end-transverse colostomy was created 3 weeks prior to resection and anastomosis of the distal colon.

Bacteriologic data on the 2 days antibiotic-treated and control animals on cultures taken the day of surgery are shown in Table 1. Cultures taken at other time intervals gave essentially the same results. Germfree animals remained uncontaminated throughout the course of the experiment. Assays of stool specimens for antibiotic activity in the drug-treated rats showed levels of 450 $\pm$ 45 mcg neomycin per gram feces and 226 $\pm$ 43 units bacitracin per gram feces.

The postoperative course was uneventful, with one exception. All but one of the conventional animals which were used as controls in the germfree experiment, died postoperatively primarily as a result of peritonitis. This can be attributed to technical difficulties in performing the colonic anastomoses in the germfree tanks, and to the use of a different and smaller strain of rat (Fisher vs. Walter Reed) for this experiment. All but 3 of the germfree animals survived indicating a degree of protection provided by the germfree state. In addition, one of the bacitracin treated animals suffered an abdominal wound dehiscence on the second postoperative day and had to be sacrificed. The small number of minor complications (partial intestinal obstruction, peri-anastomotic abscesses) appeared to be evenly distributed between the treated and untreated control groups.

At sacrifice, there was a slight tendency toward a decrease in peritoneal reaction at the anastomotic site, adhesion formation and anastomotic edema in both the neomycin and bacitracin groups as compared to their controls. In the combined neomycin-bacitracin group, this difference was marked indicating at least some beneficial effect of antibiotic therapy.

The results of biophysical measurements of wound strength as determined by bursting pressure (intraluminal) and breaking strength (linear) techniques are shown in Table 2. The apparent differences noted in the bursting pressure in both colostomy and neomycin-bacitracin tests are not considered meaningful in terms of anastomotic healing. In most cases, the bowel ruptured away from the anastomotic line, which is in contrast with earlier observations. At that time, the rupture was found to occur at the anastomotic line up to 10 days postoperatively. The difference could be related to increased skill in anastomotic construction. It is possible that histological examination, which is only partially complete, will provide more information on structural differences.

Pending histologic studies, results can be summarized as follows: Neomycin, in average dosage range, appears to have little influence upon the pattern of healing in the anastomosed rat colon. Higher doses of neomycin appear detrimental to the healing process. Bacitracin alone likewise appears to have no beneficial effect. The combined use of neomycin and bacitracin has a definite beneficial influence on the degree of peri-anastomotic inflammation, but does not result in increased strength of the healing anastomosis. Both neomycin and bacitracin have a marked effect on the gram-negative and gram-positive aerobic fecal flora; however, neither agent nor combination of the two appreciably affects the predominant intestinal organisms, the anaerobic bacteria. No currently available antibiotic suitable for intestinal "sterilization" is proven to have appreciable effect upon anaerobic enteric bacteria.

Differences in thickness of structure of the bowel wall in both germfree and fecal diversion experimental animals make comparative evaluation of gross and physical healing difficult. Histologic examination in this case is of paramount importance. Moreover, the germfree animals cannot be compared to control animals from other experiments, since a different strain of rat was used. It is interesting to note, however, that the strength of the colonic anastomoses in germfree animals had almost reached normal strength in 7 days as contrasted with 2 weeks in the conventional rat.

c. Neomycin present in the feces of treated animals was bacteriologically assayed. It was found that only 5% of the administered neomycin could be recovered in the feces. Serum and urine assays showed that intra-enteric neomycin was not absorbed. Subsequently it was shown that neomycin was inactivated even when mixed with feces in vitro. The inactivation was found to be directly proportional to the concentration of fecal matter in suspension. Suspensions of human, dog, and guinea pig feces caused similar inactivation of neomycin. In fact, a wide variety of substances including starch, cellulose, dextran, albumin, and bacterial suspensions depress neomycin antibacterial activity. Pre-sterilization of feces had little effect on suppression of activity. Removal of the fecal particulate matter by filtration markedly lessened the inactivation; in addition, at least part of the unassayed neomycin was recovered from fecal suspensions by centrifugation and resuspension. Although

the exact mechanism of inhibition of neomycin cannot be elucidated at this time, a reversible physical absorption of the antibiotic to a wide variety of organic substances appears likely although enzyme or other degradation of drug cannot be excluded.

Table 1. The Effect of Neomycin and Bacitracin on the Fecal Flora of the Rat.

Organisms[1]	Concentration of Organisms per Gram of Wet Feces[2]			
	Control	Neomycin 160 mg/kg/d	Bacitracin 20,000 u/kg/d	Neomycin plus Bacitracin
Total Aerobes	2×10^9	2×10^6	6×10^9	4×10^6
Total Anaerobes	3×10^{10}	3×10^{10}	6×10^{10}	1×10^{10}
Anaerobic Spores	4×10^3	1×10^3	1×10^3	1×10^3
Coliforms	3×10^5	10^3	8×10^8	3×10^4
Non-lactose Fermenting Enterobacteria	5×10^5	10^3	1×10^9	10^3
Total Gram (+) Organisms	8×10^8	1×10^5	5×10^5	10^3
Staphylococci	6×10^3	10^3	1×10^4	10^3
Enterococci	3×10^6	3×10^3	8×10^4	10^3
Yeasts	8×10^2	3×10^3	2×10^5	1×10^5

[1]Bacteriological criteria used for classification or cataloguing and identification of organisms.

[2]Values represent the mean of multiple determinations. Standard error of the mean approximated ½ log in most cases. Control values did not differ significantly between experiments.

Table 2. Healing Strength of Colonic Anastomoses in the Rat (7 Days)

Group	No. of Animals	Bursting Pressure (mm Hg)	Breaking Strength (grams)
Neomycin Rx	10	210 ± 15	54 ± 3.6
Control	10	228 ± 28	62 ± 5.0
Bacitracin Rx	10	260 ± 14	71 ± 3.9
Control	10	261 ± 17	59 ± 3.8
Neomycin plus Bacitracin Rx	9	253 ± 12	49 ± 3.7
Control	10	208 ± 7	53 ± 4.3
Fecal Diversion	10	141 ± 24	68 ± 5.
Control	10	196 ± 14	69 ± 5.2
Germfree	10	217 ± 24	41 ± 4.5
Germfree Normal Unoperated Bowel	10	217 ± 10	48 ± 4.4

(2) Effects of orientation on the healing of incisional wounds in rats: Previously the breaking strength, skin thickness, and tensile strength of transverse and longitudinal dorsal skin incisions two and three weeks postoperatively were reported. Long-term studies now have been completed which further delineate the factors of incisional orientation as a parameter of healing strength. Breaking and tensile strength values for longitudinal and transverse orientated incisions were compared with normal skin and to each other. The results for 1 and 3 weeks postoperative specimens showed that longitudinal dorsal skin incisions (perpendicular to normal skin "wrinkle lines") were stronger than transverse incisions (parallel to "wrinkle lines"). The microscopic pattern of junctional repair between newly formed and pre-existing collagen was strikingly different in the two planes, and could be a possible explanation for the observed results.

The long-term effect of orientation on the strength of healing incisional wounds was extended to 6 and 15 weeks postoperative. The thickness, breaking strengths and tensile strengths of transverse and longitudinal skin incisions of experimental and control animals were compared. The differences in incisional strength between the transverse and longitudinal incisions were even more striking than in younger wounds. Longitudinal incisions were less thick yet stronger. The tensile strength at 15 weeks reached the strength of normal skin, while the transverse incision had achieved only 65% of normal skin strength. By extrapolation,

it would appear that approximately one year would be required for transverse incision to achieve normal skin strength.

These studies confirm earlier findings that orientation of skin incisions is important in the healing of incisional wounds in rats. Therefore, in addition to time, age of animal, and thickness of skin, orientation of incision with respect to skin lines must be taken into consideration. The thinner, stronger longitudinal incisions form smoother interdigitations between new and old fibers, which probably account for their increased strength.

(3) Evaluation of a vascular stapler for anastomosing small blood vessels: During the last year, we have been testing an American-made vascular stapler. The instrument, modified from the Russian instrument, uses staples from a pre-loaded sterile cartridge. The stapler has been tested on vascular anastomoses in renal transplants of primates and contrasted with surgical sutures and tissue adhesives on the aorta of the dog.

Use of the vascular stapler for renal transplantation in primates, conjointly with the Department of Surgical Physiology, was done. The stapler was used to construct arterial and venous anastomoses incident to auto- and homotransplantation of kidneys in 22 monkeys. The kidneys were placed in the pelvis. Arterial (1.0-2.2 mm) and venous (2.8-5.0 mm) anastomoses were constructed utilizing common iliac vessels. In the 16 animals which survived longer than 24 hours vascular anastomoses were successful in only 5 instances. The high rate of failure (70%) was attributed to discrepancies in size between renal and iliac vessels, the extremely small size of the renal arteries, and the short length of the renal vessels. The physiologic and pathologic data derived from the successful transplants is reported by the Department of Surgical Physiology.

A program was initiated to contrast the gross and histologic characteristics of small vessel anastomoses constructed with the stapler, hand suture, and adhesive polymer. The trifurcation of the dog's aorta was used as a test site so that all three methods of anastomosis could be constructed in a single animal. To date, there has been a high incidence of thrombosis associated with use of the plastic adhesive for anastomosis. The stapler and manual suture methods appear uniformly successful. The stapled anastomosis, however, was much easier and quicker to perform. The use of autogenous vein grafts to bridge vascular defects, is planned as an extension of these studies.

IV. Bilirubin metabolism and excretion during biliary diversion and obstruction:

All the bilirubin constantly excreted via the hepatic ductal system into the alimentary canal cannot be quantitatively accounted for by fecal urobilinogen or other known metabolic products. Experimental models have

been designed to study bilirubin metabolism, and to determine the nature of the breakdown products and their possible reabsorption. The initial studies were performed in dogs with constructed circular intestinal loop which were anastomosed to their gallbladder; later studies were conducted in monkeys with induced complete common duct obstruction.

(1) Studies on bilirubin metabolism in dogs with biliary diversion: The metabolic pathways of bilirubin metabolism and its products have not been determined. Experiments were designed using radioactive C^{14} bilirubin to ascertain whether that bilirubin, unaccounted for by fecal bile pigments, is reabsorbed from the gastrointestinal tract or is broken down to heretofore unknown metabolites. Dogs were prepared surgically by the construction of a circular intestinal loop which was anastomosed to the gallbladder. During the same operation, the common ducts were ligated and their gastrointestinal tracts reconstructed. The bile, therefore, was shunted into an intestinal loop, and the only route of egress was the vascular-lymphatic system. The serum bilirubin of these animals was consistently below 0.8 mg%. Radioactive C^{14} bilirubin was injected intravenously into the animals 2 weeks postoperatively. The tagged bilirubin was cleared rapidly from the blood stream; its biological half-life fell between 2.5 and 5 hours. After 12 hours, little or no radioactivity remained in the blood, and only small amounts were detected in the urine and feces. The animals were sacrificed one week later, and at that time 49% to 65% of the injected radioactivity was found in the contents of the circular loop. Attempts to extract and identify the radioactive metabolites are presently underway.

(2) Studies on bilirubin metabolism in the primate with complete common duct obstruction: The metabolism of bilirubin in the primate, under conditions of complete common duct obstruction, has been reported to resemble closely that of man, and to differ significantly from that of the dog. The primate with complete common duct obstruction shows a gradual increase in serum bilirubin until a plateau which is stable is reached. The dog, by contrast, shows an increase in serum bilirubin, a transient plateauing, then a return to near normal pre-obstruction levels. This species difference has been attributed to distinctive enzyme systems.

Monkeys were prepared for experimentation by ligating the common duct. If the gallbladder was left intact, it was found that it takes an average of seven days to produce a serum bilirubin elevation of 1 mg%. If the gallbladder is removed, however, the bilirubin level is elevated 24 hours following common duct ligation. The stable bilirubin level is reached in about two weeks and is in the range of 10 mg%. Urine and stool urobilinogen, bilirubin, and pentdyopent determinations performed at this time accounted for only one-half the theoretical bilirubin production. Experiments are underway in which intravenously injected radioactive bilirubin will be used to follow the degradation of bilirubin and the characterization of its metabolites.

V. The effects of the sulfonamides on metabolic processes:

Sulfonamide therapy can affect markedly the metabolism of the recipient. The organ particularly susceptible is the thyroid gland, and especially its production of thyroxine. The nature of sulfonamide inhibition of thyroid function has been the subject of continuing investigation, but the mechanisms involved remain obscure. Since the sulfa-drugs are widely used therapeutically, the Department had conducted further studies on their pathophysiology and metabolic effects. Investigations directed toward the effect of sulfonamides on the thyroid gland included: (1) mechanism of inhibition, (2) time required for demonstrable histologic and biochemical effects, (3) reversibility of inhibition, and (4) compounds to protect against goitrogenicity. In addition, other studies were done on the effects of sulfaguanidine and iodide on the submaxillary gland. As a result of these studies, an improved method of chromatographing the iodoaminoacids of the thyroid gland was developed.

(1) The mechanism of sulfaguanidine inhibition of the thyroid gland: The antithyroid activity of sulfonamides has been postulated to result from the formation of molecular complexes with iodine. To test this postulate, iodinated compounds of sulfaguanidine were synthesized in the Department (Progress Report 1962-63) in an attempt to distort the normal electron configuration, and hence to minimize the formation of iodo-organic complexes.

Sulfaguanidine (SG) (2% in basal diet), monoiodosulfaguanidine (MIS), and diiodosulfaguanidine (DIS) were fed in equimolar quantities to groups of rats. Controls received 20 g daily of unaltered basal diet, and all groups received tap water ad lib. Body weight and food consumption were recorded daily. Urine and feces were collected separately for each 24-hr. period. After 4 collection periods, the animals were sacrificed by carbon dioxide asphyxiation, and the thyroid, submaxillary glands, adrenals, pancreas, kidneys, and testes were removed for histologic examination. Sections were stained with hematoxylin-eosin and PAS-hematoxylin. Urine and feces of both control and experimental animals were analyzed concurrently for sulfonamides.

The inclusion of SG, MIS, and DIS in the basal diet did not affect food intake or body weight. Intestinal absorption of the different compounds, as indicated by urinary excretion, was altered substantially by iodination. Urinary excretion of sulfaguanidine averaged 26% of the injected dose; in contrast only 2% of the MIS, and 0.5% DIS appeared in the urine.

The thyroid glands showed no significant increase in weight in any group. Histologic examination of the thyroids, however, revealed morphologic dissimilarities. Thyroid glands from the SG animals revealed characteristic early hyperplasia with interstitial vascularity, colloid resorption, and reduced follicle size. Follicular epithelium was

hypertrophic with large pale nuclei and occasional papillary projection. In contrast, the thyroids of control, MIS and DIS animals showed larger, more uniform-sized follicles, retained colloid, flatter epithelium, denser nuclei, and less vascularity. Testes, kidneys, adrenals, submaxillary glands, and pancreas of all groups were indistinguishable.

The low intestinal absorption of MIS and DIS precluded assessment of their goitrogenicity. Similar experiments utilizing more soluble sulfonamides are planned. It was found, however, that MIS and DIS were not diiodinated in vivo as demonstrated by spectroscopic analyses on the reacted extracts of excreta. The spectral absorption of the reacted sulfaguanidines are maximal at 545 mu (MIS) and 505 mu (DIS) compared with 550 mu for SG.

(2) Time required for sulfaguanidine to produce histologic and biochemical changes in the thyroid glands: Sulfaguanidine (SG) (2% of diet by weight) rapidly produced observable histologic changes in the thyroid gland. In just 2-3 days histopathologic changes are noted like those described in Section V (1). To possibly correlate histologic changes with the biochemical mechanism associated with thyroxine synthesis, experiments were designed in which groups of rats were sacrificed after varying times on SG, and the histology and production of iodoaminoacids studied. Rats were fed SG and then groups were sacrificed 2 to 7 days. Control groups on basal diet were sacrificed at the same time. One-half of each lobe of the thyroid was removed and sectioned for histologic study. The portion remaining was homogenized, hydrolyzed, extracted, and chromatographed as described under methods, Section V (5).

The histopathologic changes were as noted previously. These changes were evident after 2 days and were even more pronounced with longer therapy. The inhibition of thyroxine synthesis was evident after 3 days by a decrease in the amounts of performed thyroxine (T_4) and triiodothyronine (T_3). There was a progressive loss from 3-7 days in the gland's ability to synthesize iodoaminoacids; the formation of T_4 and T_3 was inhibited first followed by diiodotyrosine (DIT), and finally monoiodotyrosine (MIT). By the seventh day only one compound was found on the chromatograms. This previously unidentified compound was studied intensively. It was the only substance found after prolonged sulfaguanidine treatment, and it increased as sulfaguanidine administration was continued for longer periods of time. Chemical analyses and paper chromatography ultimately revealed it to be tyrosine. The progressive increase of tyrosine in the thyroid of sulfa-drug treated rats suggests either that thyroglobulin synthesis is continuing or that the gland accumulates free tyrosine. Further experiments are planned.

(3) Reversibility of sulfonamide effect on the thyroid gland: Sulfaguanidine completely inhibits iodoaminoacid production by the thyroid gland in 7 days, and causes hyperplasia and histological changes. The permanency of this goitrogenic effect has not been determined.

Groups of 8 rats each were fed either a basal diet or a basal diet containing 2% by weight of sulfaguanidine. Groups of control rats and sulfaguanidine fed rats were sacrificed after 7 and 14 days respectively, while another group was fed sulfaguanidine diet for 7 days then basal diet alone for 7 days before sacrifice. The thyroids were removed and weighed; one portion was removed for histologic studies and the remaining portion processed for iodoaminoacid chromatography. The histological sections have not yet been studied.

Sulfaguanidine administration for 7 days completely inhibited the production of iodoaminoacids, and the gland weights were twice those of controls. After 14 days the gland weight had tripled, and still no iodoaminoacids were observed. The thyroids of rats used in the reversibility experiments showed a slight increase in gland weight, but only two iodoaminoacids, MIT and DIT, were present in the gland. If T_3 and T_4 were present at this time, they occurred in amounts too small for detection. One would conclude from these results that the gland could return to normal function after the administration of sulfaguanidine for 7 days. Further experiments investigating the reversibility of the sulfaguanidine effect are planned.

(4) Studies on compounds which antagonize the sulfaguanidine effect on the thyroid gland: The goitrogenicity of the sulfonamides has been attributed to their structural similarity to tyrosine. It was found, however, that l-tyrosine given orally was totally ineffective in reversing the effects of sulfaguanidine (Progress Report 1962-63). Tyrosine ethyl ester-HCl (TEEH), on the other hand, when given orally in equimolar quantities to animals on a 2% sulfa diet, partially prevented and/or reversed the goitrogenic action of the sulfa drug. The experiment was repeated using larger numbers of animals and the TEEH was increased to double the amount used initially. The results, both histological and biochemical, indicate that an increase in the amount of TEEH, instead of being antagonistic, increased the adverse effects of the sulfa drug. Further work is planned to discover the dynamics of this observed difference.

(5) Two-dimensional chromatography of iodoaminoacids on DEAE cellulose paper: During investigations of inhibition of thyroxine synthesis, by sulfaguanidine, it was found that existing chromatographic methods were inadequate. They gave either incomplete resolution of the iodoaminoacids, or especially in two dimensional systems, compounds that were diffused and difficult to locate. This led to the development of the technique of two-dimensional chromatography on diethylaminoethyl (DEAE) cellulose paper.

The iodoaminoacids of the thyroid gland were released by homogenization in either 0.9% NaCl or 0.1M phosphate buffer pH 8.5, hydrolyzed for 6 hours in a sealed tube with 4N HCl or with pancreatin for 48 hours, and then clarified by centrifugation. The hydrolysate was adjusted to

pH 3 and extracted 3 times with butanol. The extracts were evaporated to dryness, and the residue dissolved in 1 ml of a solution of propylene glycol, diethanolamine and ethanol. The solution of iodoaminoacids was applied to the DEAE paper, and chromatographed ascendingly for 7 hours in each direction. Solvent I was the organic layer of a solution of butanol-acetic acid-water (4:1:5); solvent II was a solution of butanol-ethanol-2N ammonia (5:1:2). The iodoaminoacids were located by spraying with ceric sulfate-arsenious acid, and viewing under ultraviolet light (360 mu). A permanent record was obtained by laying the wet chromatogram, after spraying, on Kodak Verifax Matrix paper and exposing it to ultraviolet light for 3 minutes. The Matrix paper was treated with Kodak acid fixer, washed with water, and dried. The iodoaminoacids appeared as brown spots on a tan background. This chromatographic method, because of the fine resolution of the iodinated compounds of the thyroid, and the compactness of the spots, lends itself well to I^{131} tracer experiments.

(6) Effects of sulfaguanidine and sodium iodide on rat submaxillary glands: The administration of goitrogens has been reported to induce reduction in the relative and absolute gland weights and atrophic degranulation of granular tubules in the rat submaxillary gland. Also, inflammation of salivary glands often follows administration of iodide to those animal species which concentrate iodide in the salivary glands. Since the sulfonamides are unique goitrogens in that their antithyroid activity is potentiated by iodide, studies were performed on the effects of sulfaguanidine, iodide, and sulfaguanidine plus iodide on the submaxillary gland of the rat.

In adult male white rats fed 2% sulfaguanidine diet, augmented in goitrogenic effect by the addition of sodium iodide, no degranulation of the distal convoluted tubules of the submaxillary gland was seen. Animals were examined after one, two and four months. This effect contrasts with that seen following propyl-thiouracil administration, in which degranulation has been demonstrated. When thyroidectomy preceded sulfa administration, tubular degranulation occurred. Submaxillary weights correlated poorly with the observed morphological responses.

These results confirm that at least minimal circulating thyroid hormone is required to maintain submaxillary granular tubules and suggest that the distal convoluted tubule of the submaxillary gland requires thyroid hormone for maintenance which can be synthesized and released by the sulfaguanidine-inhibited rat thyroid.

In this same study isolated submaxillary gland sialadenitis was produced in the male adult white rat, a species which has been shown not to concentrate iodide in the saliva, by diets containing 0.5% or 4% NaI, administered for 28 days. Squamous metaplasia of distal submaxillary gland ducts accompanied the acute inflammatory response. The sialadenitis was ameliorated by concomitant inclusion of 2% sulfaguanidine in the diet.

The study suggests that the rat submaxillary gland responds in a similar way to those of other species to high levels of iodides, and that such levels are produced by massive iodide dosage.

VI. Studies on the sanitization of occupied patient isolators:

The Department of Surgical Metabolism and Pathology provided personnel, equipment, and consultation service to the Dept. of Nursing, WRAIR, for sanitization studies on patient isolators. The prolonged confinement of patients in the isolator system presents a difficult problem of maintaining stable bacteriological conditions. This problem includes the prevention of endogenous contamination in situations where patients have lowered resistance, as well as the protection of attendants and others from infected patients within the isolator. Studies were conducted on the (1) evaluation of germicides for sanitization of patient isolators while in use, (2) determination of the relative antibacterial efficiencies of ultraviolet germicidal lamps used in pass-through locks of the isolators, and (3) monitoring the bacterial flora of patients and isolators during prolonged isolater confinement.

(1) Evaluation of germicides for sanitization of patient isolators during prolonged patient confinement: The physical limitations imposed by the configuration of the tent portion of the patient isolator are such as to preclude routine methods of cleaning or sanitization within the isolator during occupancy. Attendants during such operations are limited in both degree of access and extent of contact to many areas within the tent. Accordingly, routine methods of germicide evaluation were not applicable to this study. New procedures had to be devised that most closely approximated the conditions under which germicides would be employed and the techniques by which they would be applied.

Germicidal agents were first screened for chemical and physical compatibility with the polyvinyl chloride material which comprises the isolator structure. The agents which met this criteria and were at the same time nontoxic, included: Wescodyne, an iodophor (West Chemical Co. Products, Inc.), Tergisyl and Amphyl, phenolic based preparations (Lehn and Fink Products Corp.), SBT and SBT-24, salicylamide based preparations (Lever Bros. Co., Ind. Div.), and Zephiran chloride, a tertiary amine (Winthrop Laboratories). The germicidal activities of the agents were assayed against the following microorganisms; Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Streptococcus faecalis, Candida albicans and Bacillus subtilis. Concentrations of the agents were those recommended for general purpose cleaning and disinfection by the manufacturers.

The procedures used were as follows: A pure culture of the assay organism, usually 24 hours old and containing in excess of 1×10^{10} colony forming units (cfu) per ml was atomized onto sterile polyvinylchloride plastic strips. Both sides of the strip 1 x 18 in. were

inoculated for uniformity. The number of viable organisms on the strip after drying usually averaged approximately 5 x 10^5 cfu per square inch. The agents were assayed by dipping a sterile cotton swab into a solution of the test germicide and, with the excess moisture expelled, wiping the swab over a 5-inch section of the strip with a single forceful back-and-forth motion so that no point on the section was by-passed. Both sides were treated in an identical fashion. The same procedure was repeated on another section of the strip with a swab moistened in saline, thus providing a control for the determination of the effects of the mechanical cleansing action of the technique. The remaining portion of the strip served as the unswabbed control.

After drying for 10 minutes, the three sections of the strip were cut apart, and each section, in turn, cut into five standard 1" x ½" sections. Each section was placed in sterile broth and the viable organisms eluted from the strips by agitation. Each sample tube was then taken through a ten-fold serial dilution series, with aliquots of the different dilutions being plated on infusion agar for subsequent bacterial colony counts. Assay data of the germicidal agents are complete for three of the organisms, E. coli, S. aureus and Ps. aeruginosa.

Tests indicate that a simple mechanical cleansing with saline is sufficient to remove approximately 93% of the organisms. Replacement of the saline with a disinfectant or germicide solution increases removal to approximately the 98-99% level. The combined detergent and germicidal properties of the tertiary amine class of compounds, represented by benzalkonium chloride, proved most efficient in sanitizing the isolator tent.

(2) The sanitization and sterilization of pass-through locks by ultraviolet germicidal lamps: The patient isolator includes two pass-through locks for the introduction and removal of materials. The function of the locks and chambers is to provide (a) surface decontamination or sterilization, and (b) sterilization of air introduced during manipulations at doors. The chambers consist of stainless steel shells containing ultraviolet opaque doors. Each shell is equipped with four General Electric G-8T5 germicidal bulbs, two on the lower surface and two mounted in the top. Maximum direct exposure distance between radiation source and target surface is 12 inches. Experiments were designed which tested the relative killing efficiencies and 100% kill time of the two ultraviolet lamps installed in the top of the shell.

Cultures of S. aureus, E. coli, B. subtilis, S. faecalis, Ps. aeruginosa, C. albicans and P. vulgaris in trypticase-soy broth were assayed by decimal plating procedures and diluted to contain 500 - 1,000 cfu per ml. Dried trypticase-soy agar plates were flooded with 0.5 ml of the diluted inoculum and allowed to dry for 30-45 minutes. These plates were then exposed, in triplicate, to the direct ultraviolet radiation of the upper lamps at a distance of 12" in the chamber. Exposure times ranged from 1 second to 150 seconds for vegetative cells and to 30

minutes for spore suspensions. For spore suspensions, a 48-hour culture of B. subtilis was concentrated by centrifugation, washed with sterile saline and heated to 80°C. for 15 minutes in order to destroy the vegetative cells; the suspension was then assayed for the viable spore cfu count and diluted to a suspension of 500 - 1,000 spores per ml.

Results of the ultraviolet irradiation studies were determined with quantitative plate counts of survivor colonies after 48 hours' incubation at 37°C. Total cfu counts from six unexposed plates, representing the non-irradiated culture controls, were averaged to give the 100 per cent survivor level. The three experimental plates from each exposure period were then counted and averages taken to give the per cent survival level, per unit time of ultraviolet light exposure. This was contrasted with the 100 per cent survivor level of the control plates and the results then expressed in terms of per cent survivals as compared with the unexposed controls.

Ultraviolet exposure for 60 to 90 seconds is sufficient for surface sanitization except for the yeast-like fungi. Candida albicans requires a minimum exposure of 240 seconds under these experimental conditions. Total surface sterilization, including destruction of all viable yeast-like organisms as well as bacterial spores will require an exposure in excess of 30 minutes.

(3) Bacteriological monitoring of patient and isolator flora during prolonged patient confinement: Numerous cultures, which were taken during trial patient-care program, are in the process of isolation and identification. Results will be reported at a later date.

Summary and Conclusions:

The Department of Surgical Metabolism and Pathology has initiated, expanded, or continued a diversified program on the metabolic and nutritional problems associated with injury. Long term investigations have been initiated on the identification of the factors responsible for lethality in generalized peritonitis. Two experimental models have been examined; one based on quantitation of the intestinal microflora of peritonitis, the other based on a system utilizing a known combination of E. coli and hemoglobin. The results suggest that ideal vehicle for mouse and rat peritonitis studies would be hemoglobin, and a bacterial challenge of E. coli, Proteus spp., A. aerogenes, and A. faecalis. Repeated experiments failed to demonstrate a significant role for anaerobic species of bacteria.

The role of bacterial endotoxins in lethal peritonitis has been investigated, and an egg embryo assay method for endotoxin evaluated. Preliminary experiments on the physicochemical properties of lethal peritoneal exudate implicate endotoxin. Other physiological and biochemical parameters associated with peritonitis are under investigation.

The use of adhesive plastics for the coaptation of wounds was also evaluated. The self-polymerizing plastics, alkyl-2-cyanoacrylates, were found to possess the essential properties, and the less toxic higher ester homologues were particularly promising. Further studies are required, however, to fully examine the biological behavior of these plastics.

The Department has extended and broadened its investigations into the basic mechanisms of wound healing. The roles of endogenous microflora and oral antibiotics in colonic healing have been continued. Oral doses of neomycin, bacitracin, or a combination of the two had no striking effects on the speed of healing or the strength of colon anastomoses. However, the combination of neomycin and bacitracin may have been influential in decreasing peritoneal reaction at the anastomotic site. The predominating fecal organisms, the anaerobic bacteria, were not suppressed by the antibiotics studied. Results in germfree animals did not differ significantly from antibiotic-treated or conventional animals in respect to rate of wound healing. Neomycin activity was found to be substantially reduced by binding with fecal matter.

The dermal incisional wounds, when orientated longitudinally (perpendicular to skin "wrinkle lines"), were thinner and stronger than those of transverse incisions. In addition, they reached maximum strength in a much shorter time than comparable transverse incisions. The parameter of orientation of dermal incisions, therefore, must be considered in the assessment of wound healing studies along with time, age of animal, and thickness of skin.

An American-made vascular stapler proved to be effective when used for anastomosis on the trifurcated aorta of the dog. The anastomoses were technically easy and rapid. Limitations are imposed on its use with smaller-caliber vessels and those of insufficient length. Further studies are in progress.

Studies on the pathways of bilirubin degradation were initiated using dogs with a cholecysto-ileostomy and primates with common duct obstruction. The fate of intravenously injected radioactive C^{14} bilirubin and identification of the formed metabolites is being investigated.

Studies on the mechanisms of sulfonamide inhibition of thyroid gland function were continued. The goitrogenic properties of these drugs result from their inhibition of the production of the iodoaminoacids used to synthesize the hormone, thyroxine. Their action is exceedingly rapid; histologic changes in rats are evident in 2 days and biochemical changes are noted in 3 days, after oral therapy. The production of iodoaminoacid is apparently eliminated by 7 days. Upon removal of the sulfa from the diet, after the 7th day, the thyroxine synthesizing function of the thyroid appears to return to normal. Tyrosine ether ester HCl, which at low concentrations appeared to modify the sulfaguanidine effect, had no effect

when used at higher concentrations. Sulfaguanidine was shown also to ameliorate sialadenitis of the submaxillary gland caused by high concentration of iodine.

The sanitization and/or sterilization of patient isolators were studied while in use. Solutions of several commercial germicides were tested as cleansing agents for the plastic tent, and were found effective in removing 98-99% of the test organisms. Ultraviolet germicidal lamps were evaluated also for their effectiveness on the surface sterilization of material introduced or removed via pass-through locks of the isolators. The germicidal chambers of the locks appeared capable of providing adequate sanitization in 4 - 6 minutes. However, total sterilization of viable yeast-like organisms and bacterial spores apparently requiresan exposure time in excess of 30 minutes.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36155

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8020106

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C. 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C. 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
A Walter Reed Army Inst of Rsch
Walter Reed Army Medical Center
Washington, D. C., 20012
723-1000, Ext 3552
49

5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER
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576-3614 or Interdepartmental Code 198, Ext 3614 See Continuation Sheet 49

6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Wound Healing (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME'

(U)This subtask is directed at the development and evaluation of clinical procedures for the management of trauma. Mechanisms of tissue repair and techniques of tissue and organ replacement are major areas of interest. Studies in tendon repair have been extended, demonstrating that freeze-dried homografts of digital flexor tendons in the dog are incorporated in a manner at least equal to that of fresh autografts. Structurally and functionally the homografts appear to be normal tendon, grossly and microscopically. No rejection phenomenon is seen. Tissue adhesives as aids in wound repair are being studied. Members of the alkyl alpha-2 cyanoacrylate group of adhesives are being investigated in peripheral nerves, in bile duct prosthesis and anastomosis, in vascular repair, and in bone. The cyanoacrylates appear to be efficient soft tissue adhesives, but the bond formed is not sufficiently strong for bone gluing. Bile duct repair procedures are being studied using acrylate-amide prostheses, as well as pre-formed collagen tubes. Peripheral nerve repair was studied in chimpanzees using processed collagen as a wrapper at the repair site. The repair process in the collagen wrapped nerves appeared more orderly than in the controls and stimulation thresholds were lower. An American vascular stapler is being evaluated.

9. KEY WORDS
Tendon, wound, healing, adhesives, prosthesis, biliary tract, collagen, cyanoacrylates, artery, stapler.

10. SUPPORTING PROJECTS
Not applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36155	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	11–22: 3A012501A802 · 23 24: 01 · 25 26: 06 · 27 28: · 29:
14. DATE OF REPORT (30–33)	30–33: 0664
15. SECURITY OF WORK (34)	34: 4
16. TYPE OF REPORT	35: 3 · 36–47 48 49 50 51: · 52–55: 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56–61) b. Functional Class (62–64)	56–61: 011000 · 62–64:
18. OSD CLASSIFICATION (65–66)	65 66: AR
19. R&D CATEGORY (67)	67: 1

Card "D"

20. CONTRACT NUMBER	11 12: DA · 13 14: · 15–17: · 18–21: · 22–26: · 27:
21. GRANT NUMBER	28 29: DA · 30–33: · 34 35: · 36–38: · 39 40: · 41–45: G · 46:
22. ESTIMATED COMPLET. DATES	47–51: 1CONT · 52–56: 2 · 57–61: 3 · 62–66: 4 · 67–71: 5

Card "E"

23. PRIORITY (11–14) 24. PROGRAM ELEMENT (15–26)	11–14: 1 · 15–26: 6•11•25•01•1
25. CMR&D CODES	27–29: N/A · 30–32: · 33–35:
26. CDOG REFERENCE a. Paragraph No. (36–44) b. Functional Group (45)	36–39: 1412 · 40: · 41 42: a · 43 44: · 45: 6

Card "F"

27. FUNDING

a. Est. Total Cost (11–15)	11–15: · 16–18: 1 · 19–21: 2 · 22–26:
b. % Spent Intern. (16–18)	
" " Extern. (19–21)	
c. Total Obligation (22–26)	
d. Progrmd. Cur. FY (27–33)	27 28: · 29–33: · 34 35: · 36–40:
e. " " " +1 (34–40)	
f. " " " +2 (41–47)	41 42: · 43–47: · 48 49: · 50–54:
g. " " " +3 (48–54)	
h. " " " +4 (55–61)	55 56: · 57–61: · 62 63: · 64–68:
i. " " " +5 (62–68)	
j. " " " +6 (69–75)	69 70: · 71–75: · 76–78:
k. Total Man Years of Effort (76–78)	

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ACCESSION NUMBER	ARMY RESEARCH TASK REPORT
36155	Continuation Sheet

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576-3477 or Interdepartmental Code 198, Ext 3477 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Potenza, A. D.: Prevention of Adhesions to Healing Digital Flexor Tendons. JAMA 187(3) 187-191, 18 Jan 1964.

Potenza, A. D.: Critical Evaluation of Flexor-Tendon Healing and Adhesion Formation Within Artificial Digital Sheaths. The J. of Bone and Joint Surg., 45-A, No. 6: 1217-1233, Sep 1963.

Kline, D. G. and Hayes, G. J.: An Experimental Evaluation of the Effect of a Plastic Adhesive, Methyl 2-Cyanoacrylate, on Neural Tissue. J. of Neurosurgery, XX, No. 8: 647-654, 1963.

Project No. 3A012501A802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma

Subtask No. 06 Title: Wound healing

Description:

Tendon Healing: The study of tendon healing has been a continuing project. The healing of canine digital flexor profundus tendons has been investigated as a model of the clinical situation. Freeze-dried homografts have been introduced using the atraumatic method previously described.

Investigation of Alpha-2 Cyanoacrylates as Bone Adhesives: A substance capable of gluing together bone fragments so securely that normal activities might be carried out during the period of fracture healing is obviously most desirable. The substance must also be nontoxic, non-carcinogenic, and incapable of interfering with fracture healing chemically or mechanically. Many substances have been investigated since 1935, without discovery of a suitable agent. The present study involves the use of a series of a-2 cyanoacrylate derivatives.

Experiences with the American Vascular Stapler: Since Androsov's original work, published in 1955, the interest in stapling systems has grown throughout the world. The Russians are said to have over 50 different stapling systems, including end-to-end and end-to-side vascular staplers, staplers for pneumonectomy, for closing duodenal stumps and for gastrointestinal anastomosis. Subsequently, the Japanese have, under Nakayama, produced a successful stapler as well. Recently, a stapler has been produced in this country which is said to be a more delicate instrument, having additionally the advantage that the staples come presterilized in mounted bushings which facilitates the loading of the stapler. It has been suggested that surgeons of average talent without previous experience in vascular surgery could, with this stapler do a meritable job in the mass casualty situation. It was of particular interest, therefore, to the military to test this thesis.

Peripheral Nerve Injury: Efforts to improve the technique of peripheral nerve injury repair have indicated the potential advantages of processed collagen wrappers at the repair site. Further studies have been performed using collagen wrappers on uninjured nerves to assess the effects of the collagen alone.

Dural Replacement: The closure of dural defects using processed collagen membrane has been investigated. Comparative studies of collagen membrane, collagen laminate, and autologous temporalis have been performed. Histologic data is completed.

A Method of Nerve Staining: A standardized method for staining nerve structures by modifications of the classic Bodian stain has been developed.

Wound Healing, Preformed Collagen Tubes as Common Bile Duct Replacements: The multiplicity of injuries to the structures in the hepatic porta and especially the common bile duct in combat casualties, civilian trauma and iatrogenic instances necessitate the development of a fitting substitute for this structure in injuries in which its length is compromised so that direct anastomosis cannot be made. In the past, attention has been directed toward end-to-end anastomosis or the use of the gall bladder as a substitute. However, work done in recent years at other institutions has suggested that preformed tubes of fibrocollagen can be manufactured in animals and used both as autografts and homografts. This report will describe an attempt to produce these preformed tubes, to alter their antigenicity, and to provide a safe and sterile method of storage until the time of use.

Use of Acryl-amide Prosthesis in Replacement of a Section of Common Bile Duct and Use of Eastman 910 Adhesive and Other Derivatives in the Anastomosis: In an attempt to study a nonsuture prosthetic anastomosis of the common bile duct, a defect in the common duct was replaced with an acrylate-amide prosthesis and the anastomosis effected using a monomer adhesive, octyl 2-cyano-acrylate.

Progress:

Tendon Healing: The initial series of 68 tendon grafting procedures has been completed. The donor tendons have been preserved by freeze-drying only, with no other mechanical or chemical processing. After introduction into recipient animals, the tendon grafts were removed for examination when the animals were sacrificed at intervals up to 160 days. Contrary to expectation, the grafts were tolerated very well, and there was no evidence of host rejection. Grossly, the grafts appeared to be normal tendons in structure and in function. Microscopically, the initial inflammatory changes were rapidly followed by revascularization, repopulation with living fibroblasts and the deposit at the junction site of longitudinally oriented collagen fibers, just as is seen in tendon healing in general. In the dogs sacrificed at later stages, it was difficult to identify the tendon junctions except by microscopic examination. Further procedures have been done to allow examination at intervals up to one year.

Investigation of Alpha-2 Cyanoacrylates as Bone Adhesives: A series of cyanoacrylate adhesives has been synthesized by the Army Medical Biomechanical Research Laboratories. These are the ethyl to decyl analogues of the commercially available methyl alpha-2 cyanoacrylate. Using isolated dog femora, fractures were produced and subsequently glued. Static loading tests were performed and indicated that the glue joint was approximately one eighth as strong as intact bone in the best gluings. While it seems apparent that these agents alone are not effective bone glues, it is still important to

demonstrate their effect on fracture healing, either to allow for the possibility that their strength may be improved, or to develop a model for the investigation of future candidate glues. A series of dogs is now under study. A device has been developed for producing transverse fractures. The left femur is fractured under anesthesia, and open reduction is performed. After introduction of the adhesive into the fracture line, plate and screw fixation is accomplished. Dogs will be sacrificed at appropriate intervals to determine the effect of the cyanoacrylates on fracture healing.

Experiences with the American Vascular Stapler: The overall experimental design called for careful comparison of the standard suture anastomosis versus the stapler by concurrent operations on a series of dogs. It was decided to study the femoral artery, then the brachial artery, followed by an appropriately small vein, the common duct, the ureters and the vas deferens. Each one of these was pilot tested to judge the general feasibility and problems in technique which needed to be met. In the femoral artery series, the only one as yet completed, the vascular stapler was an improvement over the suture technique only after the sixth case in the series of ten, at which time it halved the occlusion period and gave an anastomosis comparable in other respects to the standard 6-0 merselene suture technique. Though the stapler seemed adequate for this simple vascular anastomosis, there were problems in anastomosing other tubular structures which had a thick wall in proportion to lumen size, such as the ureter, common duct and vas deferens.

Peripheral Nerve Injury: Protection of a neurorrhaphy site from infiltration with fibrous tissue and prevention of neuroma formation by the use of wrappers, cuffs or tubes of various materials has been practiced since 1888. The majority of previously used materials were non-resorbable and therefore, necessitated a secondary procedure to remove them after repair had been achieved. A resorbable material composed of commercially processed and purified "collagen" was made available in thin, transparent membranes and was used in this study. Chimpanzees were selected as the experimental animal because of their species' response to various types of peripheral injury as found in previous studies.

The peroneal nerves were exposed in the lower limbs from the sciatic nerve origin to the head of the fibula. Functional threshold of stimulation was determined. Lateral epineurial sutures were placed so that the nerve could be sharply divided between them. After severance of the nerve, the lateral sutures were tied. Several epineurial sutures were then placed anteriorly and posteriorly. The severed area was then wrapped over a 2½ to 3 cm. distance with a transcollagen membrance of 1 mil thickness. The collagen membrane was flexible and when moistened in saline could be easily wrapped around the repaired nerve. The wrapper was then secured by circular ties. The animals were biopsied at 24 hours, 2, 6, 8, 14, 24 and 32 weeks following functional stimulation measurements. The tissue specimens were stained by H&E, Masson, Bodian, Morgan's myelin and Nissl's stains.

The nerves were studied grossly and histologically for persistence of cuff, subepineurial inflammation, gross neuroma, disorganization, axonal carry-through, connective tissue proliferation, remyelinization and conduction. They were graded on a 0 to 4 basis by comparison between each specimen, 0 representing the least or absence of characteristic and Grade 4 the most or strongest results. Bilateral foot drops were present and persisted with the exception of the 8-month chimpanzee in whom dorsiflexion of the feet returned at approximately 6 months. There was lower leg atrophy and several animals developed persistent trophic ulcers on the dorsum of the foot. Exposure of control operative sites revealed heavy adhesions about the repaired nerve. Although difficult to quantitate, the wrapped nerves did not appear to have as many adhesions as the control repairs. Cuff tears, although infrequent during the first few weeks after operation, resulted in early adhesions to the uncovered portion of the repair. The control nerves had the gross appearance of a neuroma. The wrapped nerves were not so swollen, but had a more mottled appearance. Histologic study of the control repair sites revealed central suture line disorganization. Although the axons crossed the repair area and entered the distal stump within several weeks, they regenerated in many directions, taking on a disorganized pattern. Several of the control specimens had histologic evidence of extra-neural spread of axons. The repair areas of the collagen wrapped nerves had some areas of axonal disorganization, but the predominant axonal pattern was a longitudinal one. Connective tissue framework, as well as regenerated axons maintained a longitudinal orientation and disorganization was reduced to a minimum. As a result, axon carry-through of the distal stump was orderly, except in one animal who had more axonal disorganization in the repair area than the control specimen. Faulty alignment of the severed stumps was responsible for the disparity. This finding served to stress the primary importance of careful suture in gaining an adequate peripheral nerve repair. Remyelinization of the control as well as the wrapped nerves did not differ and indeed was sparse until 32 weeks after the operation. Although a functional threshold of stimulation did not return to the postoperative level until 24 weeks or more after repair, the wrapped nerves required a lower voltage than the non-wrapped control nerves.

The collagen wrappers underwent a process of gradual resorption. The resorption time of the wrappers varied and appeared related in part to the degree of tanning the wrappers had achieved. The moderately tanned collagen wrapper was not completely resorbed, even at 24 weeks. The lightly tanned collagen wrapper was resorbed by 8 to 14 weeks. Cellular reaction to the wrapper was also varied. Epineurial infiltrates of lymphocytes, plasma cells and eosinophils surrounded the lightly tanned wrappers at two weeks. As the wrapper was resorbed, these infiltrates decreased, so that by 8 weeks, the scattered fragments of the lightly tanned wrappers were surrounded by a small number of round cells. The 14-week specimen was free of reactive cells and the collagen wrapper was completely resorbed. In comparison with the lightly tanned collagen wrapper, those nerves wrapped with moderately tanned collagen had collections of round

cells around persistent wrapping material and portions of the wrapper were present even at 24 weeks. When it became apparent that collagen was technically easy to handle, and appeared to limit the tendency to form a neuroma, further studies were designed to delineate the process of resorption and attempt to reduce the cellular response. A series of chimpanzee median nerves were exposed in the middle third of the upper arm and wrapped with a collagen membrane over a distance of 2 cm. by a technique similar to that above. This series of wrapped nerves extended up to 24 weeks and included nerves wrapped with non-tanned collagen and lightly tanned collagen. Although the non-tanned collagen seemed to stimulate less cellular reaction, experience using otherwise intact nerves did not confirm this. The non-tanned wrappers were rapidly resorbed within 2 to 6 weeks and this was accompanied by a cellular response which was greater than that seen in the lightly tanned collagen. Two specimens wrapped with non-tanned collagen showed infiltrates of lymphocytes, plasma cells and eosinophils in the perineurial, as well as ipineurial areas. This is accompanied by small scattered areas of tubular demyelinization and axonal change in the peripheral fascicles of several specimens. Thresholds of stimulation were increased in these cases. The lightly tanned collagen wrappers resulted in an epineurial cellular response which was maximal at two weeks and resolved with the resorption of the wrapper over an 8-to 14-week period. Thresholds of stimulation remained unchanged throughout the lightly tanned series and histologically by 14 weeks, the nerve appeared normal except for a slightly thickened epineurium. There was no evidence of distal segment damage in the specimen.

Further studies: A series of 8 chimpanzees was used to determine the antigenicity of the collagen material. Those animals which had previous collagen wrapping around nerves were rechallenged with collagen wrapped around their ulnar nerves. Studies of these specimens extended from 2 to 24 weeks. Gross and histologic findings were similar to those in the primary series of repairs. There was no appreciable change in the gross or histologic characteristics, no change in the resorption time of the wrapper and there was no increase in the amount of inflammatory cell reaction. In several of the two-week specimens, eosinophil response seemed to be somewhat greater, and thus stimulated further studies along these lines. The peroneal and radial nerves of a group of five baboons were surgically exposed and wrapped with collagen. At the end of two weeks, the operative sites were reexposed and the segments of wrapper nerves were removed for histologic studies. At that time the peroneal and radial nerves of the opposite side were surgically exposed and wrapped with collagen. Two weeks later, the second set of nerves were removed for histologic study. Thus, each animal had two sets of nerves for histologic study and comparison with a two-week interval between each set. Comparison of the two sets revealed several interesting findings. The functional threshold of stimulation remained unchanged in all the nerves studied and there was no histologic evidence of damage to the functional portions of the nerves. The epineurial cellular response in the primarily and secondarily

challenged nerves, however, differed. Animals who had previously had collagen film wrapped around their peripheral nerve, reacted to a second implant with an increased cellular response in the epineurium. This response was composed of infiltrates of lymphocytes, plasma cells and eosinophils, and was most concentrated in the secondarily challenged nerves. There were occasional giant cells seen in the specimens in the secondary challenge. These findings suggested that the collagen of the type used in these investigations might stimulate an acute hypersensitive response. Serial implants of collagen were placed in the skeletal muscle of primates and dogs and confirmed the preliminary histologic observations made on peripheral nerves. These observations, however, must await confirmation by more refined immunologic techniques.

Dural Replacement: Dural defects have been closed with a large number of inert, nonresorbable materials, and with a number of resorbable materials such as amniotic membrane, allantoic membrane and fibrin film, which showed an inflammatory response as they underwent resorption. This resulted in fibrosis and meningocerebral adhesions. Because of the need of a suitable material for this procedure, the collagen material provided for a previous study on peripheral nerve repair and cuffing was used as a grafting material to replace dural defects.

Three groups of dogs were used. The first group consisted of nine animals and had moderately tanned collagen film, one mil in thickness. The second group consisted of 13 dogs which had a collagen laminate used for the dural graft. The third group of animals consisted of six dogs and had autologous temporalis fascia used as a graft. A left frontoparietal craniotomy was performed on each animal; a segment of dura measuring 2 x 3 cm. was resected on alternate animals. A segment of left frontal cortex was removed so that the graft would span an injured area of cortex, as well as an intact area of cortex. The line of resection was just rostral to the motor strip in the tip of the left temporal lobe. Each graft was carefully sutured in place and the bone flap reinserted but not wired into place. The animals were observed for periods of two days to twelve months and then sacrificed. An en bloc dissection of the craniotomy site was performed, removing the overlying temporalis muscle, bone flap, meninges and entire brain. These specimens were studied by H&E, Masson, Nissl, Morgan's myelin and Rinehart's acid mucopolysaccharide stains.

Collagen film grafts: Nine dogs in this group were studied at 2 and 12 months. Gross inspection of the area demonstrated good spanning of surgically created dural defect. In the specimens where frontal lobectomy had been carried out, dense connective tissue filled a portion of the frontal fossa, the expanded brain filling the remainder of the defect. The dural graft was often adherent to the edge of the resected portion of the frontal lobe. Fine adhesions over the intact cortex specimens were present, but the dura could be peeled back without discernible damage to the cortex. Within 48 hours, the film was surrounded by blood clots, small numbers of round cells,

consisting of lymphocytes, plasma cells and occasional polymorphonuclear leukocytes. At two weeks, fibroblasts and collagen were seen to be lining both the cortical and bony surfaces of the graft. Fibrosis was heaviest in those areas of the graft adjacent to muscle. By 8 to 12 weeks, the collagen graft was fragmented and surrounded by a zone of round cells and connective tissue. The moderately tanned collagen was completely resorbed by 6 to 8 months, but traces of collagen graft were found in one of the specimens at 12 months.

Collagen laminate: 13 dogs had collagen laminate and were sacrificed over a period of 2 days to 8 months. Gross inspection of the brains revealed a thicker fibrotic layer overlying the dural defect than was observed with collagen film. In several cases the grafted portion of the dura was adherent to the cortex even when a lobectomy had not been performed. In most cases, the graft was much more adherent to the bone flap than to the underlying cortex. In two specimens, chronic inflammatory foci extended from the area around the collagen laminate to involve the subarachnoid and superficial layers of the cortex. The collagen laminate, when used as a dural graft, appeared to promote a heavier round cell, as well as connective tissue response, and meningocerebral adhesions were more abundant than had been observed in the moderately tanned film series. Due to its laminated structure, the collagen laminate presented more collagen for resorption and thus fragments of collagen were persistent for longer periods of time than in those of the collagen film animals.

Autologous temporalis fascia: Autologous fascia was used as a dural graft in six dogs and these animals were studied over a period of 2 to 6 months. The autologous fascia resulted in minimal inflammatory reaction and the fascia underwent gradual resorption. There were no cerebromeningeal adhesions except in those cases with cortical trauma. Most of the autologous fascia was resorbed in two months and a thin, fragile appearing neomembrane lined the inner surface of the bone flap. In four- and six-month specimens, the neomembrane was thicker and histologically appeared stronger. The replacement, fibroblast and collagen were oriented in the direction of the cortical and bony surface.

A Method of Nerve Staining: During the histologic study of the tissues from the neurosurgical investigation of peripheral nerve injuries the supply of German-made protargol became exhausted. A suitable substitute was found after several commercial brands were tried. The use of the substitute (Roques of Paris) required modification of the standard Bodian solution. The strength of the protargol solution was increased from 1% to 2%, with incubation at 37° C. for 48 hours or longer. The reducing solution was used as a pure 1% solution of hydroquinone, the sodium nitrite and formaldehyde being eliminated. The developing time in oxalic acid was increased to 20 minutes. With this technique excellent, standardized, reproducible results were obtained.

Wound Healing, Preformed Collagen Tubes as Common Bile Duct Replacements: Sheets of fine mesh stainless steel gauze which contain approximately 20 squares to the centimeter or 400 squares to the cubic centimeter were cleaned and sterilized for surgical use. They were formed by means of wire suture into tubes approximately 1 cm. to 0.75 cm. in diameter and about 7 cm. in length. Four tubes were then implanted subcutaneously in the backs of each of six dogs and were left in place for the formation of a fibrocollagen layer. These dogs have now been maintained for some two months. The harvesting of these tubes has begun and the products removed from one dog. It was found that a yield of approximately 50% occurs. The lumen of some of the tubes appears to be destroyed by centripetal ingrowth of the fibrous tissue, but in the remainder, a preformed fibrocollagenous tube may be extracted from the middle of the wire mold with an adequate lumen for bile flow. For storage purposes, these tubes are frozen in liquid nitrogen immediately after extraction and then are dried under high vacuum and stored in sealed glass containers. The freeze-drying process is also used in an attempt to alter the antigenicity of the tube that has been selected. This technique has been suggested by work done here on methods of altering the antigenicity of tendon transplants. We plan to continue to harvest these tubes and store them until such time as we have completed the harvest. At this point, homo and autotransplantations will be made in the site of the common bile duct.

Use of Acryl-amide Prosthesis in Replacement of a Section of Common Bile Duct and Use of Eastman 910 Adhesive and Other Derivatives in the Anastomosis: A series of long-term survivor dogs has been completed and will be sacrificed at various intervals over a two-year period to study the effectiveness of this type of bile duct replacement. Ten dogs have ducts replaced with a plain acryl-amide prosthesis and ten have this prosthesis plus a dacron cuff over the prosthesis. In addition, a series of short-term surviving dogs will be completed to study the immediate histological and gross effects of the prosthesis and adhesive. The monomer adhesive, octyl-2-cyano-acrylate, was used in both the proximal and distal anastomosis with no other form of suturing attempted.

Summary and Conclusions:

Tendon Healing: The use of freeze-dried homografts of digital flexor tendons in the dog has produced excellent results. The grafts are accepted without attempt at host rejection and come to appear normal in structure and function. If these results can be duplicated clinically, the advantages in the realm of tendon surgery are truly significant.

Investigation of Alpha-2 Cyanoacrylates as Bone Adhesives: A series of tests of cyanoacrylate adhesives is being undertaken to determine their suitability as bone glues. Static load tests indicate that in their present state of development, they are ineffective. Animal investigation is proceeding to determine their effects on fracture healing.

Experiences with the American Vascular Stapler: In spite of the obvious advantages, the following drawbacks to the stapler have presented themselves:

1) Can do only end-to-end anastomosis.
2) Vessels must be of roughly equal size, whereas suture technique is more accommodating to such disparities.
3) The present stapler has a very limited range of sizes, 3.8 - 1.5 mm.
4) Judgment beyond simple measurement is needed to estimate appropriate size of staple bushings.
5) One loses more vessel length than expected in the cuffing procedure.
6) The need to preserve collateral branches may limit the use of the stapler, e.g., popliteal artery.
7) There appears to be more suture line bleeding with the stapler than by standard suture techniques.
8) Cuffing slightly compresses the anastomosis.
9) The present instrument is unsuited for deep or narrow operative fields.
10) Staples can slip out of tilted bushing unnoticed.
11) The method of cuffing is still crude.

Peripheral Nerve Injury: Processed bovine flexor tendon collagen was used as a resorbable wrapper around the peroneal nerves in chimpanzees. Gross, histologic and functional studies were performed. There was less disorganization in the repair site and axonal carry-through was more orderly and concentrated in those nerves wrapped with collagen, compared to the control nerves which were not wrapped. When a lightly tanned collagen wrapper was used, the collagen was completely resorbed by 8 to 14 weeks. When a moderately tanned collagen cuff was used, resorption was incomplete even at 24 weeks. Resorption was accompanied by a reactive cellular response, composed of lymphocytes, plasma cells and occasionally, eosinophils. The cellular response disappeared when complete absorption of the collagen cuff was achieved. Fibrosis was minimal to absent in the areas where the nerves were wrapped. Because of the protein nature of the collagen and the presence of eosinophils in the inflammatory infiltrates, a secondary challenge procedure was developed. The rechallenge with collagen on other nerves did not result in a heightened cellular response or change in wrapper resorption time. However, more accurate rechallenging experiments demonstrated a heightened cellular response.

Dural Replacement: Processed collagen and autologous fascia were used as a dural graft in 28 dogs and a left frontoparietal craniotomy with resection of a 2 x 3 cm. segment of dura was carried out in each dog. Alternate animals had left frontal lobectomy. The first group of dogs had moderately tanned collagen used as dural graft, the second group had collagen laminate and the third autologous temporalis fascia. Collagen film was found to be a satisfactory dural replacement, resorption was slow and was attended by round cell response, which resolved as the collagen

was completely resorbed. Collagen laminate presented more collagen for resorption and also had a microscopically uneven surface for fibroblastic invasion. This resulted in an inflammatory response in the early months and disorganized fibroblastic replacement of the laminated graft in the later months. Autogenous temporalis fascia was resorbed with minimal inflammatory response and was replaced by a neomembrane closely resembling intact dura.

A Method of Nerve Staining: A new modification of the classic nerve stain was developed. The use of this stain in the preparation of tissue sections in the study of peripheral nerve injuries was most satisfactory.

Wound Healing, Preformed Collagen Tubes as Common Bile Duct Replacements: Preformed fibrocollagenous tubes may be successfully grown in the period of one to two months in wire molds in subcutaneous tissue of the back of the dog. The yield of useful prosthesis appears to be about 50%. We have demonstrated that these tissues may be stored by means of the freeze-drying technique. Harvesting of the product of the implants continues with storage until such time as sufficient quantity to evaluate their effect as common bile duct prosthesis is available. The value of this method lies in the use of a natural substitute which in the case of chronic disease may be grown as an autograft in the patient. Antigenicity can be altered by freeze-drying techniques so that successful homotransplantation following prolonged storage may take place.

Use of Acryl-amide Prosthesis in Replacement of a Section of Common Bile Duct and Use of Eastman 910 Adhesive and Other Derivatives in the Anastomosis: A total of 33 prosthetic replacements have been attempted, with survivors ranging up to three and one-half months in duration. In this series, 16 dogs have plain acryl-amide duct replacements and to date there have been no deaths in this group. One of these dogs was sacrificed at 82 days with a functional duct and no clinical evidence of biliary or hepatic difficulty. Histological sections showed a patent prosthesis surrounded by dense fibrous connective tissue. No bile concretions were present; however, there was slight dilatation of intrahepatic ducts. Serum bilirubin studies show the remainder of this series, except for two dogs, to be doing well. In 17 dogs with dacron cuffed prosthesis there were six deaths, five of which occurred in the early stages of this series and which can be attributed to faulty technique. The remainder are doing well with no loss of weight and no clinical or laboratory signs of hepatic or biliary damage. Continued studies of serum bilirubin will be carried out along with studies in serum alkali-phosphotase. Although it is too early to determine the degree of success of this prosthetic replacement, it is believed that the preliminary results do indicate the merits of a nonsuture prosthetic replacement of the common bile duct.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R1)

ACCESSION NUMBER
36156

PROJECT, TASK, OR SUBTASK NO.
3A012501A8020107

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Responses to trauma (U)

7. DATE OF REPORT DAY 30 MONTH June YEAR 1964

8. RESUME'

(U)The major objective of this subtask is to identify the phenomena associated with injury and to devise methods of treatment thereof. Current studies are continuing in hemorrhagic shock with special attention to the role of intravascular clotting in hindering adequate capillary perfusion. The prevention of irreversibility of shock is a prime objective. The use of vasoconstrictor and vasodilator drugs in shock has been investigated. Vasodilators increased survival rates three-fold over controls with a significant decrease in clotting abnormalities, while vasoconstrictors produced no improvement over controls. Studies on the role of pH changes in the clotting mechanism indicated that the clotting tendency increased as pH fell, so that heparinized blood at pH 6.8 clotted more rapidly than unheparinized blood at normal pH. This has led to studies of the influence of buffer systems in shock to combat the associated acidosis. The fibrinogen half-life has been investigated after hepatectomy and evisceration to elucidate further the role of individual factors in intravascular clotting. The study of wound infections in rabbits indicates that delayed debridement of massively contaminated wounds may increase mortality. Further studies on clostridial protection continue. The study of hepatic encephalopathy after portacaval shunting continued.

9. KEY WORDS
Shock, clotting, coagulation, fibrinogen, liver, debridement, trauma, vasoconstrictors, vasodilators.

10. SUPPORTING PROJECTS
Not applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

DA FORM 1 June 63 1309R PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____

ACCESSION NO. 36156

ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER — 3A012501A802 | 01 | 07

14. DATE OF REPORT (30-33) — 0664

15. SECURITY OF WORK (34) — 4

16. TYPE OF REPORT — 3 | 1263

Card "D"

17. SCIENTIFIC FIELD
- a. Topical Classific. (56-61) — 011000
- b. Functional Class (62-64)

18. OSD CLASSIFICATION (65-66) — AR

19. R&D CATEGORY (67) — 1

20. CONTRACT NUMBER — DA

21. GRANT NUMBER — DA

22. ESTIMATED COMPLET. DATES — 1 CJNT | 2 | 3 | 4 | 5

Card "E"

23. PRIORITY (11-14) — 1

24. PROGRAM ELEMENT (15-26) — 6.11.25.01.1

25. CMR&D CODES — N/A

26. CDOG REFERENCE
- a. Paragraph No. (36-44) — 1412
- b. Functional Group (45) — a | 6

Card "F"

27. FUNDING

- a. Est. Total Cost (11-15)
- b. % Spent Intern. (16-18) — " " Extern. (19-21)
- c. Total Obligation (22-26)
- d. Progrmd. Cur. FY (27-33)
- e. " " " +1 (34-40)
- f. " " " +2 (41-47)
- g. " " " +3 (48-54)
- h. " " " +4 (55-61)
- i. " " " +5 (62-68)
- j. " " " +6 (69-75)
- k. Total Man Years of Effort (76-78)

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Previous Editions are Obsolete

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Project No. 3A012501A802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma

Subtask No. 07 Title: Responses to trauma

Description:

Hemorrhagic Shock: Previous work indicated that disseminated intravascular coagulation (DIC) is an important factor in the production of irreversible hemorrhagic shock. The present studies were carried out in order to indicate what factors precipitated the intravascular coagulation and how the intravascular coagulation could be prevented.

Study of Experimental Clostridial Infections in Massive Open Wounds: Previously, this department and the Dept. of Bacteriology have studied gas gangrene in a standard rabbit preparation simulating massive open wounds. The wound is made by excising a one-inch square of skin on the shaved and prepped left lateral thigh of a rabbit, deeply incising the muscle through this opening, thoroughly crushing the incised muscle and mixing in one gram of "Washington street dirt" known to contain 14 clostridial strains, including all the major pathogenic strains for gas gangrene plus clostridia tetani.

Histological and Biochemical Studies on Portosystemic Encephalopathy in Dogs with Graded Portacaval Shunts: The etiology of portosystemic encephalopathy remains obscure. It is rarely seen in patients with good liver function with surgical portosystemic shunts; for example, children with cavernous degeneration of the portal vein. To evaluate contributory factors, graded portacaval shunts were performed in dogs, since these were previously shown by Doberneck in this lab to vary the degree of another port shunt complication, hemachromatosis, a correlation being made with the amount of hepatic deterioration after the shunt.

Progress:

Hemorrhagic Shock: It was found that by careful handling of the blood removed in producing hemorrhagic shock that dogs could be maintained at a mean arterial pressure of 40 mHg for four hours and then retransfused with a mortality of only 13%. To achieve this the blood was exposed only to a plastic surface. If the blood touched air, metal or glass it became toxic, probably through activation of the Hageman factor. Using this preparation as a model the effect of hemolysis was determined. It had previously been shown that hemolysis was produced by mallet trauma to a dog's thigh, hemoglobinemia becoming most marked 48 hours after the injury in spite of rapid renal clearing of hemoglobin. If the animal was bled at this time the mortality rate was high. It was thought that perhaps the hemolysis was the key factor in mortality. It was found that a mild hemolysis produced by hemolysis by freezing of 20 cc of the animal's own blood and administered just before hemorrhage increased the mortality from 13% to 91%. This amount of hemolysis also caused a great increase

in the coagulation changes which had previously been noted in irreversible hemorrhagic shock. These include prolongation of silicone clotting time, prothrombin time, decrease in fibrinogen and platelets, and activation of endogenous heparin. Control animals given up to 100 cc of hemolyzed blood without hemorrhage were completely unaffected and had no mortality. That the toxic factor in the hemolysis was not hemoglobin was indicated by the fact that purified hemoglobin had little effect on the clotting mechanism. The toxic factor was in a fat soluable fraction and is probably a lipoprotein. It was theorized that fibrinolysin should be able to dissolve the intravascular thrombi and prevent mortality. It had previously proved effective in hemorrhagic shock without hemolysis. Therefore, fibrinolysin was administered during the shock period and was successful in reducing the mortality rate from 91% to 38%. All of these changes were highly statistically significant.

Previous work had shown that for thrombin to be toxic when injected intravenously, trauma in the form of a laparotomy must precede it. Similar results had been shown with the injection of incompatible blood. It was postulated that the trauma caused a vasoconstriction with capillary dilatation producing capillary stasis and that this capillary stasis was necessary to encourage coagulation. If vasoconstriction could be prevented by drugs, then capillary flow would be speeded up and coagulation prevented. To test this theory a vasodilator -- hexamethonium -- was administered in paired experiments to animals in irreversible hemorrhagic shock. In a parallel series a vasoconstrictor -- methoxamine -- was administered to other animals. In the control group the mortality was 70% and the coagulation changes were marked. In the group treated with a vasodilator the mortality was reduced to 25% and coagulation changes significantly decreased. In the vasoconstrictor treated group mortality was increased to 94% and coagulation changes increased.

In other experiments anesthetized dogs were subjected to mallet trauma of one thigh. It was found that fibrinogen levels increased in 48 hours from an average of less than 300 mg% to over 600 mg%. Removal of fibrinogen with intravenous thrombin also caused a dramatic increase in fibrinogen. Cortisone and the mental stress of being put in a cage also increased fibrinogen levels. Fibrinogen is very high in survivors of hemorrhagic shock experiments. It was found that in the early hours of this response fibrinogen could increase at the rate of 50 mg% per hour. Decreases in fibrinogen as a result of intravascular coagulation, therefore, were made in the face of this tremendous fibrinogen activation.

Considerable work was done on the influence of pH on shock. It was found that a rapidly developing severe metabolic acidosis partially compensated by a marked respiratory alkalosis occurred in hemorrhagic shock. Mixed venous pH's as low as 6.2 were recorded. Venous pH's decreased much more than did arterial pH's. It was postulated that this was in part due to the slow capillary flow enabling increased amounts of lactic acid to accumulate in the capillaries. Later the conversion of cells to anaerobic metabolism increased the lactic acid output. It was found that vasodilators had a marked effect in raising the venous pH, probably by improving capillary flow. In an attempt to see what effect this marked

acidosis might have on blood coagulation, blood from both heparinized and unheparinized humans and animals was adjusted to various pH's in vitro with lactic acid. It was found that as the pH of blood dropped below 7.3 that the blood became increasingly coagulable. Heparinized blood at pH's of 6.8 clotted before normal unheparinized blood. Unheparinized blood clotted almost instantly. Apparently an acid pH neutralizes both exogenous and endogenous heparin. This may have wide clinical importance in the use of heparin. It also points up the influence of acidosis on disseminated intravascular coagulation.

An attempt to correct acidosis with the buffer THAM and thus influence mortality in hemorrhagic shock has been carried out. It was found that THAM causes an almost complete aglycemia and death in hypoglycemic convulsions. This was corrected with intravenous glucose. It was also found that THAM in quantities sufficient to prevent marked acidosis would result in marked alkalosis after retransfusion of blood with pH up to 7.9. This was accompanied by apnea and also increased the susceptibility of the animal to citrate intoxication due to conversion to nonionized calcium. These factors were minimized by adjusting the dose and time of the THAM administration and by adding sodium bicarbonate to the infusion. It was found that the use of THAM resulted in a diminution in the coagulation changes and a better appearance of the animal and his organs at the end of the shock period. However, after retransfusion animals frequently became worse and died with an exceptionally high pulmonary artery pressure. As yet no significant mortality salvage has been achieved with THAM. Work is continuing.

A marked drop in fibrinogen has been noted in the hemorrhagic shock preparation. To eliminate from consideration the possibility that decreased production of fibrinogen by a damaged liver could be significantly responsible, the rate of decay in fibrinogen when the hepatic production was totally eliminated, by hepatectomy, was studied. Fibrinogen levels following one-stage hepatectomy showed a small, gradual decline for the first 4 to 8 hours. Then, at an average time of about five hours, there was a marked acceleration of the fibrinogen drop, giving a two-sloped line. The probable explanation to the second slope is the superimposition of disseminated intravascular clotting, presumably set off by the shock and metabolic acidosis encountered a few hours after hepatectomy. This concept is supported by a series of heparin-treated hepatectomy dogs in which this secondary sharp drop failed to appear. Subsequently, repeating the hepatectomy series with improved technique, it was noted that the second phase decline was not always apparent. Conceivably, improving technique with the decrease in portal occlusion times delayed the time of the onset of shock and acidosis, and disseminated intravascular coagulation failed to appear during the length of these experiments. In further studies, evisceration of the dog at the time of hepatectomy also is successful in preventing this second stage decline and apparently prolonging survival.

A consolidation of the concept of disseminated intravascular coagulation has been developed over the past several years and is as follows:

Disseminated intravascular coagulation (DIC) is the transient but widespread coagulation of blood in the microcirculation predominantly in the capillaries. It is important in the etiology of a large number of clinical syndromes in both humans and animals including the following:

1) Shock: (Inadequate capillary perfusion)

   a) hemorrhagic
   b) septic or endotoxin
   c) reversible
   d) irreversible
   e) traumatic
   f) anaphylactic
   g) burn

2) Syndromes involving red cell hemolysis:

   a) hemolytic transfusion reaction
   b) paroxysmal nocturnal hemoglobinemia
   c) malaria
   d) favism
   e) cold hemoglobinuria and march hemoglobinuria
   f) sickle cell disease
   g) trauma
   h) hemolysis due to hypotonic solution

3) Syndromes of late pregnancy:

   a) eclampsia
   b) pituitary necrosis
   c) premature separation of the placenta
   d) defibrination syndrome
   e) amniotic fluid embolism
   f) acute renal failure
   g) sepsis
   h) venous thrombosis
   i) acute necrosis of the liver

4) Extracorporeal circulation

5) Surgical bleeding

6) Clotting abnormalities due to cancer

7) Snake bite

8) Pseudomembranous enterocolitis

9) Hemorrhagic gastritis

10) Hemorrhagic enteritis

11) Acute pancreatitis

12) Acute renal failure

13) Fat embolism

14) Heat stroke

15) Epidemic hemorrhagic fever

16) Hepatorenal syndrome

17) Swartzman reaction

Mechanism

The mechanism of DIC is as follows: Initiated by hemorrhage, trauma, infection, etc. there is vasoconstriction of arterioles brought about by the sympathetic and adrenomedullary systems. Accompanying this is the opening of arteriovenous shunts and the simultaneous opening of all capillaries. All of these factors contribute to an extremely slow capillary flow. The slow capillary flow allows more lactic acid to accumulate in the capillary blood resulting in a rapidly developing acidosis. Slow capillary flow, and acidosis plus other factors as listed below result in clots forming in the capillaries. This completely halts perfusion in the clotted vessels and may result in cellular death by microinfarction. Usually these capillary clots are washed out by dissolution of the clots by endogenous fibrinolysin. However, if they may remain long enough focal tissue necrosis may result. This may be lethal if enough parenchyma of vital organs such as the liver and kidney is destroyed.

Cause

The causes of DIC involve a combination of two major factors: (both must be present)

1) Slowing of capillary flow caused by

   a) arterial hypotension
   b) arteriolar vasoconstriction
   c) capillary dilatation of all capillaries simultaneously (Histamine mechanism)
   d) opening of arteriovenous shunts.

Usually all of these occur simultaneously.

2) A stimulus to clotting of the blood. These include the following:

   a) acidosis
   b) hemolysis

c) high level of clotting factors (fibrinogen and others)
d) bacterial toxins particularly endotoxin (also cause vasospasm)
e) surface factor activation as in extracorporeal circulation. Glass, air (or any gas), or metal surfaces activate Hageman factor, whereas activation by plastic surfaces is minimal.
f) cancer or necrotic tissue
g) particulate matter introduced into the blood (amniotic fluid)
h) snake venom
i) thrombin.

Manifestations

The manifestations of DIC are immediate and delayed. The immediate manifestations include the following:

1) An occult to clinically obvious bleeding tendency.

2) An acute systemic arterial hypotension, pulmonary artery hypertension, vena cava hypotension and portal hypertension.

3) Possible immediate death.

The bleeding tendency may be evidenced by nothing more than a prolonged silicone clotting time. Or it may be the cause of intractable, copious, unrelenting and fatal hemorrhage. Treatment may be complicated and may include fibrinogen, epsilon amino caproic acid and heparin simultaneously. The bleeding tendency is due to:

1) A decrease in clotting factors due to consumption in an intravascular clotting episode. These include platelets, fibrinogen, prothrombin, factor V, factor VII and others. The decrease in clotting factors cannot be accounted for on the basis of dilution, liver insufficiency or endogenous fibrinolysin.

2) The endogenous activation of heparin and fibrinolysin. This may be looked on teleologically as a protective mechanism attempting to halt and reverse the intravascular clotting. The combination of intravascular coagulation and endogenous fibrinolysis results in a complete afibrinogenemia.

The arterial hypotension is due to obstruction in the microcirculation particularly in the liver and lungs. This results in a decreased venous return to the heart and a decreased cardiac output. This hypotension may disappear with washing out of the clots by endogenous fibrinolysin. Immediate death may result as in anaphylaxis.

The delayed manifestations of DIC appear if the organism survives the immediate manifestations. They consist of tissue and organ failure

secondary to focal necrosis caused by microinfarction. Organs frequently involved are:

1) kidney (acute renal failure)

2) liver (liver failure)

3) pancreas (pancreatitis)

4) gastrointestinal mucosa (hemorrhagic enteritis).

Death may result from any or all of the above.

Treatment

DIC can be treated or prevented in experimental animals by the following methods:

1) Improving capillary flow by vasodilators. This is effective treatment. It is being used effectively in clinical shock.

2) Preventing coagulation with exogenous heparin. Heparin is effective only in prevention. It is inactivated by acidosis which accompanies DIC. These limitations are severe restrictions to its use.

3) Dissolving clots with exogenous fibrinolysin. It is effective in treatment of animals. Its clinical use is yet untried.

4) Preventing activation of Hageman (surface) factors in extracorporeal circulation by avoiding contact of blood with air (or any gas), glass or metal by using membrane (not disk or bubble) oxygenator.

5) Preventing acidosis by means of buffers or by vasodilators. The use of buffers is difficult, complicated and not yet ready for clinical use.

Study of Experimental Clostridial Infections in Massive Open Wounds: The effect of tetanus immunization on gas gangrene was studied. It became evident that some of the rabbits dying after five and ten days were dying not of gas gangrene, but of tetanus and although it was thought that the number was small, it was decided to evaluate its significance by protecting the rabbits with tetanus antitoxin. This measure was expected to change the LD70 to an LD50, but allow us to more particularly deal with gas gangrene itself. Surprisingly, this preparation was only 10% fatal. To rule out an effect from the horse serum in the tetanus antitoxin, a tetanus toxoid control was run, along with a no treatment and T. A. T. series. Again, in both cases the mortality was 10%, compared to 70%, in the control. In retrospect, although never published or followed up, similar findings were made in 1958 in this institution in the Dept. of Bacteriology. It was then elected to sterilize this dirt and repopulate it with four major pathogens causing gas gangrene. To date, only one such series has been run and the repopulated dirt was too lethal,

killing all the controls and all the tetanus antitoxin treated rabbits. The plans are to "cut" the dirt to give an LD50 to LD70 control and then retest for the cross-protection by tetanus immunization.

The second aspect studied was the effect of the timing and extensiveness of debridement. Rabbits were wounded in the standard manner and debrided at intervals of six, twelve, eighteen and twenty-four hours. The control mortality, performed with every experiment, was 55%. The mortality at six hours was 10%, at 12 hours 40%, but at 18 hours had overshot to 80%, and dropped somewhat at 24 hours to 70%. While mortality of 18-hour debridement was statistically greater than the mortality without treatment, the 24-hour mortality - though 15% greater than the controls - was not significantly greater statistically. More radical debridement at 18 hours, namely hind quarter amputation, did not improve the mortality.

Histological and Biochemical Studies on Portosystemic Encephalopathy in Dogs with Graded Portacaval Shunts: In this series, both total end-to-side portacaval shunts and end-to-side portacaval shunts performed below the level of the gastroduodenal vein were performed. By leaving this collateral flow through the portal vein the dogs were seen to thrive and not develop the symptoms nor the histopathologic changes typical of hepatic encephalopathy that the complete portacaval shunt dogs did. The latter lost weight steadily and died in 8 to 12 weeks, approximately, and showed a marked gliosis and Alzheimer's cells. However, if these same dogs with a gastroduodenal collateral intact were fed a meat diet and challenged once weekly with ingestions of blood, these same pathologic changes were seen, although less marked. Ammonium curves on these animals after ingestion of blood were much flatter than the completely shunted controls group.

Summary and Conclusions:

Hemorrhagic Shock: A small amount (20 ml.) of endogenous hemolyzed blood causes an increase in mortality of dogs in hemorrhagic shock from 13% to 91%. This lethal effect is due to the stimulation by hemolyzed blood of disseminated intravascular coagulation which is also stimulated by hemorrhage and shock. The lethal factor is a clotting factor in the red cell. Large amounts (100 ml.) of hemolyzed blood alone are harmless, but even a small amount (20 ml.) is lethal in the presence of shock. Hemolyzed blood causes the conversion of fibrinogen to fibrin. Fibrinolysin will prevent death in irreversible hemorrhagic shock both as produced by hemorrhage alone or as influenced by hemolyzed blood. Endogenous heparin is stimulated by disseminated intravascular coagulation which is produced either by hemorrhage alone, hemolyzed blood alone, or by a combination of both. It is probably a protective mechanism. A markedly prolonged silicone clotting time occurring during hemorrhagic shock is an accurate prognostication of irreversibility. Irreversibility is correlated with a fibrinogen fall, when the fibrinogen fall is due to consumption in disseminated intravascular coagulation. Central venous pressure is not elevated till preterminally even though the shock is already

irreversible. However, pulmonary artery pressure is high in shock. Irreversibility is due to an episode of disseminated intravascular coagulation.

"Irreversible" hemorrhagic shock can be prevented by administration of a ganglionic blocking agent after hemorrhage. This must be accompanied by blood volume expansion to prevent immediate death in extreme hypotension. A ganglionic blocking agent administered during hemorrhagic shock will prevent the changes in the blood coagulation mechanism which have been shown to be associated with disseminated intravascular coagulation and a fatal outcome. Characteristic changes in the clotting mechanism were again shown to be associated with disseminated intravascular coagulation and a fatal outcome. These include prolonged silicone clotting time, lengthened prothrombin time, and decrease in blood coagulation factors including fibrinogen and platelets. A vasodilator prevented in large part the severe venous blood acidosis. A vasoconstrictor had no beneficial effect in hemorrhagic shock and appeared to be detrimental.

Heparinized blood begins to clot in pH's below 7.3 and at pH's of 6.6 and below clots more rapidly than unheparinized blood. Venous and capillary pH's in this magnitude are frequent in experimental and clinical conditions. Acidosis may play a part in promoting intravascular coagulation. In pH's below 6.1 heparin appears to be effective again. The use of the buffer THAM to treat the acidosis of shock is complicated and dangerous and needs further study.

Stress in the form of trauma, hemorrhage, cortisone, pregnancy, clinical disease or mental anxiety causes an elevation in blood fibrinogen levels. A high blood fibrinogen level may be correlated with hypercoagulability and an increased susceptibility to the syndromes of disseminated intravascular coagulation. Decreases in fibrinogen in DIC are made in the face of a massive fibrinogen manufacture.

Study of Experimental Clostridial Infections in Massive Open Wounds: Early debridement was shown to protect against the mortality of massive clostridial wounds. Later debridement, however, was shown not only to fail to protect the animals but actually increase the mortality. This failure of late debridement could not be reversed by more radical procedures, namely, hind quarter amputation. Initial experiments suggest that immunization against tetanus favorably affects the outcome in massive clostridial wounds even though the mortality is nearly all from gas gangrene.

Histological and Biochemical Studies on Portosystemic Encephalopathy in Dogs with Graded Portacaval Shunts: Portosystemic encephalopathy is probably dependent upon three factors: portosystemic shunting, hepatic disfunction and protein intake. In this experiment, preserving a small portal collateral to the liver at the time of shunting, maintained normal liver function and morphology while only slightly decreasing total portosystemic shunting. This was sufficient to prevent the histopathologic changes and symptoms of portosystemic encephalopathy, except when fed a high protein diet.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER 36189

PROJECT, TASK, OR SUBTASK NO. 3A012501A8020108

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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49

5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER
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49

6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Experimental anesthesia (U)

7. DATE OF REPORT DAY 30 MONTH June YEAR 1964

8. RESUME'

(U)Continuation of the HDL-WRAIR team approach is proving profitable in development of reliable equipment and improved methods for resuscitation. The Army Artificial Heart Pump has been improved by increasing the output and reducing the inter-dependency of controls. Continued experiments testing the effects of pulsatile blood flow in extracorporeal circulations have been conducted, comparing the Army Pump with roller pumps. Lower mortality occurs using pulsatile flow in dogs. Effects of pulsatile flow on the clotting mechanism are being carried out. Concurrent examination of arterial wall electrolytes shows no significant changes during pulsatile flow vs. steady flow. Use of the Army Pump for synchronized counterpulsation shows a decrease in mean left ventricular pressure with an increase in mean aortic pressure. In vitro tests with human blood show the prototype HDL-WRAIR membrane oxygenator to exceed the efficiency of any membrane oxygenator studied to date. Anesthetic agents were not damaged or altered after exposure to gamma radiation. No positive evidence of liver necrosis was found in 22,701 patients receiving halogenated anesthetic agents. A simple air-volatile agent system for field anesthesia was shown to be feasible. Two ventilators and a mechanical closed chest cardiac assister are in advanced developmental stages. A study of ventilatory obstruction by placement of oxygen catheters in tracheostomy appliances reveals that significant resistance occurs even with small catheters.

9. KEY WORDS

Heart, pump, oxygenator, ventilator, anesthesia, radiation, resuscitation.

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☐ NO ☒ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

DA FORM 1 June 63 1309R PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____

ACCESSION NO. 36189	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	3A012501A802 / 01 / 08 / /
14. DATE OF REPORT (30-33)	0664
15. SECURITY OF WORK (34)	4
16. TYPE OF REPORT	3 / 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	011000 /
18. OSD CLASSIFICATION (65-66)	AR
19. R&D CATEGORY (67)	1

Card "D"

20. CONTRACT NUMBER	DA
21. GRANT NUMBER	DA / G
22. ESTIMATED COMPLET. DATES	CONT / 2 / 3 / 4 / 5

Card "E"

23. PRIORITY (11-14)	1
24. PROGRAM ELEMENT (15-26)	6.11.25.01.1
25. CMR&D CODES	N/A
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	1412 / a / 6

Card "F"

27. FUNDING

a. Est. Total Cost (11-15)	
b. % Spent Intern. (16-18)	1
" " Extern. (19-21)	2
c. Total Obligation (22-26)	
d. Progmd. Cur. FY (27-33)	
e. " " " +1 (34-40)	
f. " " " +2 (41-47)	
g. " " " +3 (48-54)	
h. " " " +4 (55-61)	
i. " " " +5 (62-68)	
j. " " " +6 (69-75)	
k. Total Man Years of Effort (76-78)	

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REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Wright, E. A. and Kahn, R. L.: Electronic Analog Simulation of the Mammalian Cardiovascular System, Part I. TR-1173, Harry Diamond Laboratories, Army Materiel Command, 10 October 1963.

Joyce, J. W., Jr.: Engineering Analysis of the Model 1 Army Artificial Heart Pump. TR-1191, Harry Diamond Laboratories, Army Materiel Command, 27 December 1963.

11. Engineer, research, tests, prototypes and plans by Harry Diamond Laboratories, Army Materiel Command, Fluids Systems Branch, Washington, D. C.

ANNUAL PROGRESS REPORT

Project No. 3A012501A802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma

Subtask No. 08 Title: Experimental anesthesia

Description:

This report deals with current efforts to resuscitate the soldier following trauma, surgery, or any condition producing collapse and shock. These efforts are manifest by progress in developing equipment, methods and an expansion of the educational program. Efforts are being concentrated on means for cardio-respiratory support until return of normal function can be achieved. Emphasis is being placed on the concept of a more prolonged support of vital functions than is currently being practiced with existing equipment and methods.

Progress:

A combined effort with the Harry Diamond Laboratories* in developing medical equipment and methods for immediate as well as long-term resuscitation and support of the injured or wounded soldier is continuing. A group of devices using the principle of fluid amplification as a simple, reliable and economical source of power and control is being developed.

Heart Pump: The Army Artificial Heart Pump is being evaluated both at WRAIR and in the field by civilian investigators. Progress this year has included alteration of fluid amplifier design to increase the output to better meet circulatory requirements. Some investigators found the output low when perfusing animals through small femoral catheters. This has been corrected and a problem of interdependency of controls has also been resolved. Packaging of the pump has been improved. The body of the pump has been changed to Lexan to improve its ability to withstand sterilization.

Reliability tests have been performed and show valves to have operated 16 months continuously without failure. Similar tests on ventricles are showing high reliability for silastic over natural rubber.

Medical testing has shown the superiority of pulsatile flow over steady flow in a series of 20 dogs pumped for 2 hours on total bypass. This is based both on survival times and hemolysis.

*K. E. Woodward, MEA, Research and Development Supervisor
H. Straub
J. W. Joyce
G. Mon
E. A. Wright

A series of 40 dogs using alternately pulsatile (AAHP) and essentially nonpulsatile (Roller Pump) flow in the extracorporeal circuit has been completed. The accrued data shows that pulsatile flow is beneficial in heart-lung bypass.

A similar series of 20 dogs is in progress utilizing a renal arterial flow probe, renal venous blood gases, and microscopic study of the kidney in an attempt to further delineate the effects of pulsatile versus nonpulsatile blood flow delivered to the kidney during heart-lung bypass.

A concomitant study on the effects of extracorporeal bypass on the clotting mechanism indicates widespread intravascular clotting during bypass despite anticoagulant administration.

A study is in progress on a form of synchronous arterio-arterial pumping for relieving a failing myocardium of its work load. This method referred to by many as counter-pulsation, extracts blood through a femoral artery during systole and returns it immediately after systole. This process should decrease myocardial work and increase coronary flow and tissue perfusion by increasing the total pulsatile energy available to the peripheral circulation. This study uses the Army Artificial Heart Pump without valves and controlled by an electronic ECG synchronizer. The R wave of the dog triggers the heart pump through a delay circuit which places the pump's output back into the aorta during diastole.

The completed series of dogs showed that mean left ventricular pressure is decreased and mean aortic pressure is raised but only when the shock is due to myocardial failure. This method of support does not appear to be effective in hemorrhagic shock. An advantage of this direct support of a failing circulation is that the chest need not be entered as in other forms of pump assistance to circulation. The effects of counterpulsation on arterial and venous pH, pO_2, pCO_2 and free hemoglobin are being evaluated.

A study was undertaken to determine if arterial wall electrolyte levels changed during steady flow as compared with pulsatile flow.

Some workers have noted that vessel tone is related to the ratio and amount of Na^+ and K^+ in the vessel wall.

Samples of arterial walls were taken from normal dogs and again after they have undergone pulsatile and nonpulsatile extracorporeal bypass. Quantitative analytic study shows that the changes in Na^+, K^+ and H_2O content are not significant to incriminate as a cause for cardiovascular failure after prolonged nonpulsatile perfusion.

Pressure Cycled Ventilator: A simple, dependable ventilator has been developed using the principle of fluid amplification. It is a compact 2" x 4" x 1" plastic block with a place for a standard mask on one side and entrance for compressed air or oxygen at the end. There are no moving parts. This unit will either assist or control ventilation. In tests on dogs weighing 20-44 pounds, this unit will adequately ventilate or hyperventilate, keeping blood gases within normal limits under varying conditions of perfusion. By simply changing input flows, a range of tidal volume from 60 to 1300 cc. with positive pressure from 2 - 20 cm.H_2O is produced. A negative phase is also obtainable.

Further testing is in progress using anesthetized humans. In these cases, it has proved satisfactory for assisted and controlled ventilation during anesthesia with nitrous oxide and oxygen.

Volume Cycled Respirator: A volume cycled respirator is ready for testing. This respirator is especially designed to cope with problems of bronchospasm and obstruction by secretions such as occur with certain G agents. It has a minimum of moving parts and will be used mainly as a resuscitator. It can use any pressure source for its driving power, including contaminated air or motor vehicle exhaust.

Mechanical Heart Massage Assister: A prototype mechanical heart massage assister has been delivered and a pre-production model is being prepared.

Membrane Oxygenator: The first prototype membrane oxygenator has been tested both with animals and with an in vitro test circuit. The animal tests were to a degree successful but with variations in the perfusion beyond control, it was felt that test conditions could be held more constant by using an in vitro pump and a de-saturation unit. These tests are very encouraging in that a design goal of 200 cc/minute was approached. From the information gained, a new prototype has been constructed which should meet and possibly exceed this design goal.

Advances in silastic membrane fabrication and a unique pulsing system for the gases entering the oxygenator are felt to be responsible for the progress in this area. The goal of a small, simple device for bypass procedures under field conditions is close to becoming a reality with this unit coupled with the Army Artificial Heart Pump.

The use of this membrane oxygenator as a dialysis unit replacing the artificial kidney is also contemplated.

Radiation Effects on Anesthetic Agents: The possibility of radiation-induced changes in volatile anesthetic agents is under investigation to determine if stockpiles would be rendered useless or toxic by the effects of nuclear weapons. Gamma irradiation was performed with analysis of the results. Five volatile agents, di-ethyl ether, di-vinyl ether, Halothane, Fluroxene and Halothane-ether azeotrope, have been exposed to one million roentgens of gamma irradiation over a 290 minute period. Each sample was then analyzed against a standard by gas chromatography. No qualitative or quantitative changes could be found.

The second phase of this study involving neutron bombardment will be accomplished as facilities and equipment now under preparation become available.

Breathing Resistance: Ventilation studies show a great increase in resistance to breathing through tracheostomy appliances when an O_2 catheter is in place. Measurements were made using a sinusoidal breathing curve as produced by a Moerch respirator as well as by peak expiratory flow rates of 25 liters per minute. The results ranging to 34.7 cmH_2O show clearly the hazard to the patient if the optimal size catheter and O_2 flow is not selected. Further study on patients with tracheostomies will be done to show the degree of CO_2 retention by this obstruction.

Hepatotoxicity of Anesthetic Agents: All general anesthetics administered at Walter Reed General Hospital for the years 1958 through 1962 have been screened for cases of massive hepatic necrosis. There were 22,701 general anesthetics administered during that period and 406 postoperative deaths. No cases of massive hepatic necrosis due to anesthesia were found by autopsy. The relation of lesser degrees of liver damage to anesthesia is being studied.

Halothane-air Anesthesia System: An evaluation of a simple non-rebreathing method of administering Halothane-air anesthesia has shown the feasibility of this system for emergency use. Although designed for possible use by relatively untrained personnel, it is felt that this system, as with other methods of general anesthesia, should be limited to trained anesthetists. This is a step in a continuing effort to develop a safe, simple anesthesia system for use in field and mass casualty situations.

Summary and Conclusions:

Several projects involving the Harry Diamond Laboratory-WRAIR team effort are progressing satisfactorily.

The (Artificial) (Army) Heart Pump has been improved by increasing output and reducing the interdependency of controls. Good results, both at WRAIR and among the civilian AAHP evaluators, are proving the importance of pulsatile blood flow in extracorporeal perfusion systems. No effect on arterial walls has been shown by electrolyte content studies during pulsatile or steady flow.

Use of the AAHP for supporting the failing circulation shows positive results when the myocardium is failing but not in hemorrhagic shock.

The combination of improved silastic membrane utilization and pulsing of the involved gases is making the HDL-WRAIR membrane oxygenator a success at this point.

Two ventilator resuscitators being developed, one pressure cycled and one volume cycled, show excellent promise. The pressure cycled ventilator has had good results in both animal and human tests. A chest massage unit has been constructed which promises to be an adjunct to treating cardiovascular collapse in the field.

No radiation induced changes in anesthetic agents can be produced with gamma radiation. Halogenated anesthetic toxicity has not been incriminated as a source of morbidity or mortality in a review of 22,701 cases receiving general anesthesia at Walter Reed General Hospital.

Halothane-air anesthesia is proving to be a feasible approach to mass casualty anesthesia.

A study of ventilatory obstruction by placement of oxygen catheters in tracheostomy tubes reveals that significant resistance is produced even with small catheters.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36157

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8020201

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
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3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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49

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6. TITLE OF: PROJECT ☐
TASK ☐
SUBTASK ☒ Blood and blood disorders (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME' (U)A blood donor program which would support the isolated task force could be more effective if lack of iron in the body were not a limiting factor in the formation of hemoglobin. By studying the mechanisms regulating absorption and loss, means are sought to increase production of blood in normal donors. There was continuation of a program to improve the quality and safety of the Army's blood transfusion service. New plasma expanders were evaluated and there was continuation of studies to investigate the bleeding tendency caused by dextran. An improved method of storing dextran was found. Studies of hemolytic disorders were continued and expanded to include investigation of the intravascular destruction of red blood cells associated with a teflon prosthesis and to provide a better understanding of the red blood cell-parasite relationship in malaria. The destruction of marrow by x-irradiation has been studied in humans receiving therapeutic radiation. The effect of various doses is being documented at intervals following therapy. Local factors limiting repopulation of marrow are being investigated. The role of megakaryocytes in the lung and peripheral blood is being studied to determine their importance in maintaining an adequate number of circulating platelets.

9. KEY WORDS
Blood, iron, absorption, loss, blood transfusion service, plasma expanders, dextran, hemolysis, teflon, malaria, radiation, megakaryocytes, platelets.

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

DA FORM 1 June 63 1309R PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____

ACCESSION NO. 36157	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	3A012501A802 / 02 / 01
14. DATE OF REPORT (30-33)	0664
15. SECURITY OF WORK (34)	4
16. TYPE OF REPORT	3 / 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	011000
18. OSD CLASSIFICATION (65-66)	AR
19. R&D CATEGORY (67)	1

Card "D"

20. CONTRACT NUMBER	DA
21. GRANT NUMBER	DA / G
22. ESTIMATED COMPLET. DATES	1 CONT / 2 / 3 / 4 / 5

Card "E"

23. PRIORITY (11-14)	1
24. PROGRAM ELEMENT (15-26)	6.11.25.01.1
25. CMR&D CODES	N/A
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	1412 / a / 6

Card "F"

27. FUNDING

a. Est. Total Cost (11-15)	
b. % Spent Intern. (16-18)	1
" " Extern. (19-21)	2
c. Total Obligation (22-26)	
d. Progrmd. Cur. FY (27-33)	
e. " " " +1 (34-40)	
f. " " " +2 (41-47)	
g. " " " +3 (48-54)	
h. " " " +4 (55-61)	
i. " " " +5 (62-68)	
j. " " " +6 (69-75)	
k. Total Man Years of Effort (76-78)	

DA FORM 1 June 63 1309R — Previous Editions are Obsolete — Page 2 of _____

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ANNUAL PROGRESS REPORT

Project No. 3A012501A802 Title: COMBAT SURGERY

Task No. 02 Title: Blood and Expanders

Subtask No. 01 Title: Blood and blood disorders

Description: A continuing study to improve the quality and safety of the Army's blood transfusion service and investigation of functions and disorders of blood and blood forming organs.

Progress:

Intestinal mechanisms regulating the quantity of iron retained by the body have been studied. Sequestration of both dietary iron and body iron in intestinal epithelial cells has been demonstrated and quantified. This permitted study of the rate of turnover of intestinal epithelium, supplied a mechanism for limiting absorption of iron from the gut lumen and provided a method of ridding the body of excess iron stores.

The search was continued to identify the factor controlling absorption of iron from the intestine. That the body-store of iron was not the primary stimulus to absorption was shown by demonstration of increased absorption of iron in bled-iron-loaded animals and by the failure to enhance absorption following ablation of a portion of the iron store by partial hepatectomy.

Study of the role of the intestinal mucosal cell in iron absorption showed that there is a rate-limited acceptance of iron from the lumen by the cell and likewise in the passage of iron from the cell into the body. Kinetic data were compiled that indicated that there was an iron accepter system in the intestinal epithelial cell. This hypothesis was supported by demonstration of competitive inhibition of iron absorption by a number of nonferrous metals; indicating that these substances shared a common pathway for absorption by the gut.

The loss of iron from animals and humans was shown to be limited but selective. In animals excretion of iron occurred in the feces while in humans appreciable losses were found to occur from the skin. The mechanism of dermal loss appears to be due to shedding of epithelium.

Rapid methods were developed for measuring the iron concentration and the unsaturated iron binding capacity in serum or plasma. The methods are rapid and inexpensive and can be used in the laboratory or in field survey.

A program to study the red blood cell-parasite relationship in malaria was initiated. Drugs which have an oxidative hemolytic effect (phenylhydrazine, alloxan, etc.) or were cardiac antiarrhythmias (pronestyl) were shown to decrease erythrocytic infestation with Plasmodium berghei. Likewise, endotoxin which has a profound effect upon the reticuloendothelial system decreases infestation. Radioautography is being employed to identify donor populations of red blood cells. This will permit identification of these cells following transfusion into nonimmune recipients. This may permit conclusions regarding immunity in malaria.

Megakaryocytes were demonstrated and quantified in the peripheral blood of humans and animals. The concentration of megakaryocytes was shown to be increased in venous blood when this was compared to arterial blood and seemed to be the source of the pulmonary megakaryocytes. This was confirmed by performing a modified Blalock procedure on dogs so that arterial blood was shunted to one lung and the other lung was perfused solely by venous blood. The lung receiving venous blood contained 50 - 80 times the number of megakaryocytes observed in the lung perfused with arterial blood.

Study of the hemolysis produced by teflon prostheses indicated that the rate of red blood cell destruction was related to activity and cardiac output; increased hemolysis was observed during daytime activity. Significant quantities of nonheme iron were observed in the urine with a similar diurnal variation.

A method of differential elution of adult and fetal hemoglobins from red blood cells was developed. This is being used to determine the survival of fetal red blood cells in the maternal circulation and may provide information regarding the immune phenomena associated with incompatibility.

A study has been initiated to establish the normal hematologic values in monkeys and apes to provide a baseline in these species for experimentation and heterologous transplantation of organs.

In humans receiving therapeutic x-irradiation periodic study of the bone marrow in radiated and nonradiated areas has been undertaken.

In humans receiving therapeutic x-irradiation the bone marrow is being studied in irradiated and nonradiated areas to determine the duration of hypoplasia and the relation of dose and fractionation of dose to recovery.

Summary and Conclusions:

1. Control of absorption of iron by the intestine appears to reside in an active transport system in the small intestinal epithelial cell. This limits absorption and provides a mechanism for excretion of iron.

2. Drugs that produce oxidative hemolysis of red blood cells, interfere with the reticuloendothelial system or are cardiac antiarrhythmics reduce malarial infestation in animals.

3. Megakaryocytes are frequently found in the peripheral blood and are trapped in the lung. Pulmonary megakaryocytes may contribute as many as 10 per cent of the platelets in the peripheral blood.

4. Intracardiac teflon grafts can produce anemia by mechanical hemolysis of red blood cells.

5. Methods for rapid determination of the iron in serum and plasma and to study the survival of fetal red blood cells in the maternal circulation were developed.

6. Normal hematologic values have been established for many species of apes and monkeys.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36158

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8030101

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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576-5121 or Interdepartmental Code 198, Ext 5121 See Continuation Sheet 49

6. TITLE OF: PROJECT ☐
TASK ☐
SUBTASK ☒ Vascular components of cardiorespiratory disease (U)

7. DATE OF REPORT | DAY 30 | MONTH Jun | YEAR 1964

8. RESUME' (U) The control of regional blood flow and metabolism has been studied in the unanesthetized resting and/or active dog by means of chronically implanted electromagnetic flowmeters (our own design) and special tubes in the aorta and coronary sinus for blood pressure and blood sampling. Left coronary flow and myocardial oxygen usage have been obtained in the semibasal state. Additional experiments have evaluated the radiographic method for determining coronary flow in humans, and have extended previous findings of the effects on the coronary circulation and systemic dynamics of exercise, excitement, intra and extracardiac reflexes, blood transfusions, and compensation of blood pH during hypovolemic shock. The controls of the splenic circulation have been outlined. Preliminary experiments have been done to determine during stress the effect of a controlled (paced) heart rate on coronary energetics and cardiac dynamics.

9. KEY WORDS
Flowmeter, circulation, heart, shock, blood, oxygen.

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36158	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	11–22: 3A012501A803 · 23 24: 01 · 25 26: 01 · 27 28: · 29:
14. DATE OF REPORT (30–33)	30–33: 0664
15. SECURITY OF WORK (34)	34: 4
16. TYPE OF REPORT	35: 3 · 36–47 48 49 50 51: · 52–55: 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56–61) b. Functional Class (62–64)	56–61: 010603 · 62–64:
18. OSD CLASSIFICATION (65–66)	65 66: AR
19. R&D CATEGORY (67)	67: 1

Card "D"

20. CONTRACT NUMBER	11 12: DA · 13 14: · 15–17: · 18–21: · 22–26: · 27:
21. GRANT NUMBER	28 29: DA · 30–33: · 34 35: · 36–38: · 39 40: · 41–45: G · 46:
22. ESTIMATED COMPLET. DATES	47–51: 1CONT · 52–56: 2 · 57–61: 3 · 62–66: 4 · 67–71: 5

Card "E"

23. PRIORITY (11–14) 24. PROGRAM ELEMENT (15–26)	11–14: 1 · 15–26: 6•11•25•01•1
25. CMR&D CODES	27–29: N/A · 30–32: · 33–35:
26. CDOG REFERENCE a. Paragraph No. (36–44) b. Functional Group (45)	36–39: 1412 · 40: · 41 42: a · 43 44: · 45: 6

Card "F"

27. FUNDING

a. Est. Total Cost (11–15)	11–15: · 16–18: 1 · 19–21: 2 · 22–26:
b. % Spent Intern. (16–18) " " Extern. (19–21)	
c. Total Obligation (22–26)	
d. Progrmd. Cur. FY (27–33)	27 28: · 29–33: · 34 35: · 36–40:
e. " " " +1 (34–40)	
f. " " " +2 (41–47)	41 42: · 43–47: · 48 49: · 50–54:
g. " " " +3 (48–54)	
h. " " " +4 (55–61)	55 56: · 57–61: · 62 63: · 64–68:
i. " " " +5 (62–68)	
j. " " " +6 (69–75)	69 70: · 71–75: · 76–78:
k. Total Man Years of Effort (76–78)	

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REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Gregg, Donald E.: The George E. Brown Memorial Lecture. Physiology of the coronary circulation. Circulation 27:1128-1137, 1963.

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ANNUAL PROGRESS REPORT

Project No. 3A012501A803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine

Subtask No. 01 Title: Vascular components of cardio-respiratory disease

Description:

The general plan of study is to obtain a broad perspective of the central and local control of regional blood flow and metabolism of the unanesthetized dog exposed to the normal, abnormal, and pathological stresses of everyday life.

Progress:

A standardized dog preparation has been developed with chronic implantation of regional blood flow transducers and special intravessel tubes which permits the appropriate hemodynamic and metabolic studies of the heart and other organs in the unanesthetized state for 8 to 10 weeks.

1. Development of instruments and methods for cardiovascular research. The various necessary instrumentation and methods for the standardized preparation were firmed up early in the year. Additionally, a new version of the pneumatic occlusive cuff for obtaining a mechanical flow zero promises a better yield, longer life, and permits registration of zero flow for as short a time as 0.1 second.

2. Hemodynamics of heart block. Technics are being developed to determine the effect of heart rate on phasic coronary blood flow and cardiac dynamics in the unanesthetized dog. Heart block has been produced by several methods including formalin injection, suture technic, and surgical destruction of the conduction system. Heart rate is controlled by means of an external pacemaker. Coronary-aortic blood flows are monitored by electromagnetic flowmeters. Aortic pressure is determined by a Statham strain gauge. These studies will serve as a baseline for further studies on the effect of exercise and various pharmacologic agents at fixed ventricular rates. Preliminary studies have shown the possibility of using a synchronous atrial pacemaker. This enables each animal to serve as his own control for the above studies.

3. Evaluation of methods for measuring coronary blood flow in man. Extension of experimentation previously reported indicates that in the open-chest dog, the relative flows in the left coronary artery, septal artery, and right coronary artery approximate 82, 8, and 15 per cent, respectively. Establishment of this precise fractionation permitted comparisons in the normal conscious dog of the total coronary flow calculated from the main left coronary flow (as measured by an electromagnetic flowmeter) with the total coronary flow estimated by the method of isotope injection (in this instance I-131), and monitoring over the precordium its rate of disappearance. In 5 chronic dogs, repeated comparisons of total coronary blood flow by radiocardiograms and electromagnetic

flowmeters gave values agreeing within a range of minus 16 to plus 14 per cent, the mean being minus 8 per cent. Although these preliminary experiments are encouraging, no statement is warranted as to the accuracy of the precordial counting technique in estimating coronary flow in beast or man because of the number of potential errors involved that must either be strictly controlled or eliminated.

4. Circulation of the spleen. An electromagnetic flowmeter was placed around the splenic artery and local pressures monitored from a chronically indwelling catheter inserted in the abdominal aorta. Preliminary efforts to obtain flow measurements from the splenic vein were unsuccessful. Splenic arterial flow decreased markedly after intravenous and intra-arterial injections of epinephrine, norepinephrine, and pitressin. The action of these drugs in the chronic animal, therefore, does not substantially differ from acute experiments. A slight reduction in arterial flow observed after sodium pentothal may reflect a release of systemic catecholamines or point to a mechanism of venous outflow obstruction as the reason for splenic engorgement after barbiturate anesthesia.

5. Reflex control of the coronary arteries. Additional attempts were made to demonstrate reflex coronary vasoconstriction. Chronic measurements were made of mean and phasic circumflex coronary flow, cardiac output, central aortic blood pressure, and coronary sinus blood oxygen thrcugh the implantation of electromagnetic flowmeters and aortic and coronary sinus catheters following 20-30 seconds occlusion of the descendens coronary artery. Coronary flow in 8 dogs showed the following sequential responses: 1) a slight transient drop, 2) a steadily rising flow, and 3) a brief overshoot upon release of the occlusion. Oxygen content of the coronary sinus blood did not significantly change during the occlusion. Mean coronary resistance during the drop phase of coronary flow did not change, but during the phase of a rising coronary flow (despite a lower coronary perfusion pressure), resistance dropped 23 per cent. Evidence of a vasoconstrictor reflex could not be demonstrated in these experiments.

6. Effects of rapid blood transfusions on cardiodynamics. Experiments were continued on the effects of intravenous infusion of blood on the cardiodynamics of the chronic dog. Very little change in aortic blood pressure or cardiac output was noted after rapid transfusions of one-half the calculated blood volume in 20-30 minutes. Heart rate and coronary flow rose slightly. It appears possible to overload the dog by infusing three-fourths of its blood volume in approximately 15 minutes, a rate of 50 cc per minute. In one dog, the cardiac output and stroke volume fell while coronary flow and heart rate increased moderately. This dog adjusted quickly to control levels at the end of the transfusion.

7. Cardiac metabolism and dynamics in canine hemorrhagic shock. Additional experiments were performed to estimate the effects of alterations in ventilating rhythm and gas mixtures on myocardial metabolism and cardiac performance, and to establish the value of a compensated blood pH during canine hypovolemic shock. This work was of negative value.

8. Energetics of the left ventricle at rest and during excitement and exercise. This work is essentially complete and written up for publication. Further experiments have confirmed and extended previous findings of the effects of excitement and exercise on systemic and coronary dynamics and energetics. Also, additional experiments in better trained dogs lying on their sides showed very low coronary blood flow values (50cc/100gm/left ventricle/min), and left myocardial oxygen usage (6cc/100gm/min). The associated systemic dynamics: heart rate of 45 to 60, aortic blood pressure of 90 mmHg, and cardiac output of less than 2,000 cc in 40 pound dogs, suggest that the coronary values may represent those of a dog in a basal state.

Summary and Conclusions:

The control of regional blood flow and metabolism has been studied in the unanesthetized resting and/or active dog by means of chronically implanted electromagnetic flowmeters (our own design) and special tubes in the aorta and coronary sinus for blood pressure and blood sampling. Left coronary flow and myocardial oxygen usage have been obtained in the semibasal state. Additional experiments have evaluated the radiographic method for determining coronary flow in humans, and have extended previous findings of the effects on the coronary circulation and systemic dynamics of exercise, excitement, intra and extracardiac reflexes, blood transfusions, and compensation of blood pH during hypovolemic shock. The controls of the splenic circulation have been outlined. Preliminary experiments have been done to determine during stress the effect of a controlled (paced) heart rate on coronary energetics and cardiac dynamics.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36159

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8030102

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
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2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
A. Walter Reed Army Inst of Rsch
Walter Reed Army Medical Center
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723-1000, Ext 3552
49

5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER
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576-3011 or Interdepartmental Code 198, Ext 3011 See Continuation Sheet 49

6. TITLE OF: PROJECT ☐
TASK ☐
SUBTASK ☒ Cutaneous function and disease (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME' (U) Development of vasculature injection techniques with synthetic rubber compounds. Study of histamine excretion in allergic and related disorders. Determination of normal histamine content of sweat. Evaluation of effects of urea on the skin with particular reference to miliaria and blistering.

Present status: Completed. Department is to be transferred to another Medical R&D activity.

9. KEY WORDS
Vasculature, injection, rubber, histamine, allergic, sweat, urea, miliaria, blistering.

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36159	ARMY RESEARCH TASK REPORT
13. PROJECT, TASK OR SUBTASK NUMBER	11–22: 3A012501A803 · 23 24: 01 · 25 26: 02 · 27 28: · 29:
14. DATE OF REPORT (30-33)	30–33: 0664
15. SECURITY OF WORK (34)	34: 4
16. TYPE OF REPORT	35: 2 · 36–47 48 49 50 51: · 52–55: 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	56–61: 010604 · 62–64:
18. OSD CLASSIFICATION (65-66)	65 66: AR
19. R&D CATEGORY (67)	67: 1
20. CONTRACT NUMBER	11 12: DA · 13 14: · 15–17: · 18–21: · 22–26: · 27:
21. GRANT NUMBER	28 29: DA · 30–33: · 34 35: · 36–38: · 39 40: · 41–45: G · 46:
22. ESTIMATED COMPLET. DATES	47–51: 10664 · 52–56: 2 · 57–61: 3 · 62–66: 4 · 67–71: 5
23. PRIORITY (11-14)	11–14: 1
24. PROGRAM ELEMENT (15-26)	15–26: 6.11.25.01.1
25. CMR&D CODES	27–29: N/A · 30–32: · 33–35:
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	36–39: 1412 · 40: · 41 42: a · 43 44: · 45: 6
27. FUNDING	
a. Est. Total Cost (11-15)	11–15: · 16–18: 1 · 19–21: 2 · 22–26:
b. % Spent Intern. (16-18) " " Extern. (19-21)	
c. Total Obligation (22-26)	
d. Progrmd. Cur. FY (27-33)	27 28: · 29–33: · 34 35: · 36–40:
e. " " " +1 (34-40)	
f. " " " +2 (41-47)	41 42: · 43–47: · 48 49: · 50–54:
g. " " " +3 (48-54)	
h. " " " +4 (55-61)	55 56: · 57–61: · 62 63: · 64–68:
i. " " " +5 (62-68)	
j. " " " +6 (69-75)	69 70: · 71–75: · 76–78:
k. Total Man Years of Effort (76-78)	

Card "C" — Card "D" — Card "E" — Card "F"

DA FORM 1 June 63 1309R — Previous Editions are Obsolete — Page 2 of _____

PRINCIPAL & ASSOC. INVESTIGATORS -- Item 5, Continued:

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REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Weiss, H.J., Demis, D.J., Elgart, M.L., Brown, C.S. and Crosby, W.H.: The Treatment of Two Cases of Hyperglobulinemic Purpura with Thioguanine. New Eng. J. Med. 268: 753-756, 1963.

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Zimmer, J.G., and Demis, D.J.: Burns and Other Skin Lesions: Microcircular Responses in Man During Healing. Science 140: 994-996, 1963.

Demis, D.J., and Weiner, M.: Alopecia Universalis, Onychodystrophy and Apparent Total Vitiligo. Arch. Derm. 88: 195-201, 1963.

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ANNUAL PROGRESS REPORT (FINAL)

Project No. 3A012501A803 Title: MILITARY INTERNAL MEDICINE

Task No. 01 Title: Internal Medicine

Subtask No. 02 Title: Cutaneous function and disease

Description:

Studies employing the use of the RTV-200 series of silicone rubber in preparing tests of the microvasculature of the skin have been undertaken with the ultimate aim of utilizing this technique in the demonstration of vascular changes in various cutaneous disease processes. Perfusion and injection techniques were evaluated in the rat as well as methods of demonstrating the specimens in permanent plastic mounting.

Urinary excretion of histamine in mastocytosis patients has been studied previously by this department and increased excretion of histamine and its metabolites was consistently observed. Ranges of 60-90 micrograms per 24 hours in normal individuals have been established by the use of chromatographic analysis. Urinary histamine assay has been carried out recently on patients with atopic dermatitis and chronic urticaria as well as patients with alopecia areata. In addition, measurement of the histamine content of sweat by chromatographic analysis has been done in an attempt to correlate levels with urinary excretion and to establish a normal range of values.

Urea content of normal skin will be compared to that in blistering skin disorders, particularly miliaria crystillina, in order to determine the relationship, if any, of urea with blister formation and spongiosis.

Progress:

The RTV-200 series of silicone rubber has been found to be desirable in preparing casts of the microvasculature of the skin by virtue of their ability to vulcanize at room temperature with very minute changes in volume and heat production, low viscosity, minute particle size, repellency toward water and blood, easy workability, and the fact that they appear to allow complete undistorted filling of microvascular beds. Due to the wide range of viscosities available in the RTV series, arterial channels may be injected, venous channels may be injected or both may be injected simultaneously. By refinement of preinjection washout techniques employing heparinized saline with the addition of 3% gelatin, and injection of the silicone rubber into the aorta or vena cava of the rat, excellent filling of the skin capillary system was found. The use of sodium nitrite as a vasodilator simplifies cannulization of the smaller vessels and gives nicer preparations. Permanent mounting of the specimens in plastic may be achieved by preserving the skin in formalin, dehydrating in alcohol and xylene and impregnation of the specimen in glycerin, methyl methacrylate or uncatalyzed plastic.

There have not been sufficient urine specimens available thus far so that valid conclusions can be drawn from the histamine assays actually accomplished in patients with atopic dermatitis, chronic urticaria and alopecia areata. However, it would appear that the urinary histamine excretion tends to remain normal in atopic dermatitis, high in chronic urticaria and high in alopecia areata. Normal range of histamine excretion in sweat has not yet been determined due to the small number of specimens analyzed.

Urea occurs in high concentration in normal sweat. It has been shown by other investigators to cause acantholosis and blistering when applied locally to the skin. Spongiotic changes occur in miliaria in the epidermis prior to occlusion of the sweat duct, changes which are not unlike the effect of locally applied urea. It seems reasonable, therefore, that urea, rather than merely stimulating the process of blister formation might be directly involved. Therefore a project was planned in which the content of urea in normal skin and the skin of patients with miliaria would be compared. The content of urea in blister fluid would be measured and the permeability of urea through skin would be determined. Recent biopsies were made of human skin to which urea was applied locally under an occlusive dressing: One-25% urea in hydrophilic ointment base, one- 50% urea in hydrophilic ointment base, both remaining in contact with the skin for 24 hours. Control biopsies of normal skin were taken from each subject at the same time. These specimens have not been processed as yet. The pathologic changes produced will be compared at a later date with normal skin and skin from patients suffering from miliaria. In addition, urea levels in normal skin, miliarial skin and blister fluid will be compared, and studies to determine permeability of urea through skin will be started.

Summary and Conclusions:

A procedure for demonstrating the microcirculation of the skin by the use of silicone rubber has been devised. With further refinement and adaption to micromanipulation techniques it should be possible to demonstrate vascular alterations in diseased skin to serve as an adjunct in the study of cutaneous circulation.

Urinary histamine assay has been accomplished on patients with atopic dermatitis, chronic urticaria and alopecia areata. While a regular pattern of levels appears to be developing, insufficient specimens have been run to establish valid conclusions. Levels of histamine in sweat in normal individuals as well as in the above conditions will be established and correlated with the urinary findings.

Studies to determine possible relationship of urea to blistering skin conditions have been started but have not progressed sufficiently as yet to be of importance.

(Subtask completed at WRAIR. Department is to be transferred to another Medical R&D activity.)

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36160

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8030103

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
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3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER
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576-2582 or Interdepartmental Code 198, Ext 2582 See Continuation Sheet | 49

6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Gastrointestinal disease. (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME' (U)A reproducible method was developed for production of experimental ulcers in rats. Gross studies indicate these ulcers result from clotting, hemorrhage and red cell clumping within the vascular system of the stomach. Concurrent studies of the intestinal microcirculation of the rat and gastrointestinal cellular turnover were done. Deficiencies of iron, folate, niacin altered both cell turnover and enzyme systems of rats before histopathologic changes were evident. Nine intestinal and gastric mucosal enzymes were evaluated histochemically in normal human jejunum and stomach. Serious enzymatic disturbances have been detected in several diseases. A method was developed for measurement of tritiated thymidine content of whole rats or entire organs. This appears promising as a measure of cellular regeneration and blood flow. Techniques were devised to characterize the electrophoretic and immunologic behavior of soluble liver cell protein. Anti-rat liver serum causes diffuse hepatocellular lesions in normal rats. The histopathologic effects of 8 different viral entities on the intestinal tracts of humans were studied. A fecal chromatographic technic was devised to determine deficiencies of intestinal disaccharide enzymes. A cooperative study of returnees from Viet-Nam for intestinal disease was done with Surgeons, U. S. Army Special Warfare Center.

9. KEY WORDS
Gastric ulcer, microcirculation, intestinal enzymes, hepatic proteins, viral enteritis, disaccharidases.

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☐ NO ☒ YES See Continuation Sheet. Resume'

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36160	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	3A012501A803 / 01 / 03
14. DATE OF REPORT (30-33)	0664
15. SECURITY OF WORK (34)	4
16. TYPE OF REPORT	3 / 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	010603
18. OSD CLASSIFICATION (65-66)	AR
19. R&D CATEGORY (67)	1

Card "D"

20. CONTRACT NUMBER	DA
21. GRANT NUMBER	DA / G
22. ESTIMATED COMPLET. DATES	1 CONT / 2 / 3 / 4 / 5

Card "E"

23. PRIORITY (11-14)	1
24. PROGRAM ELEMENT (15-26)	6.11.25.01.1
25. CMR&D CODES	N/A
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	1412 / a / 6

Card "F"

27. FUNDING

a. Est. Total Cost (11-15)	
b. % Spent Intern. (16-18)	1
" " Extern. (19-21)	2
c. Total Obligation (22-26)	
d. Progrmd. Cur. FY (27-33)	
e. " " " +1 (34-40)	
f. " " " +2 (41-47)	
g. " " " +3 (48-54)	
h. " " " +4 (55-61)	
i. " " " +5 (62-68)	
j. " " " +6 (69-75)	
k. Total Man Years of Effort (76-78)	

DA FORM 1 June 63 1309R — Previous Editions are Obsolete — Page 2 of ____

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

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(A) Hase, T., M. D., Dept of Gastroenterology
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REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

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Sheehy, T. W., and Floch, M. H.: The Small Intestine: Its Function and Diseases. Hoeber, P. B. and Co., 1964.

ANNUAL PROGRESS REPORT

Project No. 3A012501A803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine

Subtask No. 03 Title: Gastrointestinal Disease

Description:

Current investigations include studies dealing with:

1. The pathogenesis of experimental gastric ulcers in rats.

2. The cell turnover rate in various regions of the gastrointestinal tract.

3. The absorption and retention of Vitamin B-12 through the gastrointestinal tract of the rat.

4. The separation by electrophoresis of soluble intracellular proteins in liver cells and gastrointestinal mucosal cells.

5. The experimental production of hepatocellular damage by induction of antigen antibody reactions within the liver cells of rats.

6. The effects of niacin, folate, iron deficiency, protein depletion and drugs on gastric and intestinal enzymes.

Several cooperative studies are underway with other departments and groups. In conjunction with Major Legters, Special Warfare Center, Fort Bragg, North Carolina, returnees from service in Viet-Nam are being studied for diarrheal diseases and malabsorption. Studies of intestinal enzymes in experimental animals and man have been undertaken in cooperation with Dr. Anderson (Hematology, WRAIR) and Dr. Jones (AFIP). The effect of folic acid deficiency on malaria in rats and chickens is being done in conjunction with Mrs. Herman (Vet. Div. - WRAIR).

Progress:

I. Gastric Ulcer--Studies have been performed to investigate factors which may be involved in the production of experimental gastric ulcers in animals. It has been possible, by using rats which have been carefully protected from chronic stimulation and stress, to produce by fasting and restraints in wire mesh, ulceration in the stomachs of a high percentage of animals. Therefore a suitable animal study model has been achieved and duplication of work described by others has been possible. A variety of pathological changes has been observed in the stomachs of stressed rats. These include venous thrombosis in the submucosal plexus, intravascular red cell clumping in the mucosal capillaries closest to the lumen, necrosis of mucosal cells and discontinuities

extending to the muscularis mucosa, and hemorrhage from the lumen end of mucosal collecting veins. Efforts to prevent occurrence of these lesions by anticoagulants and a rheological agent, low molecular weight dextrans, have been unsuccessful.

Accidentally observed was the occurrence of ulcers in the stomachs of rats who had been isolated for two weeks after becoming adjusted to group living. A preliminary inadequately controlled study showed ulcers in 2 of 6 isolated rats. A pilot study was performed in attempt to correlate with ulcer formation changes in behavior determined by a standard open field running test. Results were inconclusive and indicated that a much larger study would have to be performed to obtain significant results. No further study is planned.

With it in mind that shunting of blood flow past gastric mucosa may play a role in acute ulcer production through rendering mucosal cells ischemic, efforts have continued by a variety of means to demonstrate the presence of significant arteriovenous shunts in the submucosal plexus in rat stomach. While injection of a variety of media has not revealed visible shunts, it has been possible to pass glass spheres of sizes up to 37 microns diameter into the portal vein after intra-arterial injection. The pathway followed by these spheres is unknown and is the subject of further study.

Formation of gastric ulcer has been accomplished in dogs by beeswax and histamine stimulation. Initial studies have shown no change in gastric enzymes after histamine stimulation.

II. Cell Regeneration and Turnover in the Gastrointestinal Tract--It has been demonstrated elsewhere that factors known to influence gastric blood flow, i.e. adrenalin, acetylcholine, fasting, digestion, etc., and artificially induced ischemia have a profound effect upon the mitotic rate of cells in the gastrointestinal tract. Current methods for study of cellular regeneration are limited by many factors; therefore, a method has been developed utilizing tritiated thymidine by which the integrated function of mitosis and intestinal blood flow may be measured for the entire stomach, intestine or bowel. Data obtained to date indicate that the uptake of thymidine is relatively constant in the small bowel and the colon, while the uptake is greater in the antral region of the stomach than in the fundus at midnight by a ratio of about 6:4, the ratio being reversed at noon. Measurement of whole rat tritium content is in progress in order to determine the biological half-life of tritiated thymidine. Data obtained to date indicate a shorter half-life than expected and is in the order of 4 days.

III. Vitamin B-12--Measurement has also been made of the uptake and whole-body retention of Co-60 labeled Vitamin B-12 in normal and gastrectomized rats with and without intrinsic factor. It was demonstrated that hog intrinsic factor would not cause absorption in gastrectomized rats while rat stomach homogenate would do so. This confirms work done elsewhere. Long range retention curves are currently under study.

IV. Intracellular Protein Studies--Techniques for the separation of intracellular soluble proteins by electrophoretic methods and their characterization by immunoelectrophoresis have been developed and preparation of reports is in progress. Extensive study has been performed of the proteins in rat liver cells and proteins have been identified which are unlike proteins in electrophoretic mobility and immunological specificity. Similar studies have been performed on the proteins of intestinal mucosal cells. It has been shown that intracellular protein content of hepatic cells is altered by fasting and re-feeding, or by causing liver cells to regenerate after damage or partial hepatectomy.

V. Immunologic Studies--Using whole rat liver homogenate protein as antigen, high antibody titers have been produced in rabbits. The antiserum has been administered to rats and serial changes in the liver have been observed. Remarkable changes result, including focal necrosis of hepatic cells, proliferation and clumping of Kupfer cells, degeneration of cytoplasm of liver cells with the appearance of inclusion bodies which varied from 1 to 10 u in size, and changes in the protein composition of hepatic cells. These changes occur within a few hours and the liver heals by regeneration within 2 to 3 days. Studies to characterize the material in the inclusion bodies are in progress. Fluorescent tagging methods demonstrate that rabbit serum proteins are present in inclusion bodies. Similar antiserum has been prepared to intestinal mucosal soluble proteins and parallel studies are in progress to determine the effect of antibody on gut cells.

VI. Intestinal Enzymes--A. Histochemical Studies: These studies have been performed on the intestinal and gastric mucosa of humans and experimental animals. Nine enzymes were monitored histochemically. These were selected to reflect the oxidative, glycolytic and nucleoprotein metabolism of the gut. In the intestine of normal man and the rat glycolytic and oxidative enzymes were found mainly in the villous epithelium and its brush border, while enzyme activity indicative of cellular regeneration was confined mainly to the crypt epithelium. A manuscript dealing with histochemical findings in the normal human bowel has been submitted for publication.

B. The effect of experimentally induced vitamin deficiencies of folic acid, niacin, riboflavin, iron, and protein depletion on rat gastrointestinal enzymes is being continued: Studies of enzymatic and histopathologic changes are being correlated with intestinal cell survival and cell regeneration under monitor with tritiated thymidine. Concurrent histochemical studies in humans with certain deficiencies (iron and folic acid) and disease (sprue, infectious hepatitis, cirrhosis, etc.) have already shown severe changes occur in the epithelial cell enzymatic systems long before histopathologic changes are apparent.

C. Disaccharidase Deficiency: Current diagnostic methods for determining a deficiency of intestinal enzymes capable of hydrolyzing dietary sugars are difficult, unreliable or time consuming. Diagnosis is imperative in children with these diseases and at present is based primarily on oral tolerance tests. We have found these unreliable in a large series of volunteers

and patients. Diagnosis is also feasible by analyzing intestinal mucosa for its enzyme content, but intestinal biopsy in children may be hazardous. In conjunction with Dr. Anderson a fecal chromatographic technic has been developed which appears to detect disaccharidase deficiency.

D. Viruses: Studies of intestinal mucosa have been carried out in patients with rubella, rubeola, varicella, infectious hepatitis, infectious mononucleosis, mumps, Adenovirus infections and non-specific enteritis. Histopathologic changes were found in infectious hepatitis and measles. Studies are underway to determine the effects of these viruses on intestinal enzymes.

VII. Diarrheal Disorders in Soldiers Returning from Viet-Nam--In association with Major Legters, Assistant Surgeon, U. S. Army Special Warfare Center, Fort Bragg, North Carolina, a cooperative study was undertaken to determine the cause of diarrhea among troops serving in Viet-Nam.

Four groups of returnees have been evaluated since November 1963. Stool cultures in 135 returnees revealed an incidence of 10 percent parasitic infestation and 0.5 percent enteric infection (Shigellosis). Tests for intestinal malabsorption revealed an abnormal D+Xylose test in 15 percent, low serum carotene levels in 8 percent, and low serum folate levels in 4 percent. Intestinal biopsies were performed on 24 returnees and histopathologic changes were found in 6. This study is continuing.

Summary and Conclusions:

1. Arteriovenous anastomoses have not been visualized in the submucosal plexus of rats, but passage of glass spheres of sizes up to 37 microns suggests that such may be present. Further study is planned.

2. Experimental ulcers have been produced successfully in rats by fasting and restraint. Pathological changes, for the most part vascular in nature, have been observed. Ulcers can also be produced in a small percentage of rats by causing them to live in isolation.

3. A method has been developed whereby the tritiated thymidine uptake (content) of whole organs or whole animals can be determined. Biological half-life is being measured in the whole rat and appears to be about 4 days. Uptake in regions of the stomach has been measured as a function of time of day, and a circadian variation in proportional uptake between antrum and fundus has been observed. Experiments using this method to study effects of various manipulations, pharmacologic and otherwise, are planned after normal values have been established.

4. Uptake and whole body retention of Vitamin B-12 in normal and gastrectomized rats has been measured Hog intrinsic factor has been found not to cause uptake in rats. Retention of absorbed B-12 has been found to be very prolonged. Excretion curves are being analyzed.

5. Electrophoretic and immunoelectrophoretic properties of rat liver cell and intestinal mucosal cell soluble proteins have been studied. Discrete proteins differing from serum proteins electrophoretically and immunoelectrophoretically have been observed. Written reports of these findings are in preparation.

6. Heterologous (rabbit) antiserum to rat liver cell proteins has been shown to react with rat liver cells in vivo causing temporary damage and the appearance in liver cells of spherical inclusion bodies containing rabbit serum proteins. Further studies are in progress to identify this material.

7. High titer antiserum has been produced in rabbits to soluble cellular proteins of rat small bowel mucosa. Studies parallel to those in (6) above are in progress.

8. A histochemical method has been used in the study of glycolytic, oxidative and regenerative enzymes in human and animal intestine.

9. A technic has been developed for determining absence of intestinal disaccharidase enzymes by stool analysis.

10. Enzymatic changes occur in the intestine of certain vitamin deficient, folate deficient and iron deficient animals before microscopic change is apparent.

11. A study of patients with several viral diseases has shown gross intestinal changes in only three--infectious hepatitis, measles and nonspecific enteritis.

12. A mild malabsorption syndrome has been found in some troops returning from Viet-Nam.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36161

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8030104

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
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3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER
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49

6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Military Nursing (U)

7. DATE OF REPORT DAY 30 MONTH June YEAR 1964

8. RESUME

(U) Investigations by Department of Nursing are directed toward systematic examination of nursing practice. The rationale of military nursing practice is being documented by identification, formulation, and testing of principles. Nursing investigations are directed toward assessing need for redefinition of the concept of Military Nursing and ultimately to revise and improve practice within the mission of the Army Medical Service. Application of various measures for nursing care of patients with decubitus ulcers is being continued. Investigation of nursing's contribution toward continuity of care of military patients follows the completed survey of Army health nurses, nurse administrators, and hospital care nurses. Study of recorded nursing observations and their usefulness is being continued at Walson Army Hospital and a guide for systematic recording will be tested. An investigation of nursing observations and judgment has been initiated. Reproducibility of varied blood pressure procedures is being investigated with normal volunteers and automatic monitoring devices. From the investigation of a prototype patient plastic isolator a new concept of isolation is being evolved and is called the RES-System (Regulated Environment for Safety.) Bacteriological testing has been completed. A pilot study with one patient in a modified isolator to refine nursing technics and gain information on bacteriologic testing has been accomplished.

9. KEY WORDS
Nursing, Decubitus, Isolator, Observations, Judgment, Blood-pressure

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36161	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	3A012501A803 / 01 / 04 / 29
14. DATE OF REPORT (30-33)	0664
15. SECURITY OF WORK (34)	4
16. TYPE OF REPORT	3 / 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	010900
18. OSD CLASSIFICATION (65-66)	AR
19. R&D CATEGORY (67)	1

Card "D"

20. CONTRACT NUMBER	DA
21. GRANT NUMBER	DA / G
22. ESTIMATED COMPLET. DATES	1CONT / 2 / 3 / 4 / 5

Card "E"

23. PRIORITY (11-14)	1
24. PROGRAM ELEMENT (15-26)	6.11.25.01.1
25. CMR&D CODES	N/A
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	1412 / a / 6

Card "F"

27. FUNDING	
a. Est. Total Cost (11-15)	
b. % Spent Intern. (16-18)	
" " Extern. (19-21)	
c. Total Obligation (22-26)	
d. Progmd. Cur. FY (27-33)	
e. " " " +1 (34-40)	
f. " " " +2 (41-47)	
g. " " " +3 (48-54)	
h. " " " +4 (55-61)	
i. " " " +5 (62-68)	
j. " " " +6 (69-75)	
k. Total Man Years of Effort (76-78)	

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ANNUAL PROGRESS REPORT

Project No. 3A012501A803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine

Subtask No. 04 Title: Military Nursing

Description: Six investigations included in the Subtask, "Military Nursing" are directed toward systematic examination of nursing practice. The rationale of nursing practice is being documented by identification, formulation, and testing of principles. It is essential that nursing investigations be conducted to assess the need for redefinition of the concept of military nursing, and ultimately to revise and improve practice in terms of the mission of the Army. Brief descriptions of the six studies are as follows:

a. Decubitus Ulcer Care.- Nursing measures for the care of patients with decubitus ulcers is being continued. The limitation of available clinical facilities has precluded the on-going evaluation of various measures on the rate of healing. An extensive review of the literature relating to the nursing care of patients with bedsores is being accomplished. The ultimate aim of the research is to isolate principles of physiology and pathology in order to furnish the professional nurse with guides upon which to base a nursing judgment in the care of patients with decubitus ulcers and pressure areas.

b. Continuity of Nursing Care.- The research is designed to investigate the contributions which the Army Nurse Corps officer makes toward the continuity of medical and nursing care provided the military man and his dependents. It is felt there is a need for a re-examination of the concept of military nursing, particularly as this may be related to health teaching with increasing numbers of out-patients. The nurse has a particularly important role in cold war activities and this role has not been studied systematically. The relationships and health teaching evolved by nurses may be more significant in some of the developing countries than the highly technical knowledge and equipment that is employed.

c. The Nursing Care of Patients Confined Within Isolator Systems.- This investigation has continued to define and study the problems associated with patient care during prolonged isolation. A new concept of isolation and of confinement techniques called the RES-System (Regulated Environment for Safety) is evolving from this research. Earlier prototype systems were an outgrowth of the flexible plastic isolators used to rear germfree animals. The system in use during FY 63 was a taut, clear vinyl, plastic tent fitted with flexible plastic jackets attached for manipulatory purposes. This model was replaced at the beginning of

FY 64. The new model is constructed of a clear vinyl plastic also, but the tent enclosure is made twice the length of the hospital bed, which allows the manipulator, using only gauntlets, to move the entire length of the unit freely; thus, the need for the inconvenient jacket was eliminated. The objectives of this investigation remain as follows: (1) to ascertain the feasibility of using a closed system for the care of patients in need of isolation; (2) to describe the nursing care of patients confined within the enclosure, and (3) to test, modify and adapt the equipment as indicated.

d. Recording of Nursing Observations.- The study to examine the usefulness of nurses' notes, assessment of a systematic guide for recording nursing observations, and the relationships of the content of nurses' notes and use by the medical team is being continued by an investigator assigned to Nursing Service at Walson Army Hospital, Fort Dix, New Jersey. The basic assumption of this study is that members of the medical team could use nurses' notes to a greater extent in planning patient care.

e. Nursing Judgments.- A study designed to gain information concerning nursing observations as a basis for judgment and guide for action in patient care situations has been initiated during the year. The purpose of this investigation is to survey responses to visual (filmed) portrayals of common patient situations requiring nursing action, from a large sample of nurses with a variety of educational and experience backgrounds. Not only must the military nurse be prepared to accept functions that may be delegated to her by the physician in combat and mobilization situations, but she must be able to extend her professional judgment and skill to the assessment of the patient's condition. Her ability to observe, evaluate, and make appropriate judgments concerning large numbers of patients' conditions, simultaneously will contribute greatly during active mobilization and/or disaster, as well as in the care of a single patient during peacetime. Skilled appraisal of a group of patients should give the nurse clues for quickly determining which patients are responding to therapy and which are deteriorating.

f. Reproducibility of Measurements of Varied Blood Pressure Procedure.- As an outgrowth of a student project entitled Blood Pressure Technique As a Stressor, a study was undertaken to validate the reproducibility of blood pressure measurements between the anesthesia and ward blood pressure procedures. Studies have reported observer errors in blood pressure measurement; therefore an investigation was continued to compare two observer methods of recording blood pressures with mechanical recording of blood pressure.

Progress:

a. Decubitus Ulcer Care: During the past fiscal year data on patients have not been collected because of the inaccessibility of local clinical facilities. An extensive review of the literature

relating to decubitus ulcers and pressure areas has been continued and a design for a laboratory investigation to study the effects of varying degrees of pressure for different time periods on skin and underlying circulation has been outlined. Because nurses massage to relieve the "effects of pressure," the effectiveness of such massage will be studied in animals in the future. No progress has been made on the clinical investigation.

b. Continuity of Nursing Care: A survey of Army health nurses, nurse administrators, and hospital care nurses was completed to gain information concerning perceptions of continuity of care, the military nurse's role and contribution to the mission of the Army Medical Corps and Army Nurse Corps, and the various components of care. The number of persons who responded to the survey are shown in Table 1.

TABLE 1

NUMBER AND PERCENT OF QUESTIONNAIRE RETURN BY GROUP

Group	No. Sent	No. Returned	Percent	No. Returned Unanswered	Percent
Army Health Nurses	90	54	60.0	0	0
Nurse Administrators	136	63	46.3	5	7.9
Hospital Care Nurses	299	69	23.1	6	8.7
Total	525	186	35.4	11	5.9

The questionnaire returns are currently being analyzed to ascertain the opinions that respondents have concerning continuity of patient care and to see if the responses show differences in perception between Army health nurses, nurse administrators, and hospital care nurses. From a gross analysis of data, hypotheses are being formulated for later testing.

c. Nursing Care of Patients Confined Within Isolator Systems:- Based on the findings of three clinical trials and laboratory work performed during FY 63, the RES-System was considered by the project officers to be as follows:

1. Acceptable to patients if they received a careful orientation prior to entry into the RES-Tent and if they received continuous diversional therapy during their period of isolation.

2. Feasible for use by nursing personnel if nursing guides were formulated insuring effective and practical use of the equipment.

Potential Feasibility for Medical Use: The new model constructed by Matthews Research, Inc., Alexandria, Virginia, presented new problems and required new nursing guides to be developed and tested.

Bacteriological Testing: Prior to using the equipment in the clinical area certain bacteriological studies were required in order to know the limits of the new equipment. All of the following bacteriological tests were conducted by or under the guidance of 1st Lt. Smith Shadomy, MSC, Bacteriologist of the Department of Surgical Metabolism and Pathology, Division of Basic Surgical Research, Walter Reed Army Institute of Research:

1. A prototype ventilation system containing an absolute filter was challenged using a 10^{13} per ml suspension of Serratia marcescens. The filter was found to provide an absolute infiltration of bacteriological aerosols containing viable cells 0.5 micra in diameter and larger.

2. A prototype pass-through lock, equipped with ultraviolet lights (GE8T5) was tested for effective surface sterilization. Reference is made to the detailed report of Metabolic and Nutritional Problems Associated with Injury, Project 3A01250[illegible]A802[illegible].

3. A series of tests were made with selected disinfectants which simulated actual cleansing procedures in the RES-Tent. The findings are shown in Table 2.

TABLE 2 - RECOVERY OF VIABLE ORGANISMS FROM TREATED PLASTIC SURFACES*

Organisms	Disinfectants		
	Tergisyl 1%	Zephiran 1:1000	Water (control)
Candida	0	0	++
Staphylococci	++++	+++	++++
E. coli.	0	+++	++++
Proteus	0	0	++++
Pseudomonas	0	0	++

Organisms (cont'd)	Disinfectants (cont'd)		
	Wescodyne 75 ppm	Amphyl 0.5%	Alcohol 99%
Candida	+	0	0
Staphylococci	++++	++++	+++
E. coli.	++++	++++	+++
Proteus	+++	++	0
Pseudomonas	0	0	+

*Reading code
0 - no colonies
+ - 1-30 colonies
++ - 30-100 colonies
+++ - greater than 100 colonies
++++ - confluent growth

The test was repeated under highly controlled laboratory conditions relating to the RES-Tent and will be reported under Metabolic and Nutritional Problems Associated with Injury, 3A012501A8020105.

4. Peracetic acid as a sterilizing agent for the RES-Tent was tested by means of Bacillus subtilis (globigii) spore strips made by American Sterilizer Company under the trade name SpordeX. After seven days, cultures of the spore strips subjected to the peracetic acid remained negative; the control strips, not exposed to peracetic acid yielded positive cultures in twenty-four hours.

5. The physical and chemical compatibility of polyvinyl chloride plastic was tested by prolonged immersion of the plastic material in concentrated solutions of different disinfectant agents. The findings of these tests are summarized.

	Changes		
Disinfectant	Flexibility	Color	Odor
Tergisyl	Decreased slightly	None	Sweet
Amphyl	None	None	Sweet
Benzalkonium Chl.	Decreased slightly	Pale yellow	None
Alcohol	Decreased markedly	None	None
Wescodyne	None	Deep yellow	Iodine
Water (control)	None	None	None

6. Various items of standard food-serving equipment were subjected to steam autoclave sterilization (ten minutes at 240°F under 15 pounds p.s.i.). This test showed that the tray, glassware, china dishes and metal utensils were effectively sterilized in this manner without adverse changes. However, all paper materials were rendered non-usable for esthetic reasons and should be subjected to other forms of sterilization if their use is required.

Clinical Trials: On 25 March, the RES-System was placed in the private room on Ward 30, Walter Reed General Hospital. The unit was sterilized with a 3% peracetic acid solution and was then ventilated for 48 hours. A patient volunteer, with the diagnosis of myocarditis, was prepared for entry into the RES-Tent on 30 March. He was given a bath and shampoo of pHisohex and dressed in sterile pajamas. He entered the tent and stayed eleven days. Daily cultures were taken of the patient, the interior facilities and tent walls, and selected exterior fomites. Nose and throat cultures were taken from all of the personnel giving care to the patient. Air sampling was done twice a day simultaneously inside and outside the tent.

The results of these cultures have not yet been completely analyzed and it would be misleading to present a partial analysis. Although no conclusions can be made as to bacteriological safety of the system, to date, the clinical trial proved invaluable in many ways. Nursing procedures have been tried and refined. Standards have been approximated for required amounts of sterile supplies. Culture procedures have been developed and standardized. The system has been

reviewed by many and spoken of favorably by all. A documentary film has been made. The system received a severe test pointing up the need for specific modifications of the RES-Tent to insure the safety and comfort of both the patient and the personnel caring for him. Most of these modifications have been made by the end of this fiscal year.

d. Nursing Observations: The guide for the systematic recording of nursing observations has been refined, based on the findings of questionnaires completed previously by nurses and physicians. A trial of the guide on an experimental and a control ward in a military hospital has not been completed because of the contamination introduced by increased interest in nursing records, and the assignment of the project officer to the Hospital Records Committee. A noticeable improvement in accuracy and completeness in the content of nursing notes has been observed. Until recording stabilizes, the guide will not be used because it may yield biased results.

e. Nursing Judgments: Fourteen, one-minute film sequences were made, portraying patient situations frequently encountered by nursing practitioners. A group of 58 ANC officers attending a short course in Medical-Surgical Nursing appraised the fourteen sequences and on the basis of their evaluation five sequences were selected to show a large sample of persons attending the biennial convention of the American Nurses Association. Volunteer responses will be obtained from a group of 6,000 to 10,000 persons. Projection equipment to show the sequences will be incorporated as a portion of a technical exhibit depicting activities of the Department of Nursing. A tentative coding system has been devised for future analysis. The results should furnish clues concerning the specific actions nurses take based on observations and relevant patterns of responses will be examined. These actions will be further examined in terms of improving patient care.

f. Reproducibility of Measurements of Varied Blood Pressure Procedures: In order to compare the blood pressure readings obtained by observers using the anesthesia and ward techniques, twelve, apparently healthy, male volunteers were studied. Fourteen blood pressure measurements were recorded at 1½ minute intervals on each subject. A Cold Pressor Test was performed during the procedure to show changes in blood pressure not present with stabilized recordings. Both the anesthesia and ward procedure measurements were recorded simultaneously by two observers and by microphone sound recording devices. Through statistical analysis, the findings showed that utilizing the two methods, anesthesia and ward, the blood pressure measurements, as recorded from sound via microphones in the earpieces of the stethoscopes, through an amplifier system and a polygraph, are reproducible. However, an assumption here was that the amplifiers and microphones were of equal sensitivity. With observers recording the measurements of the two methods, the findings were as follows: (1) a difference in method measuring systolic pressure (though the mean difference was approximately 3 mm mercury; (2) a difference in methods and between methods and observers in measuring

the diastolic pressure; and (3) a difference in methods, between observers and between methods and observers measuring pulse pressure. These findings were significant at the 1% level with the exception of the observer difference in the pulse pressure, and this was at the 5% level.

Mechanical measuring procedures apparently give equal recordings, but analysis shows a difference in methods when observers are used. The two procedures are being further compared with a tighter research design to confirm or refute the previous findings. Observer measurements are being compared with recordings obtained by an electronic physiological monitoring instrument. These data are being collected on 16 normal volunteer subjects and have not been completely analyzed to date. Findings suggest that the automatic monitoring device may not be as sensitive in recording as it is purported to be.

Publications:

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ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER 36162

PROJECT, TASK, OR SUBTASK NO. 3A012501A8030201

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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Walter Reed Army Medical Center
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49

6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Metabolic response to disease and injury (U)

7. DATE OF REPORT DAY 30 MONTH June YEAR 1964

8. RESUME'

(U) Study of the pathogenesis and treatment of acute renal failure continues. Animal studies include use of micropuncture, ultramicrochemical, enzymatic and microscopic techniques of rodents during shock and other renal insult. Dogs are utilized for study of renal hemodynamics, biochemical and morbid anatomy alterations in response to renal ischemia. Human investigation has centered on renal function during anesthesia and surgery. The results suggest hydration and infusion of mannitol prevent depression of renal function during anesthesia and surgery. Studies of peritoneal dialysis continue and suggest it is preferable to hemodialysis in many instances. Animal and human studies to determine if intraperitoneal administration of drugs, fluid, solute and calories is feasible are under way· pilot study of antibiotic, water, and fat absorption in animals is nearing completion. Use of gas chromatography and refined techniques for determination of adrenal cortical excretory products continues including observation of the response to infectious challenge in humans.

9. KEY WORDS

Renal, kidney, physiology, anatomy, uremia, dialysis, peritoneal, antibiotic

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36162

ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER — 3A012501A803 | 02 | 01

14. DATE OF REPORT (30-33) — 0664

15. SECURITY OF WORK (34) — 4

16. TYPE OF REPORT — 3 | 1263

17. SCIENTIFIC FIELD
a. Topical Classific. (56-61) — 010604
b. Functional Class (62-64)

18. OSD CLASSIFICATION (65-66) — AR

19. R&D CATEGORY (67) — 1

Card "D"

20. CONTRACT NUMBER — DA

21. GRANT NUMBER — DA | G

22. ESTIMATED COMPLET. DATES — 1CONT | 2 | 3 | 4 | 5

Card "E"

23. PRIORITY (11-14) — 1

24. PROGRAM ELEMENT (15-26) — 6.11.25.01.1

25. CMR&D CODES — N/A

26. CDOG REFERENCE
a. Paragraph No. (36-44) — 1412 | a
b. Functional Group (45) — 6

Card "F"

27. FUNDING

a. Est. Total Cost (11-15)
b. % Spent Intern. (16-18) — 1
" " Extern. (19-21) — 2
c. Total Obligation (22-26)
d. Progrmd. Cur. FY (27-33)
e. " " " +1 (34-40)
f. " " " +2 (41-47)
g. " " " +3 (48-54)
h. " " " +4 (55-61)
i. " " " +5 (62-68)
j. " " " +6 (69-75)
k. Total Man Years of Effort (76-78)

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Page 2 of ____

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ANNUAL PROGRESS REPORT

Project No. 3A012501A803 Title: Military Internal Medicine

Task No. 02 Title: Metabolism and Nutrition

Subtask No. 01 Title: Metabolic response to disease and injury

Description:

This subtask includes: (A) Development of improved methods for the prevention and treatment of renal failure. (B) Use of the peritoneal cavity for dialysis. (C) Study of the absorption from the peritoneal cavity of fluid, solute, and nutritive substances across the serosal membranes. (D) Fluid and electrolyte shifts within the body in response to acute stress which are poorly defined and more poorly understood. (E) Adrenal steroid excretion is of importance in the stress reaction. The development of more discrete techniques for fractionation in the Steroid Laboratory represents a complemental study of the metabolic response to stress.

Progress:

1. Pathogenesis of Acute Renal Failure. Studies completed last year demonstrated the ability of sustained hydration to maintain diuresis during anesthesia and surgery in patients without cardiorenal disease. Current studies of patients with cardiorenal impairment are nearing completion. Results to date indicate that hydration alone is not usually effective and that supplemental mannitol diuresis is required for these patients.

2. Peritoneal Dialysis: Treatment of Acute Renal Failure. We have continued to accumulate experience using peritoneal dialysis for renal failure treatment. Data has been tabulated on 156 dialysis procedures in over 100 patients treated during the past four years. Morbidity and mortality rates are equivalent to those observed in centers employing hemodialysis routinely. The relative safety and simplicity of the former should lead to its general acceptance as the preferred method of therapy.

3. Peritoneal Dialysis: Treatment of Acute Exacerbation of Chronic Renal Failure. Peritoneal dialysis is a valuable adjunct in patients who have suffered an acute exacerbation of chronic renal disease. In many cases, the acute reduction in renal function is secondary to an upper respiratory or gastrointestinal infection and subsequent dehydration. Fluid and electrolyte administration alone is frequently insufficient to restore adequate renal function. In addition, many of these patients have reduced myocardial reserve and the possibility of fluid overloading is always present. The gradual correction of dehydration and restoration of normal serum chemical composition by

dialysis is frequently followed by return of renal function to pre-existing levels.

4. Treatment of Intoxications with Weak Acids by Peritoneal Dialysis Incorporating THAM in the Dialysis Solution. Based on animal studies completed by Major Knochel indicating that the amine buffer tris(hydroxymethyl) aminomethane effectively maintains a high intraperitoneal pH resulting in increased peritoneal extraction of weak acids (barbiturates), a clinical protocol has been developed and approved for similar studies in intoxicated patients. One patient severely intoxicated with secobarbital was treated successfully by osmotic diuresis and peritoneal dialysis. The addition of THAM to the dialysate resulted in increased intraperitoneal pH and a slightly increased peritoneal clearance of barbiturate. Clinical studies comparing various forms of dialysis and diuresis are under way.

5. Modification of Solutions for Peritoneal Dialysis: An Approach to Treatment of Intoxication with Highly Lipid Soluble Substances. Many intoxicants of clinical and chemical warfare significance are highly lipid soluble. This characteristic limits the effectiveness of removal by renal excretion, hemodialysis and peritoneal dialysis. A common clinical example is intoxication with glutethemide. Studies in dogs are in progress utilizing intraperitoneal (Lipomul) emulsified cottonseed oil as a technique for extracting glutethemide from the body. If successful, the technique will receive clinical trial. These studies performed in collaboration with Dr. Leo Goldbaum, AFIP, will be extended to include several lipid soluble substances.

6. Studies of Other Exogenous and Endogenous Intoxications. The clinical application of osmotic diuresis and urinary pH adjustment techniques have been employed routinely at WRGH in the past year for the treatment of acute hyperuricemia. Currently, intensive clinical studies are in progress to determine whether modification of dialysis solutions can improve extraction of uric acid. Studies on the effects of Probenecid, albumin, bicarbonate, lactate and pH in altering the peritoneal clearance of urate in renal failure patients are under way and preliminary results indicate the possibility of improving urate extraction by modification of dialysis solutions.

7. Peritoneal Dialysis for Refractory Fluid Retention. Chronic edema states, i.e., cardiac or hepatic decompensation, nephrotic syndrome, are frequently associated with marked electrolyte imbalance and are at times non-responsive to cautious manipulation of fluid, electrolytes, and drug therapy. It is usually possible to restore fluid and electrolyte homeostasis in these patients promptly and safely by peritoneal dialysis. Frequently, adequate urine flow and responsiveness to drug therapy returns following dialysis.

8. Chronic Peritoneal Dialysis. In some patients with chronic renal disease, acute clinical deterioration has been irreversible despite adequate hydration and restoration of fluid and electrolyte balance. Despite dialysis, these patients have succumbed to the

inevitable advance of their renal disease. In these patients, studies of chronic peritoneal dialysis have contributed to our knowledge of pathophysiology and management of the patient with chronic uremia.

9. The Peritoneal Cannula. During the past year, numerous investigators, both in this country and abroad, have participated with us in extensive clinical trials of the cannula for both acute and chronic dialysis. To date, reports on 210 cannulae from 60 institutions have been received and the data for 162 cannulae analyzed.

10. Methods of Dialysis. Peritoneal dialysis has become the standard method of therapy for patients with renal failure at WRGH. Hemodialysis has been required only three times in the past year. Continuous evaluation of the indications for dialysis solutions of varying composition to match the specific clinical problem has been undertaken. Removal of specific solute and water from the patient can be preplanned with good precision. Peritoneal dialysis has been accomplished successfully on a number of occasions in patients with severe abdominal wounds or extensive abdominal surgery.

A new technique of peritoneal-extracorporeal dialysis with continuous recirculation of dialysate through the Kolff-Twin Coil Kidney has resulted in an increased efficiency of removal of urea, creatinine, and uric acid: higher than any published extraction rates for peritoneal dialysis. Further clinical evaluation and development of this technique is under way.

11. Studies on the Feasibility of Field Production of Peritoneal Dialysis Solution at Site of Use: Intraperitoneal Administration of Dialysis Solution from Ordinary Tap Water and Sterilized by Passage Through the Millipore Filter. The Millipore filtration results in sterility of any solution passed through it. Studies have been completed in rabbits, which are exquisitely sensitive to intraperitoneal pyrogen and bacteria. Solutions containing both acetate and bicarbonate were compared with commercially available solutions containing lactate. Though no clinical application has been made, the implications for military medicine, mass casualty situations, and chronic dialysis are clear.

12. Studies on a New Orally Effective Osmotic Diuretic. Clinical success with osmotic diuretic therapy has suggested the need for an acceptable orally effective osmotic diuretic. A metabolically inert anhydride of the hexose sorbitol has been intensively studied in animals. Studies on the effect of oral administration of the compound on inulin, PAH, creatinine and osmolar clearances in dogs have been completed. These studies show no effect on renal hemodynamics but marked osmotic diuresis. There is no gastrointestinal intolerance and very rapid absorption from the G.I. tract. The renal clearance and space of distribution have been determined in dogs. Clinical studies in human volunteers are planned.

13. Absorption of Antibiotics from the Peritoneal Cavity. Intraperitoneal doses of penicillin, streptomycin, chloromycetin, aureomycin, staphcillin were administered to rabbits. Blood levels were determined serially. Levels in the therapeutic range were obtained within one hour for all antibiotics except chloromycetin. Blood concentrations fell rapidly and there was no evidence of cumulation with repeated doses. Peritoneal histology after chronic administration of antibiotic did not reveal significant changes.

Limited studies on the absorption of antibiotics from peritoneal dialysis solutions in humans also have been undertaken. When 1 gram of staphcillin is given intraperitoneally in 1000 ml. of normal saline, therapeutic blood levels are obtained rapidly and persist at least six hours. These studies are being extended to include other antibiotic agents. The effect of peritoneal dialysis on the serum half life of penicillin and streptomycin is being investigated in dogs. Eight hours of peritoneal dialysis does not affect the half life (and therefore the therapeutic dose) of penicillin. Data concerning streptomycin currently are being evaluated.

14. Absorption of Fluids and Electrolytes from the Peritoneal Cavity. Rate of absorption of saline from the peritoneal cavity has been measured in five human subjects. The normally hydrated human absorbs 85 to 150 ml. in six hours following the intraperitoneal administration of 1000 ml. Factors influencing rate of absorption are being investigated in studies utilizing experimental animals. Preliminary studies in rats demonstrated that rate of movement of water and solute from the peritoneal cavity is increased by the intraperitoneal administration of vasopressin. More detailed studies in dogs have shown that rate of absorption is increased following 48 hours of dehydration. Data concerning the effect of intraperitoneal and systemic vasopressin currently are being evaluated.

15. Effect of Drugs on Movement of Solute Across Isolated Peritoneum. An *in vitro* system utilizing rabbit peritoneum in a leucite chamber has been developed. A series of studies have been performed to determine whether the movement of isotopically labeled sodium across the peritoneum follows the laws of simple diffusion or whether an active transport mechanism is present. Rate constants for movement of sodium across the peritoneum have been determined utilizing Ringer's bicarbonate buffer with added glucose at 37°. The effects of changing temperature and composition of the buffer and of adding Vasopressin, Oubain, and Dinitrophenol also have been studied. Data from these studies currently are being analyzed. It is anticipated that this *in vitro* system will prove valuable in determining permeability characteristics of the peritoneal membrane.

16. Radiation Nephritis in Rats*. Concentrating ability and BUN were compared serially in one group of rats which received 2500 r (Gamma radiation) to exteriorized kidneys with those in another group of sham operated controls. A standard pathophysiologic response was noted with

*Collaboration with the Dept. of Radiation Biology, Div. of Nuclear Medicine

the appearance of both concentrating defect and increase in BUN five-seven weeks following radiation. The rat thus differs from the dog in that glomerular damage appears simultaneous with tubular damage as measured by concentrating defect. Pathologically, these physiologic changes are associated with both glomerular and tubular changes.

17. Canine Radiation Nephritis*. A study is under way in which groups of dogs have received 250 r, 500 r, or 750 r to the kidneys by exposure to the reactor (Gamma rays plus neutrons) or 500 r or 1000 r of conventional X-ray. Function studies are being followed serially to evaluate tubular function (concentrating ability, T^cH_2O, ability to conserve sodium) as well as glomerular function (C_{Creat}, plasma creatinine) and renal plasma flow (C_{PAH}). In addition, the role of the loop of Henle countercurrent system is being evaluated by determining papillary Na, K and urea in animals at times of sacrifice. Histology (both by conventional light microscopy and electron microscopy) is being studied at appropriate intervals. Preliminary results indicate that significant renal damage may be produced by 500 r when supplied by neutrons. Other data are now being collected.

18. Adrenal Cortical Suppression and Stimulation. A standardized protocol utilizing ACTH stimulation, dexamethasone suppression, and SU-4885 is being used to study various groups of patients with pituitary and adrenal dysfunction. Included in this study are patients with acromegaly, Cushing's syndrome, adrenal insufficiency, adrenal androgen excess, and Klinefelter's syndrome.

19. Total Urinary Steroid Spectrum. The simultaneous measurement of 17-hydroxysteroids, 17-ketosteroids, and pregnanetriol from a steroid gum has continued to be utilized in the study of various groups of patients. In particular, these methods have been used in Klinefelter's patients and in subjects placed under stress.

20. Gas Chromatography Studies of Steroids. Gas-liquid chromatography is continuing to be utilized in the separation, fractionation and quantitation of urinary 17-ketosteroids as their tri-methylsilyl ethers. Work is being conducted on the quantitation of androsterone, etiocholanolone, and dehydroepiandrosterone in normal subjects and patients with Klinefelter's syndrome under control conditions, adrenal suppression, and chorionic gonadotropin stimulation in order to determine the use of these steroid metabolites in the evaluation of testicular and adrenal function.

21. Adrenal Androgen Biosynthesis. Studies are being conducted on adrenal androgen biosynthesis utilizing incubation of dog and monkey adrenal tissues. The incorporation of tritium labelled precursors into dehydroepiandrosterone and androstenedione is being studied under the influence of various pituitary hormones present in the media. The purpose of these studies is to delineate the factors that control adrenal androgen synthesis and release.

*Collaboration with Dept. of Radiation Biology, Div. of Nuclear Medicine

22. Endocrine Evaluation of Patients with Klinefelter's Syndrome. Further work has been done on functional evaluation of the testes. Thus far, five patients have been studied by determining testosterone production under the influence of human chorionic gonadotropin, dihydrotestosterone suppression and decadron suppression. The result of this data is pending.

23. Red Cell Metabolism. Evaluation of patients with glucose-6-phosphate dehydrogenase deficiency has been instituted utilizing 1-C^{14} glucose incubation and measuring $C^{14}O_2$ production. Thus far, two patients with a "complete" absence of G-6-PD have been shown to have a diminished but active pentose phosphate pathway. That this is not due to remnants of the Krebs cycle has been demonstrated by the lack of $C^{14}O_2$ production with 6-C^{14} glucose incubation with reticulocyte-rich blood.

24. Methods for Determination of Magnesium. Colorimetric and flourimetric methods for determination of magnesium in body fluids have been investigated and applied to the study of a salt-deprived patient dosed with aldosterone antagonists.

25. The Field Experience of a Medical Civic Action Team in South Viet Nam. The field experience of a Medical Civic Action Team in South Viet Nam has been compiled and tabulated for 20,079 patients. In addition, the incidence of hypochromia and of hemoglobin E trait have been determined in 127 central Vietnamese villages.

26. SEATO Laboratory Support. The department has continued to participate in the selection and training of personnel for the Clinical Research Center, Bangkok.

Summary and Conclusions:

The projects enumerated reflect an increased depth of effort in a more circumscribed area of interest than in previous years.

The quality and quantity of publications reflect the basic value and productivity of the current programs.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36163.

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8040101

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D.C. 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C. 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Social and preventive psychiatry (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME (U) Use of a therapeutic milieu as a treatment regime for chronic alcoholic soldiers has furnished a method for investigating related phenomena, including job-oriented units, patient government group psychotherapy behavior, various operant conditioning techniques, use of symptoms to achieve social ends and symptom disappearance when no immediate symptom-based interaction is possible. Follow-up studies, including appropriate controls, are in process with alcoholics and schizophrenics (formerly treated by Milieu Therapy). Techniques of personal interview, studies of group-interaction, analysis of family groups and larger social groups (in concurrence with demographic, content and value analysis) have been utilized in a study of Army AWOL offenders. The feasibility and utility of these methods has been established for analysis of problems of military delinquency. Data collected during the past year has culminated in an on-going phase of analysis, organization, and reduction of systematized constructs. (A thematic behavioral profile on the recidivist AWOL offender has been established). A study has been initiated to investigate cognitive and socio-cultural correlates of specific psychotic symptomatology with emphasis on language processes involved, particularly during the past year, on the language of delusions and hallucinations. Former studies of brain-injured patients have continued and application of principles made in other behavioral areas.

9. KEY WORDS
Milieu therapy, alcoholism, demography, socio-cultural, group interaction, recidivist, hallucinations, delusions, language, cognition, semantic-differential, content analysis, intelligence, fluency, connotation, metaphor, symbolic behavior, jargon, aphasia, deviant behavioral repertoire, social structure, peer group..

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

DA FORM 1 June 63 1309R PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____

ACCESSION NO. 36163	ARMY RESEARCH TASK REPORT
13. PROJECT, TASK OR SUBTASK NUMBER	11–22: 3A012501A804 · 23 24: 01 · 25 26: 01 · 27 28: · 29:
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a. Est. Total Cost (11-15)	11–15: · 16–18: 1 · 19–21: 2 · 22–26:
b. % Spent Intern. (16-18) " " Extern. (19-21)	
c. Total Obligation (22-26)	
d. Progmd. Cur. FY (27-33)	27 28: · 29–33: · 34 35: · 36–40:
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f. " " " +2 (41-47)	41 42: · 43–47: · 48 49: · 50–54:
g. " " " +3 (48-54)	
h. " " " +4 (55-61)	55 56: · 57–61: · 62 63: · 64–68:
i. " " " +5 (62-68)	
j. " " " +6 (69-75)	69 70: · 71–75: · 76 78:
k. Total Man Years of Effort (76-78)	

Card "C" · Card "D" · Card "E" · Card "F"

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ANNUAL PROGRESS REPORT

Project No. 3A012501A804 Title: Military Psychiatry

Task No. 01 Title: Military Psychiatry

Subtask No. 01 Title: Social and Preventive Psychiatry

Description:

1. Human clinical experimental studies of chronic alcoholic soldiers, 15 August 1961 to 30 June 1963, explored the feasibility of applying certain combat psychiatric principles, with ward environmental and group oriented therapeutic techniques, to a sample of one of the most disruptive forms of human behavior, in order severely to test the methods and derive reasonable inferences concerning certain aspects of "normal" behavior usually kept secret.

2. The AWOL Offender Study has continued during the year with intensive data collection by means of individual and group interviews in depth, using stockade prisoners, members of their families, members of the military community, and members of the offenders' civilian community. The subjects studied represented a cross-section of the recidivistic AWOL population of the Fort Meade stockade during this period. All of this data, originally recorded on magnetic tape, has been transcribed. The transcriptions are being subjected to standard modes of analysis. Values, symbolic usage and behavioral profiles are in the process of being established for the recidivistic AWOL group as a whole and for a selected sample in reference to equivalent analysis of discourse of their family of orientation, peer groups, and other reference groups. The relationships of these long range patterns of behavior to short range delinquent behavioral events in the military setting is also being explored and identified.

A sub-study of previously discharged AWOL offenders (1958-1960), based upon materials available in 201 files and field interviews, has also been launched. The sample utilized has been selected from the Philadelphia-Baltimore-Washington area. It is anticipated this sub-study will test specific hypotheses relating to wider implications of the behavioral patterns seen symptomatically as recidivistic AWOL in the military setting.

3. A research program has been initiated to study the cognitive and socio-cultural correlates of specific psychotic symptomatology with emphasis on investigation of the language processes involved. During the past year the study has focused on the language of delusions and hallucinations. Previously initiated studies of brain-injured patients continue and applications of principles gained are being made in other behavioral areas.

Progress:

1. A modified treatment open psychiatric ward became operational on 15 August 1961 and remained so until 15 June 1963 to study the bi-social development and the total milieu of chronic alcoholic soldiers. Individual and group psychotherapy was conducted and analyzed in concert with those aspects of group process which aid in formation of therapeutically beneficial groups. Field social anthropological studies were conducted within the military unit of the alcoholic soldier and, on a few occasions, with members of the parental family. Various attempts were made to adjust the administration of these studies so as to produce findings of increased informational value, after which the studies were re-adjusted and re-evaluated. During the year, techniques for obtaining a ten-year follow-up on the results of treatment in the experimental ward have been developed and refined. The mass of data which evolved from the study is presently being analyzed, reduced and prepared for publication.

Follow-up investigators discovered some five years ago that previously treated schizophrenic soldiers responded actively and informatively to inquiry by personal letter rather than to a "form letter." This method brought the response rate up to 100% at times, previously unheard of in this area and encouraging to the workers.

2. Collection of data from interviews in depth and from community background and demographic inventories from the basic recidivistic AWOL population has been completed. Selected sub-samples have been interviewed for the purpose of assuring adequate replication of both regularities and differences within the group.

Present evidence indicates the behavior, called recidivistic AWOL, is but tangentially related to military experience and milieu. Recidivist AWOL appears to be an expression of long-term, well-established patterns of behavior which are subculturally sanctioned and reinforced within the family and peer group constellations. The offender is normatively a Regular Army enlistee of median age 18-19. He is caucasian and of marginal working class origin. A school drop-out, he often has a history of either judicially attested or self-reported delinquent behavior. He is representative of a variant aspect of the dominant culture whose illegitimate behavior has received collusive sanction from his family of orientation and who perceives legitimate authority as founded upon special relationships, such as kinship and peer friendship, rather than from authority interest in the social structure. Often involved in a series of magically fulfilled and undone identity crisis, each major role and identity transformation usually involves AWOL from its predecessor: school, job, and military service.

Progress has thus been made in describing a behavioral profile of the recidivist AWOL. Further study hopefully will define relationships of the general profile and specific individual familial profiles with variations in value and interactional systems in the larger social structure. It is expected that guide lines for possible profitable

therapeutic intervention and retraining, as well as more selective stockade screening, will emerge from these data.

3. The continuing study of patients with brain injuries has involved investigation of language patterning and categorizing mechanisms as modes of adaptation to stress. Examinations were made of twenty (20) students in the Special Education Program of the Montgomery County Board of Education. Specific language disabilities of these children have been the focus of this work. The aim has been to determine the degree to which the disturbances in symbolic behavior may be formulated in concepts derived from adults.

A project involving psychiatric aspects of Presidential protection is being carried out in collaboration with the Protective Research Section of the Secret Service. The purpose of this project is to formulate the behavior of apparently mentally ill persons threatening Presidential security in terms of the relationships of symbolic behavior to stress, the problems that prompt the behavior, and the social value systems that determine its content. The work to date has been largely a review of files and planning of approaches.

Data from a pilot screening study of interviews with psychiatric patients admitted to Walter Reed during the period of 7 May 1963 to 7 July 1963 have been collected. This study was intended to obtain information about the overall incidence of delusions and hallucinations among the psychiatric population admitted to Walter Reed. Social background data and interviews about the current illness were collected from the patient and combined with clinical summaries from the hospital records.

From examination of induction aptitude (ACB) scores of soldiers who subsequently became hospitalized as psychiatric patients, it was found that the delusional patients who reported auditory hallucinations had achieved significantly lower scores on the induction aptitude tests than the delusional patients who did not report auditory hallucinations. This finding was examined more closely by collecting a larger series of 101 delusional patients and comparing the best test scores between hallucinators and non-hallucinators. Significant differences in test scores were maintained. Social background factors were tabulated but no significant differences could be found between the two groups. Also, there was no significant difference between the diagnostic categories which were attached to the two groups, neither on admission diagnosis nor on discharge diagnosis nor for the length of time since administration of induction aptitude tests.

A smaller series of delusional psychotics were further examined with an extensive battery of language tests to compare the level of language descriptive skill between a group of hallucinatory-delusional persons and a group of non-hallucinatory-non-delusional patients displaying markedly limited descriptive style. These were insensitive to the conventional dimensions of connotative meaning, and had lower verbal fluency.

There was also evidence of poorer performance on concept formation tests among hallucinators. In general, the tests showed an overall impoverishment of language descriptive skills in hallucinators as compared to the non-hallucinators.

On examination of hallucination content, those few hallucinating patients, who had high aptitude scores, reported their hallucinations more vividly and with more descriptive language than low scoring hallucinators. The occurrence of auditory hallucinations may be a function of the level of non-verbal concept formation skills while the vividness associated with these descriptions may be a function of verbal skills such as descriptive style, sensitivity of connotative meaning and fluency.

Currently, intensive data is being collected from a series of patients who report religious delusions. This data evolves from multiple interviews, social background inventories, language skill tests, conventional psychological tests and interviews with the relatives of the patients, and will be examined to identify the part cognitive and socio-cultural factors play in determining the occurrence and content of psychotic symptoms.

Work proceeds on the objective analysis of characteristics of delusional speech. Two different techniques are being developed to identify key words in psychiatric interview material. One technique, based on similarity of word meaning as measured on the semantic differential, requires patients to rate words-in-context taken from their own interviews. A computer program is being written which will cluster together words which are similar in meaning, and which will locate words which occupy central positions in each cluster. Raw data has been collected for this analysis.

A second method concerns inter-correlations among several physiological and psycholinguistic measures of emotionality in words. When words from an interview are presented as stimuli in a word association test, the most meaningful words can be identified by measures of orienting GSR amplitude, rate and variability of responses, and the degree of associative overlap (i.e., the extent to which two different stimulus words share common associations). Once the words are identified, using either or both of these techniques, a basis is provided for a statistical content analysis.

Summary and Conclusions:

1. A Milieu Therapy Ward, in addition to its value as a treatment method, serves as an excellent laboratory for the study of deviant behavior. Some problems of chronic alcoholism in the military have been studied by these established methods.

Follow-up studies on soldiers previously treated via Milieu Therapy

techniques are progressing satisfactorily and publication is being planned.

2. The AWOL Field Study has achieved its basic aims in respect to data collection, and has proceeded to the analysis of these data and the inception of sub-studies of increased specificity. Primary analysis of the data indicates that AWOL is not a discreet expression of deviance specific to the military, but rather is one aspect of a wider behavioral profile. It is expected that analysis of the data will be completed during the coming year as well as extended analysis of the offender and the events which control his behavior in his various milieux.

3. Investigation of language patterning in previously initiated studies of brain-injured patients continues. Disturbances of symbolic behavior in children with language disability have been studied and application of principles learned from this ongoing research are being applied in other behavioral areas.

The incidence of hallucinations and delusions in psychiatric patients has been surveyed in a pilot study. Significant difference was found in scores obtained on induction aptitude tests between hallucinatory-delusional patients and non-hallucinatory non-delusional patients. Intensive studies of these groups showed marked differences in language description skills. Further studies will focus on content of delusional patients' interviews using computer techniques.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36164

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8050101

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C. 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C. 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
A Walter Reed Army Inst of Rsch
Walter Reed Army Medical Center
Washington, D. C. 20012
723-1000, Ext 3552
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49

6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Chemical protection against irradiation (U)

7. DATE OF REPORT DAY 30 MONTH June YEAR 1964

8. RESUME' (U) Published separately.

9. KEY WORDS

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

DA FORM 1 June 63 1309R PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____

ACCESSION NO. 36164	ARMY RESEARCH TASK REPORT
13. PROJECT, TASK OR SUBTASK NUMBER	11–22: 3A012501A805 · 23 24: 01 · 25 26: 01 · 27 28: · 29:
14. DATE OF REPORT (30-33)	30–33: 0664
15. SECURITY OF WORK (34)	34: 3
16. TYPE OF REPORT	35: 3 · 36–47 48 49 50 51: · 52–55: 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	56–61: 010700 · 62–64:
18. OSD CLASSIFICATION (65-66)	65 66: AR
19. R&D CATEGORY (67)	67: 1
20. CONTRACT NUMBER	11 12: DA · 13 14: · 15–17: · 18–21: · 22–26: · 27:
21. GRANT NUMBER	28 29: DA · 30–33: · 34 35: · 36–38: · 39 40: · 41–45: G · 46:
22. ESTIMATED COMPLET. DATES	47–51: 1CONT · 52–56: 2 · 57–61: 3 · 62–66: 4 · 67–71: 5
23. PRIORITY (11-14)	11–14: 1
24. PROGRAM ELEMENT (15-26)	15–26: 6•11•25•01•1
25. CMR&D CODES	27–29: N/A · 30–32: · 33–35:
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	36–39: 1412 · 40: · 41 42: f · 43 44: · 45: 6
27. FUNDING	
a. Est. Total Cost (11-15)	11–15: · 16–18: 1 · 19–21: 2 · 22–26:
b. % Spent Intern. (16-18) " " Extern. (19-21)	
c. Total Obligation (22-26)	
d. Progrmd. Cur. FY (27-33)	27 28: · 29–33: · 34 35: · 36–40:
e. " " " +1 (34-40)	
f. " " " +2 (41-47)	41 42: · 43–47: · 48 49: · 50–54:
g. " " " +3 (48-54)	
h. " " " +4 (55-61)	55 56: · 57–61: · 62 63: · 64–68:
i. " " " +5 (62-68)	
j. " " " +6 (69-75)	69 70: · 71–75: · 76–78:
k. Total Man Years of Effort (76-78)	

Card "C" — Card "D" — Card "E" — Card "F"

DA FORM 1 June 63 1309R — Previous Editions are Obsolete — Page 2 of _____

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36188

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8050102

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER
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576-2211 or Interdepartmental Code 198, Ext 2211 See Continuation Sheet 49

6. TITLE OF: PROJECT ☐ Ionizing radiation injury - prevention and treatment (U)
TASK ☐
SUBTASK ☒ Mechanisms (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME'

(U)The aim of this subtask is understanding of the mechanisms underlying radiation effects in living organisms. Direct and indirect as well as immediate and long-term effects are being studied. Specific areas of research include (1) effects of irradiation on survival in germ-free animals, (2) studies of the levels of alpha-emitting radionuclides (from the atmosphere) in various biological materials and systems, (3) investigation of radiation effects in insect populations utilizing ESR and activation analysis, (4) characterization morphologically of reparable and irreparable damage in the bone marrow, (5) studies on initial radiation lesions in Escherichia coli using chemical and physical modifiers of irradiation as a basic tool of research, (6) delineation of the effects of irradiation on the biological activity and stereochemistry or organic compounds, (7) cytogenetic analysis of radiation damage, (8) radiationsensitivity of eleven-day chick embryos, (9) autoradiographic and histologic evaluation of vascular and parenchymal damage in the irradiated kidney, (10) metabolism after radiation injury.

9. KEY WORDS
Mechanisms, radiation effects, direct effects, indirect effects, immediate effects, long-term effects, survival, germfree animal, alpha-emitting radionuclides, insect populations, ESR, activation analysis, reparable damage, irreparable damage, bone marrow, gold Escherichia coli, x-rays, biological activity, stereochemistry, organic compounds, cytogenetics, chick embryos, kidney, manganese

10. SUPPORTING PROJECTS
Not applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

DA FORM 1 June 63 1309R PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____

ACCESSION NO. 36188

ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER — 11–22: 3A012501A805 | 23 24: 01 | 25 26: 02 | 27 28: | 29:

14. DATE OF REPORT (30–33) — 30–33: 0664

15. SECURITY OF WORK (34) — 34: 4

16. TYPE OF REPORT — 35: 3 | 36–47 48 49 50 51: | 52–55: 1263

17. SCIENTIFIC FIELD
a. Topical Classific. (56–61) — 56–61: 010700
b. Functional Class (62–64) — 62–64:

18. OSD CLASSIFICATION (65–66) — 65 66: AR

19. R&D CATEGORY (67) — 67: 1

Card "D"

20. CONTRACT NUMBER — 11 12: DA | 13 14: | 15–17: | 18–21: | 22–26: | 27:

21. GRANT NUMBER — 28 29: DA | 30–33: | 34 35: | 36–38: | 39 40: | 41–45: G | 46:

22. ESTIMATED COMPLET. DATES — 47–51: 1CONT | 52–56: 2 | 57–61: 3 | 62–66: 4 | 67–71: 5

Card "E"

23. PRIORITY (11–14) — 11–14: 1

24. PROGRAM ELEMENT (15–26) — 15–26: 6•11•25•01•1

25. CMR&D CODES — 27–29: N/A | 30–32: | 33–35:

26. CDOG REFERENCE
a. Paragraph No. (36–44) — 36–39: 1412 | 40: | 41 42: f | 43 44: | 45: 6
b. Functional Group (45)

Card "F"

27. FUNDING

a. Est. Total Cost (11–15) — 11–15: | 16–18: 1 | 19–21: 2 | 22–26:
b. % Spent Intern. (16–18) — 27 28: | 29–33: | 34 35: | 36–40:
" " Extern. (19–21)
c. Total Obligation (22–26)
d. Progrmd. Cur. FY (27–33)
e. " " " +1 (34–40) — 41 42: | 43–47: | 48 49: | 50–54:
f. " " " +2 (41–47)
g. " " " +3 (48–54)
h. " " " +4 (55–61) — 55 56: | 57–61: | 62 63: | 64–68:
i. " " " +5 (62–68)
j. " " " +6 (69–75)
k. Total Man Years of Effort (76–78) — 69 70: | 71–75: | 76 78:

DA FORM 1 June 63 1309R — Previous Editions are Obsolete — Page 2 of _____

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REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964

Knospe, W. H. and Blom, H. J.: Regeneration of Locally Irradiated Bone Marrow I. Dose Dependent, Long-Term Changes in the Rat with Particular Emphasis upon Vascular and Stromal Effects (abstract). Radiation Research 22: 205, 1964.

McLaughlin, M. M.: Effects of the Germ Free State in the Response of Mice to Irradiation I. Thirty day Survival. In press in Radiation Research.

Nelp, W. B., Wagner, H. N. Jr., and Reba, R. C.: Renal Excretion of Vitamin B_{12} and its use in Measurement of Glomerular Filtration Rate in Man. J. Lab. and Clin, Med., 63: 480-491, 1964.

ANNUAL PROGRESS REPORT

Project No. 3A012501A805 Title: Ionizing radiation injury prevention and treatment

Task No. 01 Title: Ionizing radiation injury prevention and treatment

Subtask No. 02 Title: Mechanisms

Descriptions:

1. Germfree mice exposed to x-irradiation at different ages were compared as to survival times with each other and with normally raised mice.

2. Submammalian animals have been examined with special detecting equipment to measure the amounts of alpha emitting radionuclides present internally and to deduce therefrom the fallout contamination present.

3. ESR signals have been recorded in two generic groups of ants, Pogonomyrmex and Formica, before and after irradiation. Differences in manganese concentrations were found and partially quantified.

4. Single hind limbs of rats were exposed to various doses of x-irradiation and studied in regard to hematopoietic regeneration, at first without and later with infusion of labeled donor marrow.

5. The interactions between various chemical and physical modifiers of radiation injury and the comparative effects of these treatments in irradiated and unirradiated bacteria have been studied in an attempt to clarify the nature of the initial lesions of irradiation.

6. Mandelic acid in solution was subjected to irradiation and the chemical changes studied to determine post irradiation changes in optical rotation - those changes to be then correlated with changes in physiological activity.

7. A significant amount of radiation damage to somatic as well as generative tissue is now thought to be due to genetic damage expressed when the cell divides. Some anatomic evidence of this damage can be obtained by microscopic examination of chromosomal spreads made from cells in mitosis. At the present time, characterization of the types of damage observed has been fairly well catagorized in various animals after various amounts of gamma irradiation. Much less is known about damage due to neutron or mixed irradiation. This department is now exploring this area by examining mitoses of irradiated lymphocytes after short-term tissue culture. Mitotic figures seen in bone marrow cells from irradiated animals are also being characterized.

8. Analysis of the effect of protective drugs is useful in elucidating mechanisms of radiation injury. Previous studies have indicated chick embryos have reproducible LD_{50} values after irradiation and are convenient experimental systems. It was decided to attempt extension of the usefulness of this system by determining the response of the embryos to radiation after treatment with MEA or AET (protective drugs).

9. The relative radiosensitivity of cells within organ systems has been shown to be related to mitotic rate, degree of differentiation and length of division time. The kidney has been chosen as a model with cells showing a high degree of differentiation with limited mitosis. The kidney is intermediate in degree of radiosensitivity as compared to other organ systems. Cellular turnover of various renal components is being studied by tritiated thymidine incorporation prior to and following unilateral or bilateral localized renal irradiation.

10. Alteration in metabolic pathways after irradiation may be used to elucidate the mechanism by which radiation damage occurs. At the present time activation analysis and isotope tracer methods are being developed as tools to follow these pathways. Studies have been undertaken to provide a baseline for later studies in irradiated subjects. Isotope studies have included final evaluation of radioactive, free, Vitamin B^{12} as a means for measuring glomerular filtration. A study to follow total body potassium in several children, both normals and some with growth retardation due to endocrine or cardiac lesions, has been initiated. Another isotopic study concerned volume of distribution and effectiveness of an experimental oral diuretic agent, AT-101. Methods, using activation analysis, have been developed to determine the quantitative presence in biological material of gold and manganese.

Progress:

1. Germfree Studies. Studies on the effects of the germfree state on the radiation response of mice were continued.

In order to complete the survival time curve and to substantiate the longer survival time already found for germfree mice after exposure to supra-lethal amounts of x-radiation (25,000 and 40,000 r), mice were exposed to 35,000 r. A mean survival time of 69 hours was found for germfree mice, while conventional mice survived an average of 22 hours.

A group of old (40-week) mice were exposed to 1800 r x-ray, and the survival time information was added to the data being accumulated for the age vs sensitivity study. The survival time for this age group was not significantly different than that for younger (12-20 weeks) mice. This study will be completed when mice of still younger age groups (4, 6, 8 weeks) become available.

The LD_{50} curve for the strain of mice now commercially available (ICR) was completed. The LD_{50} for germfree mice was found to be higher than that for conventional mice. This confirms the finding already reported for the ND-2 strain.

Thyroid activity was measured in both germfree and conventional mice using radio-iodine. Whole body retention of I^{131} was found to be greater in the germfree mice. This difference appeared within six hours after i.p. administration of trace amounts of I^{131}. After determination of whole body retention, the radio-iodine content of the thyroid gland and serum was measured. In addition the protein bound iodine fraction, expressed as a conversion ratio, was calculated in an effort to determine if there is a difference in thyroxine production between germfree and conventional mice. Evaluation of these data has not yet been completed.

Germfree mice have a longer survival time after x-ray doses which produce gastro-intestinal death in conventional mice within 3-5 days. Since the rate of cell renewal in intestinal epithelium is much slower in germfree mice, and since radiation damage is known to be reduced in tissues with low rates of cellular proliferation, a study was started to determine if this difference in cell renewal rate is responsible for the increased survival time in germfree mice. Tritiated thymidine was injected i.p. twelve hours before exposure to 1500 r x-ray. Animals were sacrificed at twelve-hour intervals post-irradiation. Sections of ileum, duodenum and jejunum and radio-autographs are being made from these sections. This study is not yet complete.

In an effort to determine basic differences between germfree and conventional animals (which may be related to the observed differences in radiosensitivity), biochemical, radiochemical and neutron activation analyses are planned. Serum, urine and muscle tissue from control and irradiated rats and mice have been obtained and are being preserved until these analyses can be performed.

2. Ecology of Alpha-emitters. A special electron-pulse alpha spectrometer (Eon, Model 400) was installed and calibrated and is now in use for determination of alpha-emitting isotopes in different materials. This high-sensitivity instrument (which can detect down to 10^{-12} - 10^{-14} curies/gram of isotopes in various materials) is used in connection with a semi-conductor counter so that a wide range of alpha activities can be detected from amounts close to those given in the radiation protection guides to minute amounts as given above. The equipment has been used also in the study of fish and insects (bees and ants) which in certain cases are collectors of natural fallout from foliage, flowers, etc. These studies may give additional results in connection with the usual methods of radio assay of fallout.

3. Free Radical Studies. Some relationships between ESR signals, growth of plants and protective compounds have been established. Ants

show distinctive ESR signals, the form and shape of the signal varying with species and genera. The genus Formica shows a atrong manganese signal, the genus Pogonomyrmex shows practically no manganese signal at all but only a well defined narrow signal in the free radical (g=2.00) area. Neutron activation analyses showed Formica (stingless) ants to contain 1 microgram per gram of manganese in the body, while in Pogonomyrmex (sting bearing) ants practically no manganese could be detected. Since the ESR signal is caused by manganese ions; the combination of ESR and NMR technics should allow measurement of the ratio of Mn^{++}/total Mn and its dependence on environmental factors.

4. Bone Marrow Repopulation. Studies have been completed evaluating the role of changes in the stroma and fine vasculature in the regeneration of heavily irradiated bone marrow. The upper limit of hemopoietic regeneration of locally irradiated rat bone marrow falls between 2000 and 4000 r. Furthermore, this process of regeneration appears to depend upon the regeneration of a sinusoidal system approximating that present in normal bone marrow. In turn, sinusoidal regeneration appears to depend upon the sensitivity of reticulum cells to radiation. Beyond 4000 r, reticulum cells do not give rise to a sinusoidal system capable of supporting the metabolic exchanges of the rapidly proliferating hemopoietic cell lines.

Studies are now in progress to evaluate the possibility of repopulating locally irradiated rat bone marrow with donor cells after doses of 2000, 4000 and 6000 r. After permitting the lesion to evolve for 3 months, isogenic tritium labeled donor bone marrow is administered intravenously. These animals will be compared with irradiated and non-irradiated controls utilizing standard histologic and autoradiographic techniques.

5. Modification of Radiation Injury. The purpose of these experiments is to elucidate the mechanisms by which radiation damage occurs in Escherichia coli and how these mechanisms are altered by certain chemical and physical agents.

Beta-mercaptoethylamine (cysteamine, MEA), a well known modifier of radiation effects in microorganisms has been used pre- and post-irradiation as an aid to the identification and study of the mechanisms of damage.

MEA treatment before irradiation was found to significantly enhance the colony forming ability of bacteria exposed to irradiation under nitrogen gas (anoxia).

MEA was found to have an additive effect in increasing the survival of aerobically irradiated bacteria which were incubated under optimal post-irradiation conditions of temperature and nutrition.

MEA was further found to have either a bacteriostatic or a bactericidal effect on unirradiated cells depending on how the cells were grown and/or assayed.

Preliminary results indicate that MEA given post irradiation prevents the release of the 260 m μ absorbing material frequently observed in untreated irradiated cell suspensions.

6. Radiation Damage to Biologically Active Molecules. The biological activity of optical isomers of some organic compounds is often stereospecific. The biological effectiveness may vary by orders of magnitude depending upon the optical form. If racemization occurred under the influence of ionizing radiation, profound biological consequences might result. Mandelic acid has been reported to undergo such a change and therefore was selected for the initial study. De-aerated aqueous solutions of pure D-mandelic acid have been irradiated with a cobalt 60 source. The reaction products are complex and have not all been conclusively identified. Benzaldehyde, a major component has been studied and the G value for the production has been determined as a function of dosage at a fixed concentration and as a function of concentration at a fixed dosage. Two optically active substances have been isolated and their optical rotatory dispersion measured which, in each case, resulted in a simple Drude curve. The original mandelic acid was recovered. The specific optical rotation was low but it was not conclusively proved that this change was produced by racemization or by a trace of impurity.

An effort is under way to resolve the recovered product into its constituent parts.

7. Cytogenetic Studies. Most work this year was directed towards short-term culture of rat lymphocytes. Limited success was obtained in that multiple cultures were made and only sporadically good mitotic spreads obtained. More attention is now being directed towards chromosomal characteristics of mouse bone marrow cells after exposure to either mixed irradiation from the nuclear reactor or irradiation from the 300 KV x-ray machine.

8. Radiation Sensitivity of Eleven Day Chick Embryos. Eleven-day embryos were selected for study. All irradiations were performed with a General Electric 300 KVP Maxitron unit. Embryos were innoculated intravenously with .1 ml of drug diluted in saline (controls received saline only) 15 to 20 minutes prior to irradiation. Acute toxicity studies were also performed on the drugs used. Acute radiation-induced deaths occur rapidly and mortality was recorded by candling 18 hours after irradiation.

Results of this experiment are presented in the following table.

Radiation Dose (R)	% Death (saline control)	% Death (4 doses MEA)				% Death (4 doses AET)			
		.25mgm	.5mgm	1mgm	2mgm	.25mgm	.5mgm	1mgm	2mgm
0	0.0	0.0	0.0	0.0	25.0	0.0	0.0	16.6	33
200	0.0	---	---	0.0	40.0	---	---	---	--
300	3.8	---	---	0.0	--	0.0	---	---	--
400	1.4	---	100	81	100	10	0.0	6	0
500	7.5	57	85	100	88	20	10	13	20
600	62	100	100	100	100	40	44	15	22
700	88	100	100	100	100	89	100	67	75
800	100	100	100	100	100	100	100	92.9	80

It is of interest that MEA resulted in 100% mortality. On the other hand AET reduced radiation mortality compared to saline controls.

9. Autoradiographic and Histologic Evaluation of Vascular and Parenchymal Damage of the Irradiated Kidney. Male Wistar rats have been given unilateral or bilateral acute renal irradiation with 300 KV x-ray at a dose of 1000 rad. Both irradiated and control animals have received intraperitoneal injections of tritiated thymidine 24 hours prior to sacrifice. Serial sacrifices have been made at weekly intervals for 90 days post-irradiation. Both autoradiographs and routine histologic sections with Hemotoxylin and Eosin and the Periodic Acid Schiff stain are in progress.

10. Metabolism after Irradiation. Total body water and muscle mass determination have shown no difference between 10 control dogs and 30 long-term survivors of massive acute radiation. Red blood cell kinetics, studied in 40 surviving dogs, utilizing $Iron^{59}$ and $Cromium^{51}$ were normal.

It was found that presaturation of binding sites with stable B_{12} made renal clearance of free radioactive Vitamin B_{12} an accurate measurement of glomerular filtration in humans and dogs. This was true through the full range of glomerular flow.

Using the liquid scintillation whole body counter, four pituitary dwarfs, 2 children with growth retardation secondary to cyanotic heart disease and three selected normals have had total body potassium determinations. These determinations will be repeated in the future.

The mean renal clearance value for C-14 AT-101 was 34.1 $\pm$ 3.4 (1S.D.) ml/minute. The mean inulin clearance was 42.7 ml/minute with a mean AT-101/inulin clearance ratio of 0.80 $\pm$ .09 (1S.D.). Therefore,

in the hydrated state there was a net tubular reabsorption of approximately 20% of the filtered diuretic.

The AT-101 space was 54.2 ± .2 (1S.D.) per cent of body weight and therefore was much greater than the extracellular space (determined to be 21.5 per cent) and approached that of total body water (found to be 59.9 per cent).

The mean fractional plasma clearance rate of AT-101 was 0.26/hour.

Methods have been developed, utilizing activation analysis, which allow quantitation of manganese and gold in insects and the organs of very small animals. Both methods are now routinely used with 5-10 mgm tissue samples and 1 mgm samples can be utilized when necessary.

Amounts of gold and manganese in the order of 10^{-3} micrograms are being measured which makes the method as sensitive as any competitive method. Furthermore, with a neutron source available, this method in selected cases is much more efficient than competitive methods. In the case of gold, more than 1300 samples have been run in a 4-month period by two technicians working two days each week.

Summary and Conclusions:

1. Germfree Studies. Germfree mice survive significantly longer than do conventionally raised mice following doses ranging from 1500 r up to 40,000 r. The LD_{50} is higher and I^{131} retention is greater in germfree mice. Further studies are planned to elucidate the basic mechanisms involved in these differences.

2. Ecology of Alpha-emitters. Data on the levels of alpha emitting radionuclides in certain fish and insects have been collected to establish a method of detecting and quantifying pickup of radionuclides from fallout.

3. Free Radical Studies. ESR and NMR studies have been shown to be useful technics in studying the levels of certain biologically important ions in insects both with and without irradiation injury.

4. Bone Marrow Repopulation. Regeneration of hematopoietic elements following local irradiation of rat bone marrow appears to depend on the sensitivity of reticulum cells to radiation and their subsequent ability to give rise to a marrow sinusoidal system.

5. Modification of Radiation Injury. The ability of MEA to give protection over and above that given by anoxia during irradiation suggests that it is acting on a mechanism of damage different from that related to oxygen tension. This protective action of MEA is not influenced by post-irradiation incubation temperatures or nutrition.

6. Radiation Damage to Biologically Active Molecules. The G values for the production of benzaldehyde during irradiation of D-mandelic acid has been determined. Two other optically active compounds have been detected. The lowering of the specific optical rotation of irradiated optically active mandelic acid has been confirmed.

7. Cytogenetic Studies. Two techniques have been evaluated for cytogenetic determination of neutron and mixed irradiation damage. At present chromosomal spreads from irradiated bone marrow appears to be the most satisfactory technique.

8. Radiation Sensitivity of Eleven-Day Chick Embryos. MEA enhances susceptibility of chick embryos to radiation by an undetermined mechanism. AET offers protection against radiation in this system. This preliminary study shows qualitative evidence of the usefulness of the chick embryo in radiation research.

9. Autoradiographic and Histologic Evaluation of Vascular and Parenchymal Damage of the Irradiated Kidney. Preliminary evaluation of these serial studies indicates an initial vascular insult to arterioles followed by parenchymal damage.

10. Metabolism after Irradiation. Efficient and sensitive methods for activation analysis of tissue samples from irradiated subjects have been developed and are being used in the division. Isotope tracer and dilution methods have been evaluated which will be useful in post irradiation studies of metabolism.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36190

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8050103

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
A Walter Reed Army Inst of Rsch
Walter Reed Army Medical Center
Washington, D. C., 20012
723-1000, Ext 3552
49

5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER
(P) Jacobus, David P., M.D., Department of Medicinal Chemistry
WRAIR, WRAMC, Washington, D. C., 20012
576-2280 or Interdepartmental Code 198, Ext 2280 See Continuation Sheet 49

6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Phase I CIDS program (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME

(U) Phase I of the CIDS Program for the development of prototype equipment, prototype software and experimental operating experience is proceeding on or close to schedule. The prototype of the production typewriter is to be delivered in June. The manual search program, along with information on how to ask questions manually or through the chemical typewriter is available now. Completion of the programs necessary to convert the old chemical typewriter code to the Colgate program has been accomplished. These programs are being debugged. The Service Bureau program designed to handle large files of chemical structures and many questions has been flow charted and run in Fortran. Flow charts are available. Operating costs and production rates for the old chemical typewriter are tabulated in the report.

9. KEY WORDS

Chemistry, information, data systems, computers, typewriters, software, information retrieval, CIDS.

10. SUPPORTING PROJECTS

Not applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36190	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	3A012501A805 / 01 / 03
14. DATE OF REPORT (30-33)	0664
15. SECURITY OF WORK (34)	4
16. TYPE OF REPORT	3 / 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	040202
18. OSD CLASSIFICATION (65-66)	AR
19. R&D CATEGORY (67)	1

Card "D"

20. CONTRACT NUMBER	DA / 49 / 024 / MD / 01166
21. GRANT NUMBER	DA / G
22. ESTIMATED COMPLET. DATES	1096 4 / 2 / 3 / 4 / 5

Card "E"

23. PRIORITY (11-14)	1
24. PROGRAM ELEMENT (15-26)	6.11.25.01.1
25. CMR&D CODES	N/A
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	1412 / f / 6

Card "F"

27. FUNDING

a. Est. Total Cost (11-15)	
b. % Spent Intern. (16-18)	1
" " Extern. (19-21)	2
c. Total Obligation (22-26)	
d. Progrmd. Cur. FY (27-33)	
e. " " " +1 (34-40)	
f. " " " +2 (41-47)	
g. " " " +3 (48-54)	
h. " " " +4 (55-61)	
i. " " " +5 (62-68)	
j. " " " +6 (69-75)	
k. Total Man Years of Effort (76-78)	

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Previous Editions are Obsolete

Page 2 of _____

ACCESSION NUMBER 36190	ARMY RESEARCH TASK REPORT Continuation Sheet

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:
(A) Feldman, Alfred P., M.S., Dept of Medicinal Chemistry
WRAIR, WRAMC, Washington, D. C., 20012
576-3568 or Interdepartmental Code 198, Ext 3568 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. 3A012501A805 Title: Ionizing Radiation Injury--Prevention and Treatment

Task No. 01 Title: Ionizing Radiation Injury--Prevention and Treatment

Subtask No. 03 Title: Phase I CIDS Program

Description: The objective of the program is to develop an effective system for handling chemical structural information with specific capacity of generic search and recognizable input and output.

Progress: Phase I of the CIDS Program established by Letter DA, OCRD, CRD/P, Subject: Army Chemical Information and Data System (CIDS), 12 June 1963, with Inclosure 1, is for the development of prototype equipment, prototype programs and the subsequent development of experience using those programs. Some of the technical management of this program has been at WRAIR. Some work has been done on contract.

The original typewriter which was built entirely by WRAIR personnel has been operated on contract continuously for the last 8 months. There have been appreciable periods of breakdown so that the performance of this prototype machine has not been as satisfactory as the performance we anticipate from the production model. Most of this breakdown has been attributed to the fact that we have married power supplies for the coding functions with existing RemRand equipment rather than designing a machine from the beginning. The purpose of the machine was to demonstrate feasibility and to obtain initial operating costs. In Table I, a summary of costs and output is available. Our actual cost is $ 0.25 per structure. We understand, unofficially, that the contractor is paying $ 0.08 per structure. The relatively high overhead is the result of breakdowns. The new typewriter has a chemical font which operates on one writing line instead of three, an overall speed of 10 characters per second rather than 4, and hopefully will not have the multiple breakdowns that the experimental machine suffered. We expect that we will be able to achieve the same rate with the new machine as with the old machine, namely 100,000 structures/year.

In the new typewriter development the design of the type font was a very crucial decision. The type font must provide valid information interpretable by machine yet sufficiently flexible to allow the production of attractive structures and to permit multiple forms of output. The type font was designed at WRAIR. Figure I shows examples of the old Cyanamid keyboard and the new keyboard. The examples from Mergenthaler were made before the machine was aligned but these improvements shown are the joining of lines, how hetero atoms are centered and that it is possible to complete a ring lying on its side.

Chemical structures when typed on any typewriter are a combination of atoms, bonds and corners. It is necessary to program the computer to interpret corners in order to determine how many hydrogens are attached to each corner. Some combinations may signify that a double bond is joined to a carbon atom, others that only a single bond is present. We had previously provided Colgate with an extensive table of an allowable combinations of key strokes and the interpretation of such combinations. To aid in the interpretation of the new type font, a scheme has been devised in which the symbols were written on McBee cards, one symbol to a card. The cards were punched, and by holding a pair of cards together, one is visually informed through coincidence of notches of whether the combination is valid, and what is the number of hydrogens associated with the carbon. For combinations involving more than two symbols, the relationship:

$$V(A,B) = 1 = H\ (A,B)$$
$$V(A,C) = 1 = H\ (A,C)$$
$$V(B,C) = 1 = H\ (B,C)$$

where A, B, and C represent each one a card (or character), V denotes the presence of a visible validity check, and H denotes the presence of a hydrogen, as revealed by a coincidence of notches. This may be the first instance of the use of McBee cards in programming a computer.

To optimize the layout of the keyboard on the Mergenthaler machine a frequency count of 233,070 characters as made on structures typed on the old machine. The frequency of each key was determined along with its association with every other key, so that we could not only place the most frequent keys in the area where the finger action is strong, but we could also arrange for alternation of the hands used in striking frequently occurring keys.

Contracts (A summary is shown in Table II): The development of a prototype Mergenthaler machine appears to be going well. The ACS II code has been successfully used even with the random number generation resulting from the Matrix coding. Delivery of the machine is expected in June 1964.

The contract with Colgate Palmolive for the conversion of the old chemical type font to their search system is progressing well. A great deal of work has been done on the formulation of questions. By virtue of this program, it will be possible to take input generated by a chemical typewriter and search it by means of either questions generated on the chemical typewriter or questions generated manually. We plan to extend this program to include the new typewriter.

The Service Bureau Corporation program which is designed to handle a large volume of material is scheduled for delivery in September. This program is proceeding in a satisfactory manner. Program notes and system design notes are available.

Summary and Conclusions: Phase I of the CIDS Program for the development of prototype equipment, prototype software and experimental operating experience is proceeding on or close to schedule. The prototype of the production typewriter is to be delivered in June. The manual search program, along with information on how to ask questions manually or through the chemical typewriter is available now. Completion of the programs necessary to convert the old chemical typewriter code to the Colgate program has been accomplished. These programs are being debugged. The Service Bureau program designed to handle large files of chemical structures and many questions has been flow charted and run in Fortran. Flow charts are available. Operating costs and production rates for the old chemical typewriter are tabulated in the report.

Present Coding Rate	100,000 structures/year
Total Direct Cost/structure	25 cents/structure
Direct Labor Cost	8 cents/structure
Present Production Costs (20 units)	$16,000
Amortization in 1st year	16 cents/structure
Labor Costs with 1 year Amortization	24 cents/structure
Total costs with 1 year Amortization	41 cents/structure

Table 1

CIDS CONTRACTS

Development of Prototype Machine	$ 71,678.00
Cost per machine (20 units)	16,000.00
Colgate Program Small Computer	29,000.00
Service Bureau Program Large Computer	22,487.50
Present Search Costs on Colgate: 1000 compounds/min. for 10 searches Rent $45/hour = 60,000 compounds for $45 for 10 searches	
Cost of conversion of typewriter to normal table	?
Cost of Operation on 7090	?

Table 2

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER 36179

PROJECT, TASK, OR SUBTASK NO. 3A012501A8180120

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R & D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
A Walter Reed Army Inst Rsch
Walter Reed Army Medical Center
Washington, D. C., 20012
723-1000, Ext 3552 49

5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER
(P)Sadun, E. H., Sc.D., Dept of Medical Zoology, Division of Comm Dis & Immunol, WRAIR, WRAMC, Wash, D. C., 20012
576-3308 or Interdepartmental Code 198, Ext 3308 See continuation sheet 49

6. TITLE OF: PROJECT ☐
TASK ☐
SUBTASK [A] Parasitic diseases (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964 |

8. RESUME' (U) A fluorescent antibody test for visceral leishmaniasis with high specificity was developed and evaluated. Preliminary experiments to ascertain the infectibility of germ-free mice by Schistosoma mansoni were begun. The susceptibility to S. mansoni of 5 species of monkeys was compared. After resistance to S. mansoni was induced in monkeys with irradiated cercariae, tissue damages caused by the immunizing and challenging exposures were studied. Extensive quantitative serological tests were carried out. Pulmonary reactions to normal and irradiated cercariae were compared in mice, muskrats and monkeys. Immunological experiments with schistosome egg antigen-antibody complexes were done in mice and suckling rats. Flocculation tests with schistosome egg extracts and worm excretion-secretions were developed and compared with the cercarial slide flocculation test. The acquired resistance to S. mansoni of rats injected with serum from immunized monkeys was studied. Immunodiagnostic screening tests for schistosomiasis were evaluated in an Ethiopian village. The activity against S. mansoni of Ronnel, S-201 and TWSb in oil in monkeys was studied.

No marked biochemical changes were detected in rabbits infected with Dirofilaria uniformis.

9. KEY WORDS Parasite, fluorescent, leishmania, germ-free, schistosoma, immunodiagnostic, dirofilaria, antigen, antibody.

10. SUPPORTING PROJECTS
Not applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
[X] NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
[X] NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36179

ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER (11–22, 23 24, 25 26, 27 28, 29)
3 A 0 1 2 5 0 1 A 8 1 8 | 0 1 | 2 0 | | |

14. DATE OF REPORT (30–33)
0 6 6 4

15. SECURITY OF WORK (34)
4

16. TYPE OF REPORT (35, 36–47, 48 49, 50 51, 52–55)
3 | | 1 2 6 3

17. SCIENTIFIC FIELD
a. Topical Classific. (56–61)
b. Functional Class (62–64)
0 1 0 6 0 2 | |

18. OSD CLASSIFICATION (65–66)
A R

19. R&D CATEGORY (67)
1

Card "D"

20. CONTRACT NUMBER (11 12, 13 14, 15–17, 18–21, 22–26, 27)
D A | | | | | |

21. GRANT NUMBER (28 29, 30–33, 34 35, 36–38, 39 40, 41–45, 46)
D A | | | | | | | G | | | | |

22. ESTIMATED COMPLET. DATES
1 C O N T | 2 | 3 | 4 | 5

Card "E"

23. PRIORITY (11–14)
24. PROGRAM ELEMENT (15–26)
1 | 6 • 1 1 • 2 5 • 0 1 • 1

25. CMR&D CODES (27–29, 30–32, 33–35)
N / A | | |

26. CDOG REFERENCE
a. Paragraph No. (36–44)
b. Functional Group (45)
1 4 1 2 | | a | | 6

Card "F"

27. FUNDING

a. Est. Total Cost (11–15)			1	2	
b. % Spent Intern. (16–18)					
" " Extern. (19–21)					
c. Total Obligation (22–26)					
d. Progrmd. Cur. FY (27–33)					
e. " " " +1 (34–40)					
f. " " " +2 (41–47)					
g. " " " +3 (48–54)					
h. " " " +4 (55–61)					
i. " " " +5 (62–68)					
j. " " " +6 (69–75)					
k. Total Man Years of Effort (76–78)					

DA FORM 1 June 63 1309R

Previous Editions are Obsolete

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

(A) Bruce, J. I., B. S., Dept of Medical Zoology, Div of Comm Dis & Immunol, WRAIR, WRAMC, Washington, D. C., 20012, 576-3361 or Interdepartmental Code 198, Ext 3361 49

(A) Duxbury, R. E., B. S., Dept of Medical Zoology, Div of Comm Dis & Immunol, WRAIR, WRAMC, Washington, D. C., 20012, 576-3055 or Interdepartmental Code 198, Ext 3055 49

(A) Schoenbechler, M. J., B. S., Dept of Medical Zoology, Div of Comm Dis & Immunol, WRAIR, WRAMC, Washington, D. C., 20012, 576-3361 or Interdepartmental Code 198, Ext 3361 49

(A) Williams, J. S., Dept of Medical Zoology, Div of Comm Dis & Immunol, WRAIR, WRAMC, Washington, D. C., 20012, 576-3055 or Interdepartmental Code 198, Ext 3055 49

REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

Anderson, R. I., Sadun, E. H., and Schoenbechler, M. J.: Cross-absorption studies performed with Schistosoma mansoni and Trichinella spiralis antigens in sera from patients with trichinosis. Expt. Parasit. 14:323-329, 1963.

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Sadun, E. H., Duxbury, R. E., Williams, J. S., and Anderson, R. I.: Fluorescent antibody test for the serodiagnosis of African and American trypanosomiasis in humans, J. Parasit. 49:385-388, 1963.

Sadun, Elvio H.: Immunization in schistosomiasis by use of antigens, irradiated cercariae, and cercariae from homologous and heterologous strains. Annals of the New York Academy of Sciences. 63:418-439, 1963.

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Sleeman, H. K., Fife, E. H., and Bruce, J. I.: Isolation and Characterization of Serologically Active Components from Secretory and Excretory Products of Schistosoma mansoni Cercariae. J. Parasit. 49: (Suppl.) 71, 1963.

Sandt, D. G., Bruce, J. I., and Radke, M. G.: A Snail Colony Facility for the Mass Production of Schistosoma mansoni Cercariae. J. Parasit. 49: (Suppl.) 32, 1963.

Williams, J. S., Duxbury, R. E., Anderson, R. I., and Sadun, E. H.: Fluorescent antibody reactions in Trypanosoma rhodesiense and T. gambiense in experimental animals. J. Parasit. 49:380-384, 1963.

von Lichtenberg, F. and Sadun, E. H.: The parasite migration and host reaction in mice exposed to irradiated cercariae of Schistosoma mansoni. Expt. Parasit. 13:256-265, 1963.

von Lichtenberg, F., Sadun, E. H., and Bruce, J. I.: Host response to eggs of Schistosoma mansoni. III. The role of eggs in resistance. J. Infect. Diseases 113:113-122, 1963.

ANNUAL PROGRESS REPORT

Project No.	3A012501A818	Title:	Communicable Diseases and Immunology
Task No.	01	Title:	Communicable diseases
Subtask No.	20	Title:	Parasitic diseases

Description: The primary purpose of these investigations was to study the various immunological, physiological and ecological aspects of parasitic diseases toward the goal of gaining a better understanding of natural susceptibility, acquired resistance and the effectiveness of therapeutic agents for the prevention, suppression and treatment of some of these diseases. Leishmaniasis, schistosomiasis, and filariasis were used as models for these studies.

Progress:

1. Fluorescent antibody test for the serodiagnosis of visceral leishmaniasis

The current report summarizes results of studies in which the FA technic was used to stain culture forms of L. donovani for the purpose of developing a reliable test for the laboratory diagnosis of kala-azar. The procedures were evaluated with sera obtained from humans with proven kala-azar infections in endemic areas. Attempts were made to determine the degree of cross-reactivity with sera from individuals infected with different Leishmania species and from those with several other viral, mycotic and parasitic infections. Furthermore, studies were carried out to determine whether blood smears dried on paper could be used in the serological diagnosis of visceral leishmaniasis.

A marked degree of yellow-green fluorescence was observed on the leptomonad forms where immune sera were used in the indirect FA technic. This contrasted vividly with the light red fluorescence observed with normal sera in the same system.

The relative sensitivity of the technic is indicated by results obtained with sera from visceral leishmaniasis patients (Table I). Findings based on sera from individuals with other diseases and from healthy controls indicate the degree of specificity of the test. Cross-reactions

Table I

Results obtained in the fluorescent antibody test for visceral leishmaniasis in human sera

Diagnostic Status	Number Tested	Reaction	Weak Reaction	No Reaction
Visceral Leishmaniasis:	30	21	5	4
Other Infections:				
Mucocutaneous leishmaniasis	31	7	5	19
Cutaneous leishmaniasis	5	0	1	4
African trypanosomiasis	16	0	1	15
American trypanosomiasis	13	2	0	11
Malaria	23	3	2	18
Amebiasis	2	0	0	2
Trichinosis	8	0	0	8
Strongyloidiasis	5	0	0	5
Onchocerciasis	6	0	1	5
Schistosomiasis	15	1	2	12
Syphilis	12	0	1	11
Leprosy	15	1	1	13
Tuberculosis	10	0	0	10
Systemic mycoses*	27	0	1	26
Degenerative Diseases:				
Lupus erythematosus	8	1	0	7
Healthy Controls:	22	0	1	21

*All 27 were positive in one or more tests for histoplasmosis, blastomycosis or coccidioidomycosis; 5 were from patients with coccidioidomycosis proven by recovery of organisms.

were obtained with sera from patients with mucocutaneous leishmaniasis. Among individuals with other proven parasitic, viral and bacterial infections, cross-reactions occurred occasionally with the sera from malaria, American trypanosomiasis, schistosomiasis, and leprosy patients Twenty-one of 22 healthy control sera were nonreactive in the test; the remaining serum specimen was weakly reactive.

Comparison between serum samples and dried blood smears from the same kala-azar patient indicated a close correlation between results obtained with the two types of specimens. Tests performed with these dried blood smears after storage at room temperature indicated that positive results could be obtained for at least two months following collection. Similar samples from kala-azar patients tested approximately 15 months after collection gave no reaction in the test.

The results of these experiments, conducted with a total of 248 human sera, reveal that specific fluorescent antibodies are produced in man following active infection with Leishmania donovani. These antibodies are detectable in a reproducible manner by the indirect fluorescent antibody technic, using as antigen leptomonad forms of L. donovani. The limited number of cross-reactions obtained with sera from individuals with viral, bacterial, mycotic, and parasitic infections, and with lupus erythematosus indicate the relatively high specificity of this test and its potential value in routine diagnosis.

2. Species specificity studies of the fluorescent antibody test for leishmaniasis in naturally infected humans and artifically immunized rabbits.

Following the successful use of the fluorescent antibody technic in the serodiagnosis of visceral leishmaniasis in humans, further studies were conducted on the sensitivity and specificity of the test. Leishmania braziliensis antigen was employed in the technic for the first time and comparisons were made with results obtained with L. donovani antigen.

Fifteen sera from patients with proven mucocutaneous leishmaniasis and an equal number from kala-azar patients were tested along with control sera from healthy donors. The antigens used were the leptomonad forms of L. braziliensis (Guatemala strain) and L. donovani (Khartoum strain).

Results indicate a greater sensitivity in the homologous system with L. donovani than with L. braziliensis (Table II). In addition, the

titers obtained with L. donovani in the same system were higher as compared to those of L. braziliensis (Table III). Although there was considerable cross-reactivity between the two Leishmania species, a greater percentage of positive reactions and higher titers were observed with the homologous system.

Table II

Results obtained in the fluorescent antibody test using antigens and antisera of two Leishmania species

Antiserum	Parasite antigen	No. Tested	Reaction	Weak Reaction	No Reaction
L. donovani	L. donovani	15	10	4	1
L. donovani	L. braziliensis	15	5	6	4
L. braziliensis	L. braziliensis	15	5	8	2
L. braziliensis	L. donovani	15	4	5	6
Healthy control	L. donovani	4	0	0	4
Healthy control	L. braziliensis	4	0	0	4

Table III

Summary of titers obtained in the fluorescent antibody test for leishmaniasis

Serum specimen	Parasite antigen	No Tested	No. of times given titer was obtained 0	16	32	64	128	256
L. donovani	L. donovani	15	1	2	3	2	6	1
L. donovani	L. braziliensis	15	4	1	6	3	1	0
L. braziliensis	L. braziliensis	15	2	7	1	4	0	1
L. braziliensis	L. donovani	15	6	2	4	2	0	1

The initial appearance of FA antibodies and the occurrence of cross reactivity were studied in a group of rabbits artificially immunized with different species of Leishmania.

Three pairs of adult laboratory rabbits were immunized with L. donovani, L. braziliensis and L. tropica, respectively. Previous to immunization it was determined that no Leishmania antibodies detectable

Table IV

Results of fluorescent antibody tests for leishmaniasis in immunized rabbits

Serum Specimen	Rabbit No.	Day	Titer L. donovani antigen	Titer L. braziliensis antigen	Titer L. tropica antigen
	1	0	0	0	0
	2	0	0	0	0
	1	9	0	0	0
	2	9	0	0	0
L. donovani					
	1	16	16	16	16
	2	16	64	64	16
	1	21	64	256	64
	2	21	256	256	64
	3	0	0	0	0
	4	0	0	0	0
	3	9	0	0	0
	4	9	0	0	0
L. braziliensis					
	3	16	16	256	4
	4	16	0	0	0
	3	21	16	64	16
	4	21	16	64	16
	5	0	0	0	0
	6	0	0	0	0
	5	9	0	0	0
	6	9	0	0	0
L. tropica					
	5	16	256	64	64
	6	16	64	16	16
	5	21	64	64	64
	6	21	16	16	64

in the FA test occurred naturally in the experimental animals. The animals were bled for recovery of serum on days 0, 9, 13, 16 and 21. On the 21st day all animals were sacrificed for collection of larger quantities of serum, part of which was designated for use in projected cross-absorption studies.

Each of the sera collected was tested against all three Leishmania antigens in the same FA testing procedure developed for use with human sera. Results, summarized in Table IV, indicate that extensive cross reactions occurred in the three species. However, some evidence was accumulated suggesting higher titers in the homologous system. These results were in agreement with and extended those previously observed with sera from naturally infected humans.

3. Schistosoma mansoni infections in germ free mice

On the basis of a recommendation made by a panel of schistosomiasis experts convened recently in Geneva by the World Health Organization, studies were initiated to determine some of the factors responsible for the localization of the adult worms in the portal mesenteric system. In cooperation with the Department of Germfree Research, preliminary experiments were begun to ascertain the infectibility of germ free mice with sterile S. mansoni cercariae and the location of developing worms in the portal circulation.

The results of the first experiment (Table V) were inconclusive both because very few adult worms developed in the mice and because of contamination which occurred in the germ free animals exposed to infection.

Table V

Adult Schistosoma mansoni recovered from germ free mice exposed to sterile cercariae

Group	No. Mice	No. Cercariae Per Mouse	Days After Exposure	Worms Recovered Male	Female	Total
Germ Free*	10	200	40	3	0	3.0
Conventional	10	200	40	10	0	10.3

*Contaminated with S. albus, E. coli and A. aerogenes at infection with cercariae.

It was evident that the contamination of the germ free mice was due to the fact that some cercariae were still associated with bacteria (Staphylococcus albus, Escherichia coli and Aerobacter aerogenes) at the time of penetration into the skin. This occurred in spite of the fact that cercariae were obtained by sterile technic from snails which had been swabbed with 70% alcohol, placed in a penicillin bath for 24 hours, and coated with sterile paraffin through which a hole was made to permit emergence of cercariae without contamination with the snails feces. Apparently the sterilization procedure was incomplete and rendered the cercariae noninfective. These technics are being revised for reevaluation.

4. The natural history of infection with S. mansoni in different species of monkeys

In the hope of observing differences that might give some clues to the mechanisms of resistance to schistosomiasis in primates, 30 monkeys of five species, Ateles sapaios (spider), Macaca seriosis (stump tailed), Cebus apella (capuchin), Saimiri sciureus (squirrel) and Macaca mulatta (rhesus) were exposed to a single dose of S. mansoni cercariae. Ten monkeys (2 of each species) were exposed to 50 cercariae each; 10 to 250 cercariae each, and 10 to 1000 cercariae each. All animals were bled at regular intervals and the serum was quantitatively tested for schistosome antibodies. Fecal examinations were conducted twice a week and the presence and viability of eggs were determined. The degree of susceptibility of the various monkeys was determined by their survival, the percentage of parasites developing, the growth and structural development of the worms, the ability of the worms to produce eggs, the viability of eggs recovered, the infectivity of miracidia for Australorbis glabratus, the sex ratio and location of the worms in the host, the length of the prepatent period, the serological response of the host and the pathological manifestations of the infection. The results obtained to date are summarized in Table VI. Preliminary findings suggest that of those tested, the rhesus monkey might be the most susceptible and the squirrel monkey the most resistant. Infection occurred in all of the primates tested. The studies are still in progress.

5. Parasitologic, pathologic and serologic reactions to Schistosoma mansoni in monkeys exposed to irradiated cercariae

Investigations on the development of immunity following exposure to irradiated cercariae were continued and extended. The present study was undertaken to determine: 1) the degree of protection induced in rhesus monkeys by previous exposures to cercariae irradiated at different levels,

Table VI

The number and location of adult Schistosoma mansoni worms in various species of primates

Species	Monkey No.	No. of Cercariae	Days from Exposure to Necropsy	Results of Necropsy No. of Live Worms Liver M	Liver F	Mesentery M	Mesentery F	Total
Cebus apella (Capuchin)	1	1000	57	11	8	104	111	234
	2	1000	50	32	78	148	154	412
	3	250	231	13	16	25	17	71
	4	250	231	14	12	53	29	108
	5	50	231	5	6	2	3	16
	6	50	140	7	2	4	5	18
Saimiri sciureus (Squirrel)	1	1000	36	10	9	63	51	133
	2	1000	163	7	7	15	14	53
	3	250	163	0	0	0	0	0
	4	250	163	0	0	0	0	0
	5	50	163	0	0	0	0	0
	6	50	163	1	0	1	1	3
Macaca seriosis (Stump tail)	1	1000	60	31	29	212	181	393
	2	250	alive	Not yet determined				
	3	250	"	"	"	"		
	4	50	"	"	"	"		
	5	50	"	"	"	"		
Ateles sapajos (Spider)	1	1000	"	"	"	"		
	2	1000	"	"	"	"		
	3	250	"	"	"	"		
	4	250	"	"	"	"		
	5	50	"	"	"	"		
	6	50	"	"	"	"		
Macaca mulatta (rhesus)	1	1000	56	143	161	157	169	630
	2	1000	alive	Not yet determined				
	3	300	100	5	14	44	42	105
	4	300	100	12	8	25	27	72
	5	50	140	0	0	2	2	4
	6	50	alive	Not yet determined				

and 2) the antibody curve in monkeys over a 5-month period following one or more exposures with irradiated cercariae. These investigations were also directed toward a better understanding of some of the mechanisms involved in acquired immunity to schistosomes by determining whether or not a correlation exists between the results of serological tests and the degree of resistance to superinfection induced by immunizing exposures to irradiated cercariae.

Three experiments were undertaken to study the effect, if any, of immunizing exposures to irradiated cercariae on the survival, egg recovery, worm burden, gross pathological changes and antibody development in monkeys following a challenge with nonirradiated cercariae.

Parasitological observations: In the first experiment 10 monkeys were separated into two groups (Table VII). The animals of the first group received, at weekly intervals, 5 doses of 5,000 cercariae irradiated at 4,000 roentgen equivalent physical (rep). Those of group II were not exposed to infection at this time and were used as nonimmunized controls. Thirty days after the last exposure to irradiated cercariae, all animals were challenged with 1000 normal cercariae each. This infection level was chosen since preliminary experiments had indicated that it permits most monkeys of the weight used to survive for at least two months. One monkey from the nonimmunized group died 44 days after challenge. All of the other animals survived until the end of the experiment, 60 days after challenge. The mean number of eggs per gram of feces (MNEPGF) was much smaller in the immunized group than in the controls. Also, at necropsy significantly fewer worms were recovered from the animals which had received the immunizing doses (Group I). These differences are highly significant. The liver, spleen and intestines of animals which had been previously immunized appeared essentially normal except for some occasional discoloration in the liver and spleen. Conversely, the controls had organs with marked discoloration, hemorrhages and frequently ascites.

In view of the preceding results, another experiment was designed to learn whether different levels of irradiation would induce different degrees of acquired resistance and whether this acquired resistance conferred a true protection against lethal infective doses. Preliminary experiments had indicated that an exposure of monkeys of this size to 4,000 cercariae brought about the death of all of them within two months from the date of exposure. As indicated in Table VII, in the second experiment 34 monkeys were divided into 5 groups. Those of groups III, IV and V were exposed to 5 weekly doses of 5,000 cercariae

Table VII

Worm burden, percent mortality, and egg passage in monkeys challenged 30 days after weekly exposures to irradiated cercariae

Group No.	No. of monkeys	Immunizing exposures: No. of cercariae	Immunizing exposures: Irradiation (rep)	Challenge: No. of cercariae	Challenge: Deaths	Average time of survival (days)	Time btw. challenge & necropsy (days)	Worms recovered: Mean No.	Worms recovered: Percent	MNEPGF*
I	5	5x5,000	4,000	1,000	0/5	-	60	179	17	18
II	5	-	-	1,000	1/5	44	60	352	35	64
III	5	5x5,000	2,500	4,000	0/5	-	126	184	5	29
IV	5	5x5,000	4,000	4,000	2/5	75	90	836	21	112
V	5	5x5,000	10,000	4,000	2/5	61	90	686	17	191
VI	15	-	-	4,000	15/15	52	-	1076	27	207
VII	4	-	-	450	0/4	-	124	101	22	45
VIII	5	1x25,000	4,000	4,000	2/5	61	83	1321	33	145
IX	4	-	-	4,000	4/4	50	-	1101	28	159
X	4	-	-	350	0/4	-	63	87	25	33

*Mean number of eggs per gram of feces.

irradiated with 2,500, 4,000 and 10,000 rep, respectively. Those of group VI served as nonimmunized controls. The monkeys of groups III, IV, V and VI were challenged with 4,000 nonirradiated cercariae 30 days after the last exposure to irradiated cercariae. The monkeys of group VII received 450 cercariae each. The animals of this group were exposed to a much smaller dose of nonirradiated cercariae to assure their survival up to the end of the experiment, thus making it possible at necropsy to compare the percentage recovery of worms in this group with that in the immunized groups. All animals which had not been previously immunized and which were challenged with 4,000 cercariae (Group VI) died within two months following challenge (Table VII). Conversely, all of the monkeys which had been immunized with cercariae irradiated at 2,500 rep (Group III) and 3 out of 5 of those immunized with cercariae irradiated with 4,000 (Group IV) or 10,000 rep (Group V) survived. The degree of protection induced by 5 exposures to cercariae irradiated at 2,500 rep appeared to be greater than that induced by a similar number of exposures to cercariae irradiated at 4,000 rep, and these in turn induced a greater protection than cercariae irradiated at 10,000 rep (Table VII). The number of worms recovered in the nonimmunized animals was between 22 and 27 per cent of the challenge, regardless of whether the monkeys died as a result of a massive infection (Group VI) or whether they survived (Group VII). The per cent recovery of worms in the immunized groups increased with increasing irradiation and varied from 5 per cent (Group III) to 21 per cent (Group V). The reduction of worms and eggs in the immunized monkeys (Group III) was observed up to 126 days after challenge (Table VII), thus indicating that the acquired resistance developed following exposure to irradiated cercariae is not a transitory phenomenon. As in the previous experiment, the liver, spleen and intestines of monkeys which had been immunized with cercariae irradiated at 2,500 rep appeared essentially normal (except for some occasional discoloration in the liver and spleen) in spite of the challenging infection with 4,000 nonirradiated cercariae. Conversely, the controls died with marked pathological changes in all of these organs. The gross pathological appearance of the organs of monkeys in groups immunized with cercariae irradiated at 4,000 and 10,000 rep was somewhat intermediate between these two extremes.

An additional experiment involving 13 monkeys was designed to determine whether the degree of acquired resistance observed with 5 weekly immunizing exposures would also occur when the irradiated cercariae were given in a single exposure. Five monkeys (Group VIII) received a single exposure to 25,000 cercariae irradiated at 4,000 rep. The 8 monkeys of the other two groups (IX and X) were not immunized.

Thirty days after the last immunizing exposure, all the animals of groups VIII and IX received 4,000 nonirradiated cercariae each, and those of group X received 350 nonirradiated cercarie. Prior exposure to a single dose of 25,000 cercariae irradiated with 4,000 rep failed to produce a significant degree of acquired resistance (Table VII).

Histopathological observations: Histopathological studies were conducted on tissues obtained from 4 monkeys which developed a strong immunity (Group III), 4 monkeys which developed a relatively low degree of immunity (Group IV) and one nonimmunized monkey (Group VI). In general, there was a good correlation between the parasitological and histopathological observations. The numbers of eggs and granulomata found in the liver and in the mucosa and submucosa of the colon correlated very well with the MNEPGF and with the number of worms recovered at necropsy. Likewise, there was a close correlation between the degree of protection and the size and activity of the centers of the lymph follicles in the spleen. These follicles were largest in the group immunized with cercariae at 2,500 rep and smallest in the control monkeys. In monkeys which received cercariae irradiated at 4,000 rep, the follicles were somewhat in between these two extremes. Also, there was a greater leukocytic reaction and less fibrosis and giant cell reaction around eggs in the livers of the group immunized with cercariae irradiated at 2,500 rep than in the other groups. In both immunized groups, certain specific lesions of interest were occasionally noted. In two animals there was an area of eosinophilic precipitate (Hoeppli phenomenon) around eggs in granulomata in the submucosa of the colon. Distinct arteritis and phlebitis was noted in some of the larger blood vessels of the colon. Occasionally, severe phlebitis was noted in association with dead worms lodged in large branches of the portal vein in the liver. In one of the animals immunized with cercariae irradiated with 2,500 rep there was a broad area of interlobular fibrosis, which may represent a later stage of reaction to an embolized worm.

Serological responses: Sera from monkeys exposed to irradiated and nonirradiated cercariae were tested weekly by the quantitative fluorescent antibody test (FAT). The serologic response of the immunized monkeys was followed for 21 weeks. Those of the nonimmunized animals were followed for 7 weeks at which time most of the animals had died from the infection. All the specimens were negative at the time of exposure of the monkeys to cercariae. The monkeys exposed to 5 doses of 5,000 cercariae irradiated at 2,500 rep became positive two weeks after the first exposure. The titer rose rapidly and the peak GMT was reached 10 weeks after the first exposure (1 week after challenge). Titers fell

more or less rapidly soon after the peak was reached and began rising again shortly before the appearance of eggs in the stools. The serologic response of monkeys exposed to cercariae irradiated at 10,000 rep was essentially the same as that of the previous group. The titer fell after the peak at 10 weeks and began rising again 15 weeks after the first exposure (6 weeks after challenge), shortly before eggs began appearing in the stools. The serological response of monkeys exposed to a single dose of 25,000 cercariae irradiated at 4,000 rep was similar to that of the previous groups. The GMT rose rapidly and reached the peak 11 weeks after exposure (2 weeks after challenge). This response was stronger and more rapid than that in the other groups, but the decrease in GMT was also more rapid. The secondary rise occurred 16 weeks following exposure (7 weeks after challenge) when eggs had just begun to appear in the stools. A direct relationship between the decrease in antibody response following challenge and the degree of protection against the challenging infection was observed. A similar curve of fluorescent antibody titer rise was observed during the first 7 weeks in animals exposed to nonirradiated cercariae.

The results of our experiments indicated that exposure to irradiated cercariae induced a marked resistance to a subsequent challenge with nonattenuated cercariae of S. mansoni. Detailed histopathological observations confirmed the parasitological data. Extensive quantitative serological studies showed that exposure to irradiated cercariae produced rapid antibody response. The geometric mean of titers in monkeys exposed to irradiated cercariae was similar to that in monkeys exposed to nonirradiated cercariae. No correlation was observed between the degree of irradiation and antibody levels, or between antibody titers and acquisition of immunity. All titers decreased rapidly following challenge. This decrease was inversely related to the amount of acquired resistance observed.

6. Pulmonary tissue reactions against schistosomula. I. Comparative study of the susceptible, insusceptible and immunized host

In cooperation with the Department of Pathology, Peter Bent Brigham Hospital, studies were undertaken on the pulmonary reactions of mice, muskrats and monkeys to nonirradiated and gamma-irradiated cercariae of S. mansoni with the specific aim of comparing conditions representative of host susceptibility, natural insusceptibility and acquired resistance. Special attention was given to the pathology directly or indirectly related to immunization procedures.

Mice: The number of worms recovered from mice immunized with irradiated cercariae was slightly, but significantly, lower than in the controls (747 vs 960). This represents an overall reduction of 22% in the worm burden. The lesions of the intestine and liver 42 days after challenge were similar in the immunized mice and in the controls, both showing evidence of chronic active schistosome infection. In the lung no detectable residua were found of the primary exposure to irradiated cercariae given 94 and 126 days prior to sacrifice, respectively.

The number of schistosomula found in lung sections on the 10th day after challenge were similar in the "immunized" and control mice (2.3/slide and 2.5 slide, respectively). Neither the immunized nor the control group showed morphological evidence of degeneration. While the controls showed no inflammatory reaction to schistosomula, inflammatory cells were present in the vicinity of 9 of 28 schistosomula found in the "immunized" group. Four of these foci were sufficiently large to be described as "tuft-like" foci while the remainder consisted of a few swollen endothelial cells and granulocytes. Perivascular round cell infiltrates and alveolar desquamative foci were somewhat more frequent in the "immunized" group. Forty-two days after challenge, 3 of the "immunized" mice showed focal arteriolar thickening in the lung which was absent in the controls.

Muskrats: Two schistosomula were located in 220 slides. One of these showed mild morphological damage characterized by nuclear pyknosis and swelling of the cuticle. It was surrounded by a large sharply outlined granuloma composed of epithelioid round cells and eosinophils. The other schistosomulum was partly disintegrated and surrounded by a smaller granuloma. In addition, step-sectioning of lung tissue disclosed occasional small granulomas with giant cells, but no parasites. One muskrat also showed rare foci of alveolar desquamation, and occasional ectopic bone particles in the interstitium. No "tuft-like" foci were observed. Lesions were generally scarce and widely separated from each other. Tissues other than the lung were non-contributory.

Monkeys: All monkeys showed Pneumonyssus bronchitis in mild to negligible degrees. Many showed congestion with or without slight edema which was attributed to terminal anoxia before sacrifice. Monkey A showed one intact schistosomulum in 80 slides without cell reaction. The lung tissue away from bronchi did not show inflammation. Monkey B revealed two schistosomula in 80 sections without evidence of morphological damage or significant tissue reaction in their vicinity. Grossly, the lung of this monkey showed multiple poorly defined areas of greyish discoloration, most notably in the lingula and left lower lobe, which histologically corresponded to focal interstitial pneumonitis. This was characterized by thickening of alveolar septa, infiltration with neutrophils and desquamation

of alveolar macrophages. Focal perivascular lymphoid cell aggregates were also present. The inflamed areas failed to show schistosomula and the exudate was poor in eosinophils. All animals in Group C (hyper-immunized monkeys) showed variable degrees of pulmonary inflammation in excess of the lesions attributable to Pneumonyssus. Sporadic schistosome eggs and granulomas were found in two monkeys of the series. One of three "tuft-like" inflammatory foci per 10 slides were present in all monkeys. Three of the foci contained intact schistosomula. The foci were larger than those described in the immunized mice, but smaller than those previously observed in monkeys which had become highly resistant following long term infection. Foci varied in stage of development from endothelial swelling and mantling of a group of vessels to focal consolidation with fibrin plugging of alveoli. In addition, perivascular lymphoid cell infiltration, focal alevolar desquamation and focal endothelial swelling in arterioles and capillaries were noted, the latter frequently near "tuft-like" foci. Areas of pneumonitis comparable to those in monkey B were absent. The lesions in monkey tissues other than the lung have been described in previous reports and showed comparatively mild lesions indicative of host resistance.

In order to approximate natural conditions in this series, smaller numbers of cercariae were used for challenging monkeys than in previous work; this resulted in a relative scarcity of schistosomula in the histological slides. Cercariae were usually irradiated with 2500 rep for maximal immunizing effect, but 5000 rep were employed when maximal retention of schistosomula in the lung was desired. The interval between immunizing exposure and challenge was somewhat prolonged in mice and somewhat short in monkeys compared with the immunization schedules found most effective against S. mansoni. Since the interval between challenge and sacrifice was standard for each series, the evolution and ultimate fate of lung foci could not be studied, but had to be inferred by correlating present with previous data. Susceptible hosts, such as mice or monkeys receiving primary infections, had no cell response to pulmonary schistosomula initially, i.e., after 7 or 10 days. However, by the 14th to 28th day, stray degenerating schistosomula and granulomas were found in the lung of nonimmunized mice, presumably as the result of spontaneous parasite mortality. The pattern was similar when cercariae were irradiated with 2500 rep but with some increase in the number of pulmonary foci. Monkeys A and B of this series also conformed to this pattern, although the sample was too small to be sure whether some lung granulomas might not have been present in Monkey B, which received cercariae irradiated with 4000 rep. In the muskrat, the presence of degenerating schistosomula and granulomas on the 7th day was confirmed. Presumably, in this host schistosomula succumbed sooner and in larger numbers, since they failed to reach the portal habitat. In all the above hosts, the morphology of the granulomas and their frequent association

with structural damage to the parasite suggests that they form after immobilization, possibly as a host response to the release of somatic antigens. The variations in number and distribution of such granulomas might be a function of different mortality rates of schistosomula after penetration and could provide a comparative index of host insusceptibility, or be used to assess the damage inflicted on cercariae by various procedures, including gamma radiation. The identity of the reaction patterns subject only to quantitative variations in primary infection of susceptible hosts, insusceptible hosts and hosts receiving modified cercariae is a strong argument favoring the view that natural resistance is based on "athreptic immunity."

In sharp contrast, the early cell response designated as "tuft-like" foci was limited to reinfected hosts with some degree of acquired resistance, e.g., the immunized mouse and macaque. Previously, this response had been found in Macaca mulatta made resistant to S. mansoni following long-term infection; however, "tuft-like" foci were absent in Cercopithecus sabeus when this host was challenged after multiple reinfections with S. mansoni. The green-face monkey fails to develop resistance and oviposition is observed over long periods. Furthermore, the size and intensity of "tuft-like" foci has an inverse relationship to worm recovery percentages in various types of host, i.e., the foci were smallest in the immunized mice, larger in the immunized monkeys of this series, and largest in the long-term infected, resistant macaques. A semiquantitative relationship of this kind had been demonstrated previously in monkeys but it was not evident in the mice studied here, possibly due to low-grade immunity and smallness of the sample. The "tuft-like" cell response does not seem to require structural damage or total immobilization of schistosomula, since it has been observed by the 6th day, in the absence of altered parasite morphology. At this early stage, schistosomula were frequently peripheral to the foci. Later, as the foci enlarged and gained definition, parasite damage and epithelioid macrophages were seen in the foci. Additional observations during the 2nd and 3rd week should reveal whether absorption of somatic antigen is associated with granuloma formation in the immune as well as in the nonimmune host. Since the transition from exudative to granulomatous foci is probably fluid, histological discrimination between acquired resistance and other types of host reactivity is restricted to the critical time on or before the 7th day, and requires considerable experience. Since much could be gained by verifying in vivo whether "tuft-like" foci appear prior to total immobilization, we have been observing active mobility of schistosomula in suitably prepared compression slides of infected nonimmune mouse lungs.

On the basis of these results, it was concluded that while the lung lesions caused by gamma-irradiated cercariae in mice appear to be totally reversible, their safety in primates is not established. Monkeys showed

acute interstitial pneumonitis and oviposition into the gut and lung was observed with gamma dosage levels previously considered as innocuous. The lung response to challenge in monkeys immunized with irradiated cercariae resembles that in monkeys made resistant by long-term infection, but is somewhat less intense. This is assumed to be reversible and asymptomatic, except after massive challenges.

7. Antigen-antibody complexes as immunizing agents

In cooperation with the Department of Medical Zoology, University of Puerto Rico, experiments were conducted in the attempt to immunize mice and rats using S. mansoni eggs and egg antibody complexes as immunizing agents.

In a first experiment S. mansoni eggs were obtained from infected mice and divided into two lots. One lot was incubated in pooled normal mouse serum from 18-24 hours at 37°C. After incubation they were washed in 0.85% sodium chloride several times to remove the serum. The other lot was incubated similarly in pooled serum from mice infected with S, mansoni for 60 to 70 days. Forty to 50% of these eggs were surrounded by precipitate. Following incubation this lot was washed in the same manner as the other. After suspending them in 0.1 ml of Freund's adjuvant, eggs were injected into the hind quarter of 20-25 gram mice. Two weeks after the single inoculation of eggs these mice along with uninoculated control animals were exposed to 60 S. mansoni cercariae. Some animals from each group were sacrificed at 10 day intervals from the 50th to the 80th day after exposure to cercariae. The number of adult worms was tabulated and tissues were fixed for histopathological studies.

The number of adult worms recovered from mice of the three groups at the different intervals after exposure did not vary significantly (Table VIII).

Preliminary histopathological studies of sections of liver from all animals permitted the following observations:

a. There was a reduced amount of pigment in the livers of animals inoculated with eggs with circumoval precipitate, as compared with livers of the other two groups. Furthermore, pigment was concentrated in areas near the eggs or phagocytized by large multinuclear giant cells. Pigment was not distributed diffusely throughout the Kuppfer cells as in the other two groups of animals.

b. Eggs were absorbed and disappeared more rapidly from tissues of mice inoculated with the egg-precipitate complex than from the other two groups. Frequently the eggs were invaded by giant cells and pigment.

The relationship of these findings to serum antibodies is being studied.

In a second experiment 200 milligrams of lyophilized eggs were extracted in 40 ml of ether in a Tenbroeck grinder at -20°C for 15 minutes. After centrifugation for 30 minutes at 10,000 G the supernate was decanted and the residue evaporated to dryness. It was pulverized with a stirring rod and phosphate buffer was added in the ratio of 1 ml to 10 mg of the original weight of lyophilized eggs. This suspension was hand ground at 3°C until the egg powder was dissolved in the saline. The solution was then agitated with a magnetic stirrer at 3°C overnight. Then it was centrifuged at 10,000 G for 30 minutes and the supernate was lyophilized in 1 ml aliquots. This egg antigen was applied to cholesterol-lecithin crystals.

Suckling rats were sorted into 4 groups of 12 animals each. Rats within a group were injected once daily for 12 days with one of the following substances: egg antigen-antibody complex on cholesterol-lecithin crystals, egg antigen on cholesterol-lecithin crystals and egg antigen alone. The fourth group was not injected and served as a control. One week after the last inoculation all animals were exposed to cercariae of S. mansoni. Twenty-four days later schistosomules were perfused from the rats and counted.

The number of schistosomules which were recovered are shown in Table IX. The control group averaged 18.7 worms per rat while both the egg antigen alone and the egg antigen-antibody complex on crystal groups averaged 18.5 schistosomules per rat. The group which received egg antigen on crystals had 15.5 worms per animal, but this difference is not significant. Another experiment in which the amount of antigen in the inoculum will be increased and the phenol preservative in the antigens will be omitted is being planned.

Adult S. mansoni excretion and secretion as antigen: A method of preparation of adult worm excretion and secretion antigen has been developed as follows: adult S. mansoni worms are perfused from infected mice and placed in distilled water (1 ml per 100 worms) and left at 3°C for 18 to 24 hours. The water is drawn off and centrifuged at 1000 G and

Table VIII

Recovery of Schistosoma mansoni from mice inoculated with egg antigen

Age of Infection days	Egg Antigen in Normal Serum		Egg Antigen in Immune Serum		Uninoculated	
	No. of Mice	Av. No. of Worms	No. of Mice	Av. No. of Worms	No. of Mice	Av. No. of Worms
50	10	28	10	19	10	30
60	10	24	10	21	10	26
70	8	20	8	17	9	17
80	8	16	8	14	6	16

Table IX

Recovery of schistosomules from suckling rats immunized by twelve successive daily injections of antigen

Group Number	Number of Rats	Antigen	Mg. Nitrogen per ml*	Average No. Worms per Rat
I	11	Egg antigen-antibody complex on crystals	.069	18.5
II	11	Egg on crystals	.030	15.5
III	10	Egg only	.350	18.5
IV	12	None	-	18.7

*All animals given dose of 2.4 ml.

the supernate lyophilized. This lyophilized antigen is then extracted with ether and reconstituted with phosphate buffered saline in the same manner as the lyophilized eggs (see above).

The success obtained in adsorbing egg and adult excretion-secretion antigens onto cholesterol-lecithin crystals has opened the way to: 1) the production of other serological tests employing egg or adult excretory water soluble substances as antigen, 2) the use of antigens other than cercariae for quantitative absorption, and 3) the possibility of identifying species specific antigens by quantitative cross absorption studies and by quantitative serologic tests.

Investigations along these three lines are in progress.

8. Serological reactions and antigenic analysis of extracts from different stages of Schistosoma mansoni

A slide flocculation test using a washed cercarial antigen-cholesterol-lecithin complex provided a reliable diagnostic procedure for schistosomiasis and a means of adsorbing all of the serologically reactive antibody with a single calculated volume of packed and essentially dry antigen onto cholesterol-lecithin crystals. The purposes of the current study were: 1) to determine whether slide flocculation tests could similarly be produced utilizing egg and adult products, 2) to compare reactions with the three antigenic complexes in artificially immunized and in experimentally infected animals, and 3) to detect antigen-antibody systems by gel diffusion studies and to determine the specificity of these reactions by cross-absorption.

Flocculation tests with all three antigens gave consistent results with sera from artificially immunized and experimentally infected animals. An antigen obtained from excretions and secretions of adults seemed to confer to the test the greatest sensitivity. This was evidenced by an earlier appearance of detectable antibodies, by consistently higher titers and by the persistence of positive reactions. Homologous and heterologous absorption studies revealed that specific reacting fractions may be present in the three antigens which stimulate distinct humoral factors. This conclusion was supported by agar gel diffusion studies which showed different antigen-antibody systems, some of which were common to two or three stages of the life cycle of the parasite, whereas others appeared to be stage specific.

9. Passive resistance induced in rats by inoculation of serum from monkeys immunized against S. mansoni

In earlier experiments resistance was induced in rats by previous exposure to infection and by vaccination with fresh homogenates of S. mansoni. Furthermore, a passive transfer of resistance was possible by injecting mice with serum from rabbits which had been experimentally infected or artificially immunized with S. japonicum. The current studies were performed to determine whether acquired resistance could be induced in rats by inoculation with serum from monkeys which had been immunized by prolonged and repeated infections ("immunized") or with serum from monkeys rendered resistant by multiple exposures to cercariae irradiated at 2,500 roentgens ("vaccinated").

In the first experiment, a total of 62 rats were separated into 4 groups. As shown in Table X each animal of group I was given 3 saline injections every other day beginning with the day prior to exposure for a total amount of 0.15 ml. Those of group II received the same amount of normal serum, those of group III received the same amount of immune serum and those of group IV received the same amount of serum from vaccinated monkeys. The results show that at necropsy the rats in the group receiving antisera had an appreciably lower number of worms than those receiving saline or normal serum.

In a second experiment (Table XI) 47 rats were divided into three groups. Those of the first group received 3 injections of saline, those of the second group received the same amount of normal serum and those of the third group received serum from vaccinated animals. The results indicate a marked reduction in the number of worms in the groups receiving vaccinated serum and normal serum (Groups II & III).

In a third experiment a total of 47 rats were divided into three groups as in the previous experiment. The results (Table XII) show a significant reduction in the number of worms recovered from the rats receiving serum from vaccinated animals.

In the fourth experiment 48 rats were divided into three groups as before (Table XIII). Once again the results indicate a significant reduction in the number of worms in the animals receiving serum from vaccinated monkeys.

Table X

Number of S. mansoni recovered from rats receiving 3 saline or serum injections every other day beginning one day prior to exposure

(Experiment I)

Exper. Group	Injection	Results of examination at necropsy 25-27 days after exposure to 500 cercariae		
		No. of Rats	Mean No. of Worms	Per Cent Recovery
I	Saline	15	50.0	10.0
II	Normal serum	15	57.0	11.4
III	Immune serum	15	21.3	4.3
IV	Vaccinated serum	17	20.0	4.0

Table XI

Number of S. mansoni recovered from rats receiving 3 saline or serum injections every other day beginning one day prior to exposure

(Experiment II)

Exper. Group	Injection	Results of examination at necropsy 25-27 days after exposure to 500 cercariae		
		No. of Rats	Mean No. of Worms	Per Cent Recovery
I	Saline	17	11.0	2.2
II	Normal serum	15	6.4	1.3
III	Vaccinated serum	15	6.0	1.2

Table XII

Number of S. mansoni recovered from rats receiving 3 saline or serum injections every other day beginning one day prior to exposure

(Experiment III)

Exper. Group	Injection	Results of examination at necropsy 25-27 days after exposure to 500 cercariae		
		No. of Rats	Mean No. of Worms	Per Cent Recovery
I	Saline	15	55	11.0
II	Normal serum	16	57.2	11.4
III	Vaccinated serum	16	19.0	4.0

Table XIII

Number of S. mansoni recovered from rats receiving 3 saline or serum injections every other day beginning one day prior to exposure

(Experiment IV)

Exper. Group	Injection	Results of examination at necropsy 25-27 days after exposure to 500 cercariae		
		No. of Rats	Mean No. of Worms	Per Cent Recovery
I	Saline	15	8.5	1.7
II	Normal serum	16	6.4	1.2
III	Vaccinated serum	17	1.1	0.2

The results presented here indicate that rats develop an acquired immunity to Schistosoma mansoni and that this immunity is partly, at least, serological in nature.

10. Comparative studies of some immunologic screening tests for schistosomiasis in Ethiopia

In cooperation with the Department of Epidemiology, Johns Hopkins University and the Department of Serology, WRAIR, a study was designed to determine the diagnostic strength of the fluorescent antibody test for schistosomiasis when used as a screening test in Ethiopia and to make a comparative study of various immunologic methods recommended for use as screening tests in epidemiologic studies of schistosomiasis.

The age-specific prevalence rates of the reactions observed in the various immunodiagnostic tests and the percentages of individuals from whom eggs of S. mansoni were recovered in stool examinations are shown in Table XIV. With the exception of the FA test the peaks of all the other tests are reached at the age of 10-19 years; but there are considerable differences in the percentages of positive reactions among the individual tests.

Table XV shows a comparison of agreement between immunodiagnostic tests and stool examinations. For each of the immunodiagnostic tests the present agreement was computed independently for individuals with and without eggs of S. mansoni. Furthermore, indices of agreement were computed separately for two age groups. Three general trends with increasing age can be recognized from this table: co-positivity increases; co-negativity decreases and the overall agreement also decreases. Among individuals excreting eggs of S. mansoni the probability of being correctly identified by these immunodiagnostic tests is smaller for persons under the age of 10 than for those who are older. In contrast, the probability that an individual without eggs of the trematode will have a negative test result is larger for children under 10 than for older individuals. The effect of these two differences is to exaggerate the increase with age when using the results of the immunodiagnostic tests instead of direct parasitological examinations.

The first aim of the study was to determine the diagnostic strength of the fluorescent antibody test when used as a screening procedure in Ethiopia. Table XVI shows a comparison of the percentages of positive reactions in the serologic and skin tests between persons with and without

Table XIV

Per cent of individuals with eggs of S. mansoni and with positive reactions in immunodiagnostic tests, by age

Age	No.	Per cent with eggs detected	Skin test % pos	CF test % pos	SF test % pos	SPC card test % pos	FA test % pos	Cse test % pos
0-9	26	32.0	38.4	52.1	29.2	40.0	70.8	59.1
10-19	50	50.0	74.0	91.8	63.3	69.3	81.6	84.1
20-29	12	0	50.0	50.0	25.0	33.3	91.6	45.4
30 +	12	25.0	41.7	41.7	25.0	41.6	75.0	66.7
Total	100	37.5	58.0	70.8	45.3	54.0	79.3	70.9
# with test			(96)	(100)	(96)	(97)	(98)	(97)	(86)

Table XV

Per cent agreement between the results of specified immunodiagnostic tests and of stool examinations for S. mansoni, by age

Type of test	Co-positivity		Co-negativity		Overall agreement	
	0-9 yr	10-19 yr	0-9 yr	10-19 yr	0-9 yr	10-19 yr
Skin test	75.0	80.0	82.3	33.3	80.0	57.2
CF test	100.0	100.0	60.0	16.7	73.9	58.3
SF test	62.5	79.1	87.5	50.0	79.1	64.5
SPC card test	71.0	75.0	75.0	42.0	70.8	58.3
Cse test	71.4	90.9	46.7	23.8	54.5	59.5
FA test	65.5	79.1	25.0	16.7	37.5	38.3
# in group	(8)	(25)	(81)	(25)	(26)	(50)

Table XVI

Percentage of positive skin and serologic tests among individuals with and without eggs of S. mansoni

Type of test	No. tested	S. mansoni eggs present		S. mansoni eggs absent		Difference A-B	Probability of getting as large a difference by chance
		# pos	% pos	# pos	% pos		
Skin test	96	36	77.8	60	48.3	29.5	0.01
CF test	93	35	94.3	58	62.1	32.2	0.01
SF test	94	35	71.4	59	33.9	37.5	0.01
Card test	94	35	68.6	59	45.7	22.9	0.09
Cse test	84	32	84.3	52	63.5	20.8	0.14
FA test	94	35	74.3	59	83.1	8.8	0.6

eggs of S. mansoni. For all but the FA test there was relatively good association between the presence of eggs and positive immunologic tests. The significance of the differences in the percentages of positive reactions was tested in a X^2 test. The results of this analysis are shown in the right hand column of Table XVI. They seem to indicate that whatever antibody is measured by the FA test has little relation to the presence or absence of Schistosoma eggs in the stool and, furthermore, shows poor agreement with the results of the other 5 immunologic methods indicating infection with S. mansoni.

In computing indices of agreement the presence or absence of eggs of S. mansoni in a single stool specimen was used as the reference classification. The application of concentration methods or the repetition of stool examinations will yield a higher percentage of positive findings than single direct examinations. Therefore, the results obtained with these more sensitive tests will lead to a reclassification of some of the individuals called negative by the direct stool examinations. Nevertheless, an unknown proportion of persons with true infections of S. mansoni will still remain unrecognized in the group classified as negative by the reference test. Schistosomiasis is a typical example of an infection where the value of a screening test is measured in relation to a reference diagnosis which is based on the presence of the etiologic agent. With respect to the "true" diagnosis the specificity of this reference classification is high, but its sensitivity is relatively low.

Two different levels of sensitivity of the reference test are available in the study. This permitted a comparison of the differences in the indices of agreement using as the reference classification first the results of the direct stool examinations and then those obtained independently with the MIF-ether concentration technic.

Table XVII shows for four immunodiagnostic tests the differences in co-positivity, co-negativity and the overall agreement, after 2 reference classifications were used for the same group of individuals. The percentages of individuals with eggs of S. mansoni show large differences, owing to the various degrees of sensitivity of the two methods in identifying cases of schistosomiasis from stool examinations.

These differences have an influence on the numbers in each of the four cells of a 2 x 2 table (Table XVIII), without changing the subtotals of positive and negative reactions of the immunodiagnostic tests. When the sensitivity of the reference tests is increased a proportional number of the

Table XVII

The effect of different levels of sensitivity of the reference test on co-positivity and co-negativity of selected immunodiagnostic screening tests for schistosomiasis

Type of Test	No. Tested	Per cent With Eggs Detected	Per cent In Test	Co-positivity Per cent	Co-negativity Per cent	Overall Agreement Per cent
CF test						
direct	93	7.5	74.1	100.0	27.9	33.3
M.I.F.		37.6	74.1	94.2	37.9	59.1
SF test						
direct	94	7.4	47.8	71.4	54.0	55.3
M.I.F.		37.2	47.8	71.4	66.1	68.0
Skin test						
direct	96	8.3	59.3	62.5	40.9	42.7
M.I.F.		37.5	59.3	77.7	51.6	68.0
FA test						
direct	94	7.4	79.7	71.4	19.5	23.4
M.I.F.		37.2	79.7	75.0	17.2	39.4

Table XVIII

Computation of indices of agreement between a reference and a screening test from 2 X 2 tables

		Reference test or diagnosis		
		+	-	Total
Screening test	+	a	b	a+b
	-	c	d	c+d
Total		a	a+c	b+d	a+b+c+d

co-positivity ("sensitivity") = $\frac{a}{a+c}$

co-negativity ("specificity") = $\frac{d}{b+d}$

Overall agreement = $\frac{a+d}{a+b+c+d}$

individuals in cell b of a 2 x 2 table will be transferred to cell a. Since the increase in the sensitivity of the reference test is associated with a reduction of the number of false negatives, an increase in co-negativity can be expected unless the screening test was independent of the reference diagnosis. A poor association between the results of the stool examinations and the fluorescent antibody test was shown earlier in the study. This is reflected also by the results presented in Table XVII. In this table three tests show the expected increase in co-negativity, whereas the FA test has a slight decrease of this index.

It is more difficult to understand why a change in the sensitivity of the reference classification could influence co-positivity. If the probability of detecting eggs of S. mansoni were merely a question of the sensitivity of the methods used for stool examinations, but were equal for all stages of schistosomiasis and independent of the age, sex and race of the individual, then the number of the individuals transferred from negative to positive and falling into cell "a" would be proportional to the prevalence of the reactions of the screening test in the whole group. If, on the other hand, certain characteristics of the individuals, such as age and sex or the stage of schistosomiasis, have different influences on the results of the stool examination than on those of the immunodiagnostic test, then higher sensitivity of the reference test can affect co-positivity.

After reclassification there were no or only insignificant differences in co-positivity for the CF and the slide flocculation test, but the skin test shows a relatively large increase of this index. The number of persons included in the study is too small to decide whether the observed difference is entirely the result of sample variation or real.

An increase in the overall agreement was found for all immunodiagnostic tests including the fluorescent antibody test.

In contrast to the other tests where the results of the immunological procedure and stool examinations were closely associated, the reactions of the fluorescent antibody test were independent of the presence or absence of S. mansoni eggs in the stool specimens of the individuals tested. This result indicated a lack of specificity of this test to identify infections with the human schistosomes in Ethiopia. To learn the reasons why the FA test for schistosomiasis showed low correlation with the reference test (finding of eggs of S. mansoni) further investigations are needed. It is quite possible that a large proportion of the rural population in Ethiopia has antibodies to

S. bovis resulting from inapparent infections with cercariae of this mammalian schistosome. The extensive degree of cross-reactions in FA tests using S. mansoni antigens among individuals exposed to cercariae of S. bovis was recently shown by Sadun and Biocca in Sardinia.

Although the indices measuring agreement were different for individual tests, two general trends can be recognized for all immunodiagnostic methods: co-positivity increases with age whereas co-negativity decreases.

An increase in the sensitivity of the reference test had an influence on the co-positivity, the overall agreement and to a minor degree also on co-negativity.

11. The prophylactic and suppressive activity of selected chemicals against Schistosoma mansoni in monkeys

In the following experiments the same group of monkeys served as untreated controls for all of the treated groups.

Sodium antimony dimercapto-succinate (TWSb) suspended in mineral oil. Studies with a repository form of TWSb against S. mansoni in monkeys were continued. Fourteen monkeys were sorted into 5 groups so that the prophylactic and suppressive activity of the compound could be tested. The groups were treated as follows: Group I, a total of four 25 mg doses of drug given every other day starting 2 days before infection; Group II, a single 100 mg dose on the day of infection; Group III, a total of four 25 mg doses given every 4 days starting 56 days after infection; Group IV, a total of three 100 mg doses given every other day starting 56 days after infection; and Group V, a single 100 mg dose given on the 56th day after infection. All monkeys were exposed to 400 cercariae from the same pool and were necropsied 90 days later.

All worms were eliminated in animals of Group IV, thus indicating that TWSb was highly effective when administered in three 100 mg doses to monkeys with mature worm infections (Table XIX). Smaller doses produced a lesser degree of prophylactic and suppressive activity.

Bis-(B-carbhydrazido-ethyl) sulfone (S-201). Another group of 5 monkeys (Group VI) was exposed to 400 cercariae of the same pool used above. These animals were given a daily 250 mg dose of S-201 beginning on the 56th day after infection. Ninety days after infection necropsy revealed a significant reduction in the number of worms in the treated animals as compared to the controls (Table XIX).

O, O-Dimethyl-O-(2, 4, 5-trichlorophenyl) phosphorothioate (Ronnel). Another four monkeys (Group VII) were exposed to 400 cercariae from the same pool as above. These animals were given a 100 mg dose of Ronnel on 3 successive days starting 56 days after infection. The number of worms had not been reduced in the treated animals as compared to the controls when the monkeys were examined 90 days after infection (Table XIX).

12. Studies on immunochemistry of filarial infections

New methods for performing precise biochemical analyses on a few microliters of blood made possible the study of possible changes which might derive from filarial infections.

Two experiments were set up to determine biochemical changes which might be detected in cottontail rabbits, following infection with the nematode Dirofilaria uniformis. Eight cottontail rabbits were selected for this study. All of these animals were free from naturally acquired filarial infections. One was born in the laboratory, the others were trapped as young adults. In a preliminary experiment, two animals, number 20 and 27, were examined respectively 93 and 82 weeks after infection. The results of biochemical tests did not appear to differ significantly from the values observed in normal rabbits of similar age and suggested that no marked changes occur in long-range chronic infections with this parasite.

To determine whether detectable biochemical changes occur at an earlier date during the course of infection, possibly at the time of appearance of microfilaremia, a second experiment involving 6 rabbits was undertaken. Five animals were inoculated with infective larvae varying in number from 144 to 355 per animal. The larvae were obtained from Anopheles quadrimaculatus mosquitoes. Ten days prior to dissection, the mosquitoes were allowed to feed on a cottontail rabbit which had microfilariae in the blood. Feeding was accomplished through the mesh covering of small plastic containers placed against the rabbit's shaved abdomen. The sixth rabbit was left uninfected and used as a control. All animals were bled before infection and at regular intervals afterwared for a period of 23 weeks. Parasitological examinations revealed that parasitemia developed in rabbits 58 and 62, three months, rabbit 59, four months and rabbit 64, five months after infection. One cottontail rabbit (#61) died during the third month after infection, before microfilaremia developed.

Table XIX

The effect of sodium antimony dimercaptosuccinate (TWSb) in oil; Bis-(B-carbhydrezido-ethyl) sulfone (S-201), and O-O-Dimethyl-O-(2,4,5-trichlorophenyl) phosphorothioate (Ronnel) on the number of *Schistosoma mansoni* recovered from monkeys exposed to 400 cercariae each.

Group	Monkey Number	Drug	Dose	Total Doses	Days Between Doses	Day Treatment Started	Number of Worms Recovered	Percent Recovered
I	1	TWSb	25mg	4	2	-2	50	12
	2	"	"	"	"	"	75	19
II	3	TWSb	100mg	1	0	0	99	25
	4	"	"	"	"	"	40	10
	5	"	"	"	"	"	141	35
	6	"	"	"	"	"	65	16
III	7	TWSb	25mg	4	4	+56	74	18
	8	"	"	"	"	"	100	25
	9	"	"	"	"	"	47	12
IV	10	TWSb	100mg	3	1	+56	0	0
	11	"	"	"	"	"	"	"
	12	"	"	"	"	"	"	"
V	13	TWSb	100mg	1	0	+56	8	2
	14	"	"	"	"	"	42	10
VI	1-S	S-201	250mg	6	0	+56	4	1
	2-S	"	"	"	"	"	30	8
	3-S	"	"	"	"	"	33	8
	4-S	"	"	"	"	"	14	3
	5-S	"	"	"	"	"	37	9
VII	1-R	Ronnel	100mg	3	0	+56	133	33
	2-R	"	"	"	"	"	118	29
	3-R	"	"	"	"	"	72	18
	4-R	"	"	"	"	"	131	32
VIII	15	Untreated	-	-	-	-	178	44
	16	"	-	-	-	-	97	24
	17	"	-	-	-	-	61	17
	18	"	-	-	-	-	131	33
	19	"	-	-	-	-	84	21
	20	"	-	-	-	-	84	21
	21	"	-	-	-	-	100	25

Table XX

Biochemical findings in a male (#20) and a female (#27) cottontail rabbit inoculated with 126 (#20) and 290 (#27) D. uniformis larvae

No.	No. Wks	NPN (mg%)	Crea. (mg%)	Gluc. (mg%)	Alk.Ph. (units)	Ac.Ph. (units)	TP (gm%)	SGOT (units)	Electrophoresis Results in Percent				
									Alb.	α_1	α_2	β	γ
20	93	42	2.1	86	0.8	4.6	5.9	30	57.5	3.5	11.5	19.2	8.4
27	82	42	1.4	109	2.7	2.9	5.9	14	62.9	2.6	5.7	19.2	9.4

Table XXI

Biochemical findings in a male cottontail rabbit (#58) inoculated with 225 D. uniformis larvae

No.	No. Wks	NPN (mg%)	Crea. (mg%)	Gluc. (mg%)	Alk. Ph. (units)	Ac. Ph. (units)	TP (gm%)	SGOT (units)	Electrophoresis Results in Percent				
									Alb	α_1	α_2	β	γ
58	0	35	1.9	ND	1.3	3.2	6.3	4	61.6	2.1	8.0	15.6	13.1
	4	42	1.9	162	1.1	3.3	6.9	15	62.9	4.7	6.5	16.7	9.3
	6	32	1.7	ND	1.6	3.3	6.5	12	62.0	0	5.7	21.6	11.1
	9	38	1.6	114	1.3	2.4	6.5	14	63.4	0	5.9	15.5	15.7
	10	38	ND	105	0.5	2.1	6.8	14	63.4	0	12.1	10.0	14.6
	12	35	1.6	126	1.4	3.9	6.6	8	57.3	2.9	8.5	23.2	8.2
	14	42	1.6	117	1.0	2.6	6.5	35	63.9	8.1	16.6	4.2	7.3
	18	45	1.5	116	0.5	3.6	6.8	14	79.3	0	0	16.7	4.7
	23	35	1.6	177	0.5	2.4	6.2	8	71.0	0	3.1	11.9	14.2

ND - Not done

Table XXII

Biochemical findings in a female cottontail rabbit (#59) inoculated with 206 D. uniformis larvae

Rx. No.	Nos Wks	NPN (mg%)	Crea. (mg%)	Gluc. (mg%)	Alk.Ph. (units)	Ac. Ph. (units)	TP (gm%)	SGOT (units)	Electrophoresis Results in Percent				
									Alb.	α_1	α_2	β	γ
59	0	38	1.9	ND	5.2	6.1	6.0	22	58.2	3.6	8.8	20.2	8.8
	2	40	2.2	144	4.7	5.5	6.2	41	ND	ND	ND	ND	ND
	4	38	2.2	131	5.3	4.8	6.5	35	82.7	0.3	7.0	10.5	1.3
	6	42	1.9	ND	4.5	3.7	6.3	20	62.9	2.3	6.2	20.0	8.6
	9	39	2.1	105	3.4	2.5	6.4	24	67.4	0	5.8	20.4	6.0
	10	40	ND	98	4.0	2.2	6.7	28	77.8	0	5.6	13.6	2.7
	12	40	2.6	97	2.9	3.4	6.6	25	63.9	3.2	6.3	19.4	6.9
	14	45	2.0	127	2.5	3.2	6.7	15	72.1	2.5	3.4	21.0	0.8
	18	45	2.0	112	1.8	2.9	6.6	32	85.5	0	2.2	16.2	4.1
	23	40	2.3	86	1.9	2.4	6.3	34	68.4	0	6.4	19.5	5.7

ND - Not done

Table XXIII

Biochemical findings in a female rabbit (#62) inoculated with 200 D. uniformis larvae

No.	No. Wks	NPN (mg%)	Crea. (mg%)	Gluc. (mg%)	Alk.Ph. (units)	Ac.Ph. (units)	TP (gm%)	SGOT (units)	Electrophoresis Results in Percent				
									Alb.	α_1	α_2	β	γ
62	0	34	1.7	ND	2.2	3.3	6.4	23	72.9	2.0	3.3	7.5	14.5
	2	42	1.9	114	1.6	3.6	5.8	25	ND	ND	ND	ND	ND
	4	42	1.6	125	2.3	3.4	6.9	12	68.1	1.9	3.9	15.6	10.4
	6	32	1.3	ND	2.0	2.9	5.7	10	61.4	2.5	4.9	18.3	13.3
	9	33	1.6	114	1.1	2.0	6.1	8	57.3	0	12.4	14.5	15.8
	10	34	ND	103	1.3	1.7	6.5	11	57.7	0	13.5	14.5	14.2
	12	36	1.4	98	1.4	2.1	6.0	8	54.9	6.3	6.6	21.4	10.6
	14	36	1.1	121	1.1	1.7	5.9	9	63.0	5.0	5.0	18.5	8.9
	18	42	1.5	127	0.5	2.0	6.1	7	72.2	3.8	3.4	14.6	6.4
	23	40	2.3	ND	1.6	2.3	7.0	17	54.3	4.1	10.5	16.8	14.4

ND - Not done

Table XXIV

Biochemical findings in a male rabbit (#64) inoculated with 144 D. uniformis larvae

No.	No. Wks	NPN (mg%)	Crea. (mg%)	Gluc. (mg%)	Alk.Ph. (units)	Ac.Ph. (units)	TP (gm%)	SGOT (units)	Electrophoresis Results in Percent				
									Alb.	α_1	α_2	β	γ
64	0	37	1.4	ND	5.4	5.3	6.5	23	54.4	0	10.1	21.3	14.1
	2	42	1.2	122	2.2	4.5	6.1	21	ND	ND	ND	ND	ND
	4	39	1.5	128	4.7	4.9	6.9	17	59.7	6.2	7.2	22.6	4.6
	6	39	1.3	ND	8.6	3.5	6.9	22	59.6	4.5	7.3	21.1	7.0
	9	34	1.3	118	3.4	2.5	6.5	11	59.2	0	7.8	28.2	4.8
	10	42	ND	108	7.2	2.3	7.2	28	68.2	0	8.7	11.5	11.3
	12	32	1.8	ND	7.7	3.5	6.3	24	61.6	2.5	7.4	22.0	6.9
	14	37	1.4	128	3.4	2.7	5.6	16	60.8	4.1	6.5	20.8	7.3
	18	41	1.9	119	3.8	3.8	6.7	30	67.2	2.9	7.5	19.5	2.9
	23	37	1.7	189	3.4	2.6	6.2	21	58.0	5.7	8.0	24.6	3.7

ND - Not done

Table XXV

Biochemical findings in a male cottontail rabbit (#61) inoculated with 206 D. uniformis larvae

No.	No. Wks	NPN (mg%)	Crea. (mg%)	Gluc. (mg%)	Alk.Ph. (units)	Ac.Ph. (units)	TP (gm%)	SGOT (units)	Electrophoresis Results in Percent				
									Alb.	α_1	α_2	β	γ
61	0	38	1.7	ND	3.4	5.3	6.7	24	59.3	5.0	20.5	14.3	1.0
	2	45	2.3	139	ND	5.5	6.7	44	ND	ND	ND	ND	ND
	4	39	2.2	135	2.3	4.4	7.3	33	57.2	5.5	8.6	23.2	5.7
	6	33	ND	ND	2.3	3.5	6.4	23	64.5	5.0	15.8	9.7	5.0
	9	39	1.9	114	0.7	3.7	7.0	36	28.8	17.3	17.6	27.7	8.4

ND - Not done

Table XXVI

Biochemical findings in a male cottontail rabbit (#9) which was not inoculated (normal control)

No.	No. Wks	NPN (mg%)	Crea. (mg%)	Gluc. (mg%)	Alk.Ph. (units)	Ac. Ph. (units)	TP (gm%)	SGOT (units)	Electrophoresis Results in Percent				
									Alb.	α_1	α_2	β	γ
9	0	35	2.2	95	5.0	4.0	6.0	34	71.5	4.1	6.3	15.4	2.2
	2	33	1.2	ND	5.9	6.3	5.4	19	48.2	8.3	11.6	25.6	5.2
	4	37	2.5	114	4.6	4.9	5.8	25	51.9	0	9.8	26.1	11.4
	6	44	ND	99	4.3	4.2	6.1	17	48.5	10.0	8.0	23.0	10.8
	Died												

ND - Not done

Detailed investigations in a limited number of cottontail rabbits (Tables XXV - XXVI), have indicated that even heavy infections with Dirofilaria uniformis do not bring about marked consistent changes detectable by any of the tests performed. In addition, the results indicate that a micro-technic for the performance of numerous biochemical tests can be utilized in the study of parasitic infections. This technic proved to be reliable and promises to become an ideal tool for physiopathological studies of parasitic infections, especially in small laboratory animals.

Summary and Conclusions:

1. A fluorescent antibody test was developed for the laboratory diagnosis of kala-azar. An evaluation of this test with sera from visceral leishmaniasis patients, from individuals with other diseases and from healthy controls indicated the relatively high specificity of this test and its potential value in routine diagnosis.

2. Species specificity studies of the fluorescent antibody test in patients with proven mucocutaneous and visceral leishmaniasis indicated that a greater percentage of positive reactions and higher titers were obtained with the homologous system. Similarly, although cross reactions occurred when sera from rabbits artificially immunized with the three species of Leishmania were reacted with different antigens, higher titers were present in the homologous systems.

3. Preliminary experiments were begun to ascertain the infectibility of germ free mice with Schistosoma mansoni cercariae and the location of developing worms in the portal circulation.

4. The susceptibility to S. mansoni infection was compared in monkeys of 5 species.

5. Different degrees of resistance were induced in Rhesus monkeys by previous exposure to cercariae irradiated at different levels. Histopathological observations revealed varying degrees of tissue damage induced by the immunizing exposures and by the challenging infection in previously sensitized hosts. Extensive quantitative serological studies showed that exposure to irradiated cercariae produced rapid antibody response. No correlation was observed between the degree of irradiation and antibody levels nor between antibody titers and degree of resistance.

6. Comparison of the pulmonary reactions of mice, muskrats and monkeys to nonirradiated and irradiated cercariae of S. mansoni revealed some interesting similarities and differences. In all cases the distribution of lesions appeared to be a function of parasite mortality. However, in acquired resistance, there was a characteristic early exudative cell response distributed along blood vessels which led to the appearance of characteristic "tuft-like" foci.

7. Schistosome egg antigen-antibody complexes injected into mice and suckling rats failed to produce a demonstrable degree of protection to a challenging infection. Histopathological studies indicated a reduced amount of pigment in the livers of animals inoculated with egg antibody complexes and a more rapid disappearance of eggs from the tissues.

8. Flocculation tests with schistosome egg extracts and with excretions and secretions of adult S. mansoni worms were developed and compared with the cercarial slide flocculation test. The antigen obtained from excretions and secretions seemed to confer to the test the greatest sensitivity. Homologous and heterologous absorption studies revealed specific reacting fractions in these antigens. Agar-gel diffusion studies showed different antigen-antibody systems some of which appeared to be stage specific.

9. Rats developed an acquired resistance to S. mansoni following repeated injections with serum from immune monkeys. Resistance to superinfection was observed with serum from monkeys which had become immune following an active infection, as well as serum from monkeys which had been repeatedly vaccinated with irradiated cercariae. These results indicate that acquired immunity to S. mansoni is partly, at least, serological in nature.

10. A comparative study of various immunodiagnostic screening tests for schistosomiasis was conducted in an Ethiopian village. Although the measuring agreements with results of stool examinations were different for individual tests, it was found in general that co-positivity increases with age, whereas co-negativity decreases. An increase in the sensitivity of the reference test had an influence on the co-positivity, on the overall agreement and, to a minor extent, on the co-negativity.

11. Intramuscular injections of a repository form of sodium antimony dimercapto-succinate (TWSb) resulted in an improved prophylactic and suppressive activity against S. mansoni in monkeys. Repeated daily doses

of Bis-(B-carbhydrazido-ethyl) sulfone (S-102) resulted in a significant reduction in the number of worms in treated animals. Conversely, repeated doses of O, O-Dimethyl-O-O-(2, 4, 5-trichlorophenyl) phosphorothioate (Ronnel) failed to bring about a significant reduction in the number of worms in the treated animals as compared to controls.

12. Experiments were set up to determine biochemical changes which might be detected in cottontail rabbits following infection with the filarial worm Dirofilaria uniformis. Detailed investigations indicated that even heavy infections with this parasite do not bring about marked consistent changes detectable by any of the tests performed.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36180

PROJECT, TASK, OR SUBTASK NO.: 3A0125 01A 8180121

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER
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49

6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Viral infections of man (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME'

(U) Investigations of human virus diseases and their immunology established: 1) that significant antigenic variation in influenza viruses, type A_2, occurred during the winter of 1963, 2) that when changes in alveolar gas exchange can be demonstrated in man, they are associated with severe but not milder respiratory infections, 3) that rubella virus possesses a number of unique biological and epidemiological properties which will complicate the search for immunizing preparations, 4) that specific antiviral substances can be shown in nasal secretions of normal persons; these differ from those produced during infections, 5) that CF, HI and neutralizing antibodies to arboviruses are distinct, and their activity is associated with different immunoglobulins, 6) that an attenuated dengue virus vaccine type I, can be safely administered to troops under full field duty. Such vaccine reduced the incidence of overt dengue in Puerto Rico by 50% - 67% when given to volunteer students in the face of an advancing epidemic caused by dengue virus, type III, 7) that dengue viruses are more readily recovered by a new technique employing a cell culture-interference test than by conventional mouse inoculation, and 8) that reagent grade antibody can be readily produced from ascitic fluid harvested from immune mice.

9. KEY WORDS

Virus, infection, communicable, immunology, epidemiology, arbovirus.

10. SUPPORTING PROJECTS

Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES

☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES

☒ NO ☐ YES See Continuation Sheet

DA FORM 1 June 63 1309R PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of _____

ACCESSION NO. 36180	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	11–22: 3A012501A818 · 23 24: 01 · 25 26: 21 · 27 28: · 29:
14. DATE OF REPORT (30–33)	30–33: 0664
15. SECURITY OF WORK (34)	34: 4
16. TYPE OF REPORT	35: 3 · 36–47 48 49 50 51: · 52–55: 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56–61) b. Functional Class (62–64)	56–61: 010608 · 62–64:
18. OSD CLASSIFICATION (65–66)	65 66: AR
19. R&D CATEGORY (67)	67: 1

Card "D"

20. CONTRACT NUMBER	11 12: DA · 13 14: · 15–17: · 18–21: · 22–26: · 27:
21. GRANT NUMBER	28 29: DA · 30–33: · 34 35: · 36–38: · 39 40: · 41–45: G · 46:
22. ESTIMATED COMPLET. DATES	47–51: 1CONT · 52–56: 2 · 57–61: 3 · 62–66: 4 · 67–71: 5

Card "E"

23. PRIORITY (11–14) 24. PROGRAM ELEMENT (15–26)	11–14: 1 · 15–26: 6•11•25•01•1
25. CMR&D CODES	27–29: N/A · 30–32: · 33–35:
26. CDOG REFERENCE a. Paragraph No. (36–44) b. Functional Group (45)	36–39: 1412 · 40: · 41 42: a · 43 44: · 45: 6

Card "F"

27. FUNDING

a. Est. Total Cost (11–15)	11–15: · 16–18: 1 · 19–21: 2 · 22–26:
b. % Spent Intern. (16–18)	
" " Extern. (19–21)	
c. Total Obligation (22–26)	
d. Progmd. Cur. FY (27–33)	27 28: · 29–33: · 34 35: · 36–40:
e. " " " +1 (34–40)	
f. " " " +2 (41–47)	41 42: · 43–47: · 48 49: · 50–54:
g. " " " +3 (48–54)	
h. " " " +4 (55–61)	
i. " " " +5 (62–68)	55 56: · 57–61: · 62 63: · 64–68:
j. " " " +6 (69–75)	
k. Total Man Years of Effort (76–78)	69 70: · 71–75: · 76–78:

DA FORM 1 June 63 1309R — Previous Editions are Obsolete — Page 2 of _____

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Weinberger, H.L., Buescher, E.L., McCown, J. and Gauld, J.R.: Antigenic Variation of Recently Isolated A_2 Influenza Viruses. Proc. Soc. Exp. Biol. & Med., 114:413, 1963.

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ANNUAL PROGRESS REPORT

Project 3A 0125 01A 818 Title: Communicable Diseases and Immunology

Task 01 Title: Communicable Diseases

Subtask 21 Title: Viral Infections of Man

Description: To define the etiology and ecology of human virus infections, with particular reference to those occurring in military populations; to devise and evaluate means for precise diagnosis, control and prevention of disease. Studies have applied virological, immunological, epidemiological and physiological approaches to the understanding of disease caused by respiratory, arthropod-borne, and enteric viruses, and the factors influencing their transmission among man and other vertebrates, and their survival in nature.

Progress:

1. Antigenic Variation in Influenza Viruses. During study of clinical specimens submitted for confirmation of the clinical diagnosis of influenza, January - March 1963, a variant of type A_2 influenza virus was recovered and identified. Continuing laboratory analysis of this variant has yielded confirmatory data on the nature of the variation.

It was reported in the last Annual Report that the cell culture neutralization (N) test was being adapted for the study of human and animal antibody to influenza virus. This test was found to be a reliable method for measuring specificity and development of N antibody. With rooster antisera, the test was shown to be more specific than the HI test.

a. N Antibody in Patients' Sera. Paired sera from 41 persons yielding influenza virus in 1963 were tested for N antibody to a 1957 strain (A_2 Japan 305/57) and a 1963 strain (A_2 DC 302/63) (Table I). Titers of acute phase sera were significantly lower to the 1963 (geometric mean 1:8) than to classical A_2 virus (1:36). Titers in convalescence rose to approximately the same levels for each virus. Finally, 4-fold or greater increases in N antibody were shown more frequently with A_2 DC 302/63 (36/41) than A_2 Japan 305/57 (22/41).

Although serial sera were not available for determination of actual pre-infection titers of neutralizing antibody, these data compare favorably with the significant antibody responses obtained in hemagglutination-inhibiting or complement-fixation tests (Table II).

The N test has proved to be a reliable procedure for quantitation of antibody to influenza virus. Antibody titers of both human and fowl sera are readily reproducible from test to test, because they are influenced minimally by the actual variation in the predicted dose of virus. From data obtained in

Table I Neutralizing* Antibodies in 41 Patients with Proved Influenza 1963

Virus	Serum	Number with indicated antibody titer: 5	5	10	20	40	80	160	320 or
A_2Jap.305/57	Acute+	5	3	4	4	7	6	10	2
	Conval.	-	3	-	-	2	4	8	24
A_2DC 302/63	Acute+	17	4	6	5	6	2	1	-
	Conval.	-	1	-	2	5	4	15	14

* Measured against 5-60 HAd doses$_{50}$ of A_2Japan 305/57 and 30-500 HAd doses$_{50}$ of A_2DC 302/63 viruses. Titer/0.05 ml.

+ Geometric mean titer of prebleed against A_2Jap.305/57 = 1:36; against A_2DC 302/63 = 1:8.

Table II Frequency of Antibody Responses in Patients with Clinically Diagnosed Influenza

Patient Category	No. Showing Significant Increases by Indicated Tests: HI*	CF	HI and/or CF	Neut
Virus recovered	35/42	31/42	39/42 (.93)	36/41 (.88)
No virus recovered	29/49	32/46	21/49 (.84)	13/16 (.81)

* Virus used for HI test = A_2Japan 170/62; for CF test = A_2DC 327/63; for Neut test = A_2DC 302/63.

antigenic analyses, it is apparent that the specificity of the test is equal to or surpasses the HI test with rooster antisera. While more time consuming and expensive than the HI procedure, the N test has the potential of being more readily reproduced from one laboratory to another. Because normal inhibitors apparently do not influence N reactions, the requirement for their artificial removal (as for HI tests) is obviated. The test, finally, gives a result in terms of a titer of inhibition of a hemagglutination process which, at best, is an artificial measure of serum antibody.

b. Antibody Patterns in Young Adults. Earlier attempts to distinguish human antibodies to the various A_2 viruses by HI test were unsuccessful, and the N test was found to be highly specific for this purpose. To determine whether differences in incidence of N antibody in man existed between various

parts of the country, following the 1963 epidemic in the East, tests were performed on sera from recruits arriving at 3 different bases during the summer of 1963.

Of 45 recruits at Ft. Dix, N. J., more than 90% possessed HI antibody against both the 1957 and 1963 strains in titers of 1:20 or greater (Table III). In contrast, the incidence of N antibody for the two strains varied. Whereas 91% of those studied showed N antibody titers of 1:20 or greater against A_2 Japan 302/57, only 62% showed similar titers to the 1963 strain. Further, this difference in incidence of antibody increased as serum was diluted. Recruits from the Middle West (Ft. Knox) showed essentially the same pattern of HI and N antibody as the Ft. Dix group. However, in the Far West (Ft. Ord), incidence of N antibody to the current strain was markedly lower than at the other two training areas, at all serum dilutions tested. In addition, incidence of N antibody to the 1957 strain at Ft. Ord was lower than at Ft. Dix when serum dilutions of 1:40 or greater were compared. Again, patterns of HI antibody to the 1957 and current strains could not be differentiated from one another. The N antibody status of recruits following the 1963 epidemic is consistent with the observed distribution of influenza.

c. N Antibody Response Following Immunization. To determine the pattern of N antibody responses to 1963 and 1957 viruses following immunization, the 50 men at Ft. Ord were re-bled 8 weeks after their initial samples were drawn, and paired sera were tested simultaneously. These men had been administered polyvalent influenza vaccine containing the 1957 but not the current strain of A_2 virus in their 1st week of training. Results (Table IV) showed that comparable patterns of N antibody developed in these men (to both strains tested) at serum dilutions up to 1:160. A significant difference in incidence of N antibody to the current strain was noted only in higher serum dilutions.

HI tests have consistently failed to show any differences in the incidence of antibody to the current and classical A_2 strains, which are so clearly demonstrable by the N test. Unfortunately, no controlled prospective study was available to evaluate the efficacy of standard military formula influenza vaccine during the outbreak in the Eastern United States in 1963. This vaccine contained the 1957 strain of A_2 virus, but not the current variants. The proof of the biological significance of the new variant depends on the demonstration that this variant has been associated with significant increase in clinical disease.

Table III Incidence of HI and Neut Antibody to 1957 and Current[1] Strains of A_2 Virus in Recruits, 1963

Antibody Titer	% With Antibody at Indicated Station											
	Fort Dix[2]				Fort Knox				Fort Ord			
	1957		Current		1957		Current		1957		Current	
	HI	Neut	HI	Neut	HI	Neut	HI	Neut	HI	Neut	HI	Neut
≥ 1:10	93.8	95.5	95.5	80	89.1	84.8	93.5	76.1	86	88	86	62
≥ 1:20	91.1	91.1	95.5	62.2	86.9	80.4	91.3	58.7	82	82	84	41
≥ 1:40	60	68.9	62.2	35.5	67.4	71.7	76.1	39.1	44	60	46	24
≥ 1:80	37.7	53.3	44.4	24.4	50	63.0	56.5	23.9	22	30	28	14
≥ 1:160	15.5	37.7	20	8.8	19.7	41.3	28.3	13.0	-	20	-	6

[1] HI; tested against A_2Japan 305/57
A_2Japan 170/62
Neut; tested against A_2Japan 305/57
A_2DC 302/63

[2] Fort Dix - 45 men
Fort Knox - 46 men
Fort Ord - 50 men

Table IV Neut Antibody Response of Recruits to Influenza Virus Vaccine[1]

Antibody Titer	Incidence Antibody to Indicated Viruses			
	Pre Immunization		8 Weeks Post Immunization	
	1957[2]	1963	1957	1963
= 1:10	88 %	62 %	94 %	94 %
= 1:20	82	41	94	92
= 1:40	60	24	92	90
= 1:80	30	14	90	84
= 1:160	20	6	86	72
= 1:320	-	-	76	48
= 1:640	-	-	58	24

[1] Military formula influenza vaccine contained 100 CCA units of strains A/swine/1976/31, A/PR8/34, A_1/Ann Arbor/1/57 and B/Lee/40, 200 CCA units of B/Great Lakes/1739/54, and 400 units of A_2/Japan/305/57 per ml.

[2] Test Viruses: A_2 Japan 305/57
A_2 DC 302/63

2. Effect of Acute Respiratory Infection on Pulmonary Function of Military Recruits. Acute respiratory disease (ARD) including viral pneumonia is the major cause of loss of time from duty in Army recruits. Previous studies have defined the etiology of these infections as due to Adenoviruses types 3, 4, and 7, primarily; other viruses are less frequently implicated. A preliminary study of pulmonary function in seven patients with viral pneumonia was completed during the winter of 1962-63. These patients were tested during convalescence for evidence of pulmonary function abnormality using spirometry, lung volume determinations and carbon monoxide diffusion (DLCO). Only DLCO was significantly abnormal in this group. Five of the seven were below predicted values for DLCO, the remaining two were low normal. In follow-up study one to twelve weeks later all patients were found to be within the normal range for DLCO. It should be emphasized that these abnormalities were noted at a time when roentgenological examination of the lungs and chest revealed no abnormality.

Virologic studies demonstrated evidence of Adenovirus infection in six patients and, specifically, Adenovirus type 4 in five.

The study covered in the present report was instituted to confirm and extend these preliminary findings. In order to determine lung function of a large number of recruits ill with viral pneumonia, a pulmonary laboratory was set up at Fort Dix, N. J., in facilities provided by the Post Surgeon. Ventilatory measurements were obtained using a spirometer. Carbon monoxide diffusion testing was performed by the method of Bates which does not require arterial sampling, a prerequisite for large scale field studies.

Because of limited experience with this latter test a study of normal recruits was undertaken. A group of 53 newly inducted recruits was selected by medical history and physical examination as being free of past or present respiratory disease. All subjects had had a recent chest x-ray reported as normal. Pulmonary function tests were performed during the first week of basic training and again during the final (8th) week. As a control, one member of the research team performed a diffusion test each day that recruits were tested.

a. Pulmonary Function Changes in Mild ARD. During the eight-week training period only 5 subjects reported to the dispensary with respiratory symptoms. Only one of these cases was of sufficient severity to require hospitalization. Virologic studies in all five did not reveal the etiologic agent. Six other subjects were found to have developed a significant adenovirus complement fixing antibody response during the 8-week period. The relatively low antibody levels are more consistent with vaccine induced antibody than natural infection, however (all recruits receive formalinized bivalent or trivalent adenovirus vaccine shortly after induction). These studies indicate that very little respiratory disease occurred in these recruits, thus insuring that the subjects were truly "normal". Results of pulmonary function tests on the group are presented in Table V.

Table V. Pulmonary Function Tests in 53 Recruits, Means and S.D.

		Oct. 63	Dec. 63
VC	(Vital capacity) % predicted	98±10	94±10
FEV_1	(Forced expir. vol., 1 sec) % of VC	86±7	86±7
MET	(Mid expir. time) % predicted	90±26	93±30
MVV	(Max. volunt. ventil) % predicted	95±16	92±17
DLCO	(Diffusion carbon monoxide) % predicted	109±18	101±16

Repeated diffusion tests in a trained subject are shown in Table VI.

Table VI DLCO, % Predicted, Repeated Tests in a Trained Subject

Oct. 6-10	Dec. 1-5	
109	101	
113	96	Mean = 107
117	105	
106	103	S.D. = 7
115	100	
114		

b. Changes in Pneumonitis. Recruits ill with acute respiratory disease, particularly pneumonitis, were selected from admissions to Walson Army Hospital, Fort Dix, N. J. In addition to a thorough medical history, physical exam, clinical laboratory study and chest x-ray, throat washing, stool and serum were collected for virologic studies. As soon as acute symptoms abated, pulmonary function studies were carried out in the field laboratory established at Fort Dix. CO diffusion tests were performed in duplicate (i.e, within 24 hours) in most of the subjects, and were repeated at weekly intervals for a duration of 2 weeks to as long as 3 months.

Fifty-one patients were studied initially, but 9 were dropped from consideration because pulmonary function tests were not performed. Of the 42 studied cases, 20 were shown to have adenovirus type 4 infection based upon virus isolation and serologic evidence of antibody increase. No viral agent was associated with the remaining 22 cases. Several, however, were undoubtedly of bacterial etiology. Results of pulmonary function tests are incomplete at the time of this report, but preliminary findings suggest little or no variation from the normal values established previously. Although grading of the clinical disease has not yet been accomplished, it can be stated that in general the observed illnesses were milder than those noted in previous years. Further analysis of the data will be required before definitive statements can be made.

3. Rubella.

a. Biological Properties of Rubella Virus. Studies of the characteristics of rubella in primary African green monkey kidney cell monolayers (GMK) begun at the Walter Reed Army Institute of Research, used its property of interfering with the cytopathic effect (CPE) of enteroviruses.

Utilizing this property for quantitation of virus, rubella virus infectivity was shown to be rapidly destroyed by ether, chloroform, formalin and heat. In filtration studies it appeared to be between 100 and 300μ in diameter. It differed from other human viruses with which it has these properties in common by failing to produce complement fixing of hemagglutinating antigens or CPE in other than

primary human amnion or certain continuous rabbit kidney cultures (RK_{13}). These studies have been continued in attempts to 1) determine the nucleic acid composition of rubella virus and 2) evaluate culture techniques for detection of cytopathogenic effect.

Since virus titers were inadequate for direct chemical measurements of RNA or DNA, the effect of 5-iodo-2-deoxyuridine (IDU), an inhibitor of DNA but RNA-containing viruses was used. Rubella virus propagation rates and virus titers were unaffected by IDU-containing media; under these circumstances it behaved like RNA (poliovirus type 1, rubeola) and unlike DNA viruses (herpes simplex, vaccinia) tested simultaneously as controls. The failure of IDU to inhibit growth of rubella virus suggested that it was an RNA virus.

A number of cell culture systems have been evaluated for rubella virus cytopathic effect (CPE). While a number of cell cultures support growth, including BS-C-1, LLC-MK2, Patas monkey kidney, human embryo kidney, rhesus monkey kidney, primary and continuous diploid rabbit kidney cells and WE-26, thus far only the RK_{13} line of continuous rabbit kidney cells described by McCarthy, et al (Lancet 2:593, 1963) has shown CPE. This technique has been found valuable principally for tests using virus adapted by 15 or more serial passages in the continuous rabbit kidney cell line. It was found that lowering incubation temperatures of infected cultures to 33°C and using medium without added serum, shortened the time required for appearance of rubella cytopathic effects in these cells by 3-5 days. Under these circumstances titration endpoints of adapted strains are stable by 10-12 days. Fluids harvested from RK_{13} cultures inoculated with high multiplicities of rubella virus show about 10-fold higher virus yields ($10^{5.5}$/0.1 ml) than similar harvests from GMK cultures. With this system it has also been possible to recognize CPE in cultures inoculated with throat washings from rubella patients. The sensitivity of this cell line for primary isolation of rubella viruses appears to be comparable to that of the GMK-ECHO-11 virus interference system.

Using these cell cultures it has been possible to demonstrate rubella virus plaques in agar-overlaid cultures. Studies are currently in progress to determine the utility of this plaque assay system. Preliminary observations suggest that the efficiency of plaque formation is good and that it may be useful for studies requiring critical quantitation of virus.

b. Measurement of Antibodies to Rubella Virus. To date the only tests for antibody to rubella virus are those employing rubella interference for enterovirus in grivet monkey kidney (GMK) cells or cytopathic effect (CPE) in human amnion or continuous rabbit kidney (RK_{13}) cells.

The results of these neutralization tests are influenced by a number of variables. Studies performed primarily in the GMK interference system indicate that factors important for reproducible antibody determinations include virus test dose, time of addition of the E-11 challenge virus and preincubation condition of serum virus mixtures. Results were also affected by addition of "accessory factors" contained in normal rabbit serum.

Rubella antibody titers are low in contrast with those of rubeola. With 2.0 log ID_{50} of rubeola virus, convalescent antibody titers are regularly 1:32 to 1:1024; against similar concentrations of rubella virus, antibody titers are seldom greater than 1:8 and biologically significant amounts of antibody can be missed. Lower concentrations of virus (1.3 to 1.7 log ID_{50}) allow detection of rubella antibody over a range of serum dilutions from 1:2 to 1:128. Neutralization tests performed with both animal and human sera indicated that a 10-fold increase in virus ordinarily produced a 4-fold decrease in antibody titer. The range of serum antibody titers against a similar test dose varied within 4-fold, from one test to another. This was similar to the variation observed in rubeola virus neutralization tests. Addition of E-11 challenge virus at 10 days, when rubella virus titration endpoints were stable, resulted in lower antibody levels than in tests challenged earlier.

Studies comparing the effects of preincubation on serum virus mixtures showed that at 37°C essentially all neutralization occurs during the first hour. Neither 4°C or 25°C for one hour, nor prolonged preincubation at 4°C with or without a prior 37°C period, enhanced antibody titers. The addition of unheated antibody-free rabbit serum in final volume concentration of 5% to serum-virus mixtures potentiated antibody titers by from 2- to 8-fold; the effect was evident in tests in which E-11 challenge was delayed until 10 days, but showed less enhancing effect in tests challenged earlier. In preliminary studies these factors appear also to be true for the RK_{13} cytopathic system.

From these experiments, current methods for measuring neutralizing antibody in the GMK-E-11 virus interference system employ a 37°C, one-hour incubation period, titration of serial 2-fold serum dilutions against an estimated 1.5 log InD_{50} dose of rubella virus, and addition of E-11 challenge virus after cultures have been incubated five days. Experiments are in progress to determine the relationship of results of these tests with those derived from the RK_{13} cytopathic system. Preliminary observations show that in the latter system, rubella virus doses from 1.5 to 2.5 InD_{50} can be used without sacrificing test sensitivity or reliability. These results also suggest that the RK_{13} cell line may be of considerable value for rubella neutralization test.

c. Studies of Natural Rubella Infections of Man. The reliability of the rubella virus neutralization test for detection of human serum antibody was examined by repeated testing of human sera obtained before infection and at varying times after convalescence from the disease. In neutralization tests with carefully controlled virus doses (optimally 1.0 to 1.7 log InD_{50}) sera obtained in convalescence uniformly contained antibody. In contrast, antibody was not demonstrable in sera obtained prior to either overt or clinically inapparent infection. It was possible to test the accuracy of screening tests for rubella antibody in sera collected from a military recruit training company in which frequent collection of specimens made it possible to identify all rubella infections by recovery of virus from throat washings or by serologic evidence of infection. In screening tests a 1:2 dilution of serum was tested against virus doses calculated to be 10 and 100 InD_{50}. Those without

antibody at a 1:2 dilution were predicted as susceptible, those with antibody as immune. These predictions were then compared with observed natural infections. The errors in prediction of susceptibility or immunity were increased in the dosage range less than 1.0 or greater than 2.0 log InD_{50}. Within this range, error in detecting immune status was approximately 10%. Continued experience showed that screening test accuracy was immproved by the inclusion of low and high titered antisera as well as antibody free samples as controls on the test dose of virus itself. In tests interpreted on this basis, accuracy of identification of immunologic status approached 100%.

Rubella antibody appeared promptly after infection in seven of fifteen sera collected from patients on the first or second days of rash. Low antibody levels of 1:2 to 1:4 were present. Antibody titers in these, and in patients with inapparent natural infections increased thereafter, and at 20 to 30 days ranged from 1:8 to 1:64. Persistence of antibody was suggested by findings that 1) levels in sera obtained six months after infection showed titers similar to those in immediate convalescence; 2) antibody levels in immune adults remained unchanged in sera drawn from three to eight years apart; 3) antibodies were present in the sera of elderly veterans.

These data suggest that detectable circulating antibody to rubella are associated with immunity and that immunity resulting from infection may be lifelong.

d. Immunogenicity of Formalinized Rubella Virus Vaccines. It was possible to produce rubella virus antibody by repeatedly inoculating rabbits or guinea pigs with infectious rubella virus. In these experiments three or more 1 ml inoculations of clarified tissue culture fluids containing 3.0 to 4.0 log InD_{50}/0.1 ml virus induced low levels of neutralizing antibody in all animals tested. Attempts were made to assess the antigenicity of similar preparations rendered noninfectious by treatment with formalin. In these experiments, formalin was added in 1:4000 final concentration to virus preparations; these were then filtered through Millipore membranes (300μ apd) and allowed to react at 4°C or 37°C. Inactivation rates at 37°C (normally approximately 0.3 log units/hour) were markedly increased by formalin, so that residual infectivity could not be detected after two hours in any of three different inactivation experiments. At 4°C formalin inactivation was much slower and incomplete even after 10 days.

Guinea pigs and rhesus monkeys, immunized with one to two ml doses of virus inactivated by treatment with 1:4000 formalin at 37°C for 24 hours failed to show antibody response. This was in contrast to the prompt antibody response of other guinea pigs immunized at the same time with un-inactivated virus or with preparations treated with formalin at 4°C, containing small amounts of infectious virus. Formalinized virus concentrated 10-fold or incorporated with Freund's adjuvant did not produce detectable circulating antibody in rhesus monkeys similarly immunized. Immunized monkeys were not resistant to rubella infection upon subsequent challenge with live virus.

The failure to induce antibody is probably related to lack of sufficient antigenic mass; perhaps excessive denaturation of virus protein by formaldehyde

also played a role. Studies directed toward testing more concentrated virus preparations, inactivated by a number of different methods, are currently in progress.

e. Possible rubella virus oncogenic effects. The suggestion that the morphology of rubella virus resembled that of rodent leukemia viruses by Norrby, et al (Archiv. Gesamte Virusforschung 13:421, 1963) stimulated interest in the possible production of leukemia or other tumors in experimental animals. In conjunction with Dr. John B. Moloney of the Laboratory of Viral Oncology, National Cancer Institute, a leukemia-sensitive strain of mice was inoculated with rubella virus.

Randomized litters of newborn BALB/c mice were inoculated by the intracerebral, intraperitoneal or subcutaneous routes with either rubella virus, concentrated and partially purified by ultracentrifugation, or control GMK tissue culture fluids prepared similarly. At one month of age, mice were weaned and redistributed in cages by sex and route of inoculation. In addition, litters of suckling hamsters have been inoculated intraperitoneally with the same material. Animals are being examined once weekly for evidence of apparent illness or appearance of tumors. At the present time, four months after inoculation, all animals which survived the newborn period appear healthy; no tumors have appeared. Observation of these animals is planned for one year from time of inoculation.

f. Rubella in monkeys. Since monkeys had been shown to be suitable host for a number of human viruses, including rubeola, the ability of rubella virus to produce infections in these animals was tested. Preliminary screening of sera suggested that unlike rubeola, rubella antibody occurred infrequently under usual transportation and housing conditions, since only 1 of 15 rhesus monkey sera tested contained antibody. Subsequent studies of sera from rhesus monkeys subjected to special handling procedures designed to prevent acquisition of rubeola antibody have shown a similar low incidence.

In attempts to induce experimental infections, animals have been inoculated by intravenous or intramuscular routes. No rash, lymphadenopathy, respiratory symptoms or elevations in temperature attributable to rubella virus infection have been observed in any of nine rhesus or two cercopithecus monkeys inoculated with tissue culture rubella virus at the third or fourth passage level. All inoculated animals, however, shed virus and subsequently developed neutralizing antibody, indicating that subclinical infections had occurred.

Virus was recovered from swabs taken from the nose, conjunctivae, pharynx and rectum and from whole blood or serum. Virus recovery from these specimens resemble in general, that observed with natural human infections; virus was most frequently recovered from blood or serum from the 4th to the 9th day after inoculation, but never after detectable neutralizing antibody appeared. Virus was recoverable from throat washings as early as four days after inoculation. Virus was recovered from swabs of all animals' respiratory secretions. The throat yielded virus occasionally even within a day or two after antibody appeared. Isolates from other sources were less frequent; viremia was demonstrated in one or more specimens from six of nine rhesus monkeys. Virus was

irregularly present in rectal swabs. Recovery from this source was made as long as 16 days after inoculation and thus did not appear to be related to presence of antibody as was virus in serum and throat washing.

Presence of neutralizing antibody at the time of inoculation in animals infected three months previously prevented subsequent recovery of virus from any source, even when large (4.4 log InD_{50}) inocula were employed.

4. Antiviral Activity of Nasal Secretions. Over the years, investigations of local immunity of the respiratory tract have led to a search for antibodies or antibody-like substances in respiratory secretions. Substances capable of inhibiting proliferation of viruses have been described repeatedly in respiratory mucus, although the nature of the inhibiting substances has not been clearly elucidated. Studies by Burnet et al. suggested that the substance in nasal and bronchial secretions which inactivated influenza virus was different from serum antibody. The investigations of Fazekas de St. Groth in mice and of Francis in humans suggest a serum origin for the muco-antibodies. The present studies were undertaken to investigate the spectrum of virus neutralizing activity of human nasal secretions and to attempt a definitive identification of the inhibitory substance.

a. Subjects. Ten adult male laboratory workers contributed the normal secretions. Three other men and one of the normals were studied during periods of acute respiratory disease. Three patients with chronic bronchitis in remission were also tested.

b. Collection and Processing of Nasal Secretions. In preliminary trials the use of cotton plugs, stimulation of nasal mucosa with sodium carbonate and washing of nasal cavities with distilled water were found to be less satisfactory for collection of nasal secretions than saline washes. The following technique was used throughout these studies: with neck hyperextended and glottis closed, 5 ml of sterile normal saline solution was instilled into one nostril by pipette. The solution was then forcibly expelled through the nares into a clean beaker. Washings were collected several times daily for several days; the secretions from each man were then pooled and frozen at -20°C. Nasal washings were prepared for assay of antiviral activity as follows. After thawing, the specimen was shaken vigorously with glass beads, centrifuged at $\pm$ 1000 g. for 20 minutes, dialysed overnight against distilled water, and concentrated by lyophilization. After rehydration to one tenth the initial volume, total protein was determined by the sulfosalicylic acid method. Specimens were then adjusted to a final protein concentration of 150 mg% $\pm$ 15 mg%. All samples were found to be free of hemoglobin by the guaiac test.

c. Cell Cultures and Viruses. Rhesus monkey kidney cell cultures (MKC), obtained from commercial sources, were used for tests with influenza, parainfluenza, polio and Coxsackie viruses. HEp_2 cultures were used for adenovirus neutralization tests; diploid human embryonic lung cultures were used for ECHO 28 and rhinovirus antibody determinations. The viruses were standard strains used in this laboratory, each with multiple cell culture passages.

d. Technique of neutralization tests. Preliminary studies had indicated that concentrated fresh nasal washings were invariably contaminated with fungi or bacteria. Heating at 56°C for 30 minutes sterilized them; comparative titrations showed no loss of virus neutralizing capacity upon heating. Heat inactivated (56°C for 30 minutes), concentrated nasal secretions, or serial two-fold dilutions of serum, were mixed with an equal volume of virus fluid containing 30 to 300 tissue culture infective doses (TCID 50). Following 1 to 3 hours of incubation at room temperature, two culture tubes were inoculated with 0.1 ml of each mixture. Tests were read 2 to 5 days later. Hemadsorption served as the indicator for myxoviruses; cytopathic effect for the other agents. Because of limited volume, concentrated secretions were tested for antibody without serial dilution.

Immunoelectrophoresis of nasal secretions and sera was performed by the method of Grabar and Williams as adapted to microscope slides by Scheidegger with minor modifications. Horse anti-human serum (AHS) was obtained from Dr. Elmer Becker. Goat anti-human γ-1A serum (Lot G206, Hyland Labs., Los Angeles, California) was obtained from Dr. John Fahey and was shown to be specific by immunoelectrophoresis. Goat anti-human γ-2 antiserum (Lot GP11-63) was purchased from Hyland Laboratory. Tests with this antiserum will be described in detail below.

e. Density Gradient Ultracentrifugation. The method of Kunkel was used. Continuous gradients ranging from 10 to 40% sucrose in 0.15 M NaCl were formed at 4°C by means of a mixing chamber. Serum diluted 1:2 with 0.15 M NaCl, or whole nasal wash, was layered onto the gradient in 0.5 ml amounts with an equal volume of the 10% sucrose solution. Centrifugation was performed in a Spinco Model L ultracentrifuge using the SW 39 rotor at 35,000 rpm for 18 hours. Serial fractions were collected under controlled pressure through a needle perforating the bottom of the centrifuge tube. Control runs utilizing known 7S and 19S gamma globulins have indicated a high degree of reliability in the separation of these components. It should be noted that this type of separation does not give the S rate of the antibody with accuracy; however, the term 19S will be used for the macroglobulin activity, and 7S for the lower S rate material. Macroglobulin antibodies are usually found in fractions 1 through 4, and antibody activity of 7S type in fractions 6 through 8. Antibody of the γ-1A type is found intermediate between the 19S and 7S globulins in fractions 5 to 6. Prior to antibody testing the fractions were dialyzed against phosphate-buffered saline (PBS), pH 7.36, to restore isotonicity.

f. Gel Filtration with Sephadex G-200. A modification of the method of Flodin and Killander was used. The sephadex was washed several times with distilled water and the "fines" were removed by decantation. It was finally equilibrated with 1.0 M sodium chloride, with 0.1 M Tris (Hydroxymethyl) Aminomethane, pH 8.0 buffer. A column 70 cm in length and 2 cm in width was packed with 10 gm of sephadex under gravity flow. A 2.0 ml sample of concentrated nasal secretions or serum was applied to the column and elution carried out with the Tris buffer. The flow rate was 12 ml per hour and the

eluates were collected in 2-3 ml amounts. The protein concentration of the eluates was measured by the Lowry method. Since the protein concentration of the fractions from the nasal secretions was usually very low, pools of approximately 20 ml each were made from consecutive fractions and were concentrated 10-fold with Carbowax. Prior to testing for antibodies, fractions and pools were dialyzed against PBS.

g. Antibodies in Nasal Secretions. Neutralizing activity against two or more of the eight viruses tested was found in each of the ten normal nasal washings (Table VII). The specificity of the neutralization is demonstrated by the fact that activity against closely related viruses varied; for instance, nasal secretions of three patients reacted differently to influenza A_1 and A_2 viruses. The relationship of serum neutralizing antibody titer to nasal antibody, tested simultaneously, is depicted in Table VIII. With few exceptions a high serum titer was associated with virus inactivation by nasal secretion. Conversely, absence of serum antibody was associated with lack of nasal antibody in most cases. However, 5 patients

Table VII Activity and Nature of Antibody in Normal Nasal Secretions

	Neutralizing Activity								Immunoelectrophoresis			
										Globulins		
Pt. #	Influenza A_2/62	Influenza A_1	Parainfluenza Type 3	Polio Type 1	Adenovirus Type 7	Coxsackie A9	ECHO 28	Rhino 1059	Albumin	Alpha	Y-1A	Y-2
1	0	+	+	0	0	0	0	0	+	±	+	0
2	+	+	+	+	0	+	+	0	+	+	+	0
3	+	+	+	0	0	+	0	0	+	+	+	0
4	0	0	+	+	0	0	+	0	+	+	+	0
5	+	0	0	+	0	0	+	0	+	+	+	0
6	+	+	+	0	+	0	+	+	+	+	+	0
7	+	+	+	+	0	0	0	ND	+	+	+	0
8	+	+	+	+	+	+	+	0	+	±	+	±
9	+	+	+	0	0	0	+	+	±	+	+	0
10	0	+	+	+	0	+	0	+	±	+	+	0

+ = present; 0 = not present; ND = not done; ± = trace amount.

Table VIII Relationship of Nasal Secretion Antibody to Serum Titer

Antibody to Indicated Virus in Nasal Washing		Number Persons with Indicated Serum Titer Showing Antibody in Nasal Washing							
		<10	10	20	40	80	160	320	640
Polio 1	+		1		2	1	2	3	2
	-	2		1	1		1		
Coxsackie A9	+				1	1	2		1
	-	8	3						
Influenza A_1	+		2	7	4	1			
	-		1	1					
Parainfluenza type 3	+			5		5	2	3	
	-			1					
		<2	<5	5	10	20	40	80	
Adenovirus type 7	+		1	2	1				
	-	9	1	1					
Influenza A_2	+	1		1	4	2	3		
	-	1	1	1	1				
ECHO 28	+	2 3		3					
	-	1 5	1						
		<1	1	2	4	8	≥8		
Rhinovirus 1059	+			1			4		
	-	8	1						

with undetectable serum neutralizing antibody to ECHO 28 virus (<1:2 or <1:5), and one patient with no demonstrable influenza $A_2/62$ antibody in serum (<1:5), were found to have virus neutralizing substance in nasal secretions.

Studies of seven persons with respiratory illness showed antiviral activity in nasal secretions and correlation with serum antibody level similar to that found in normals (Table IX).

Table IX Activity and Nature of Antibody in Nasal Secretions From Patients with Respiratory Diseases

		Neutralizing Activity								Immunoelectrophoresis			
											Globulins		
Diagnosis	Pt.	Influenza $A_2/62$	Influenza A_1	Parainfl. Type 3	Polio Type 1	Adenovirus Type 7	Coxsackie A9	ECHO 28	Rhino 1059	Albumin	Alpha	Y-1A	Y-2
Cold	1A*	+	+	+	+	0	0	+	0	+	0	±	±
Cold	1B	+	+	+	+	0	0	+	0	+	0	±	+
Hay fever	2	ND	+	+	+	+	0	ND	ND	+	+	+	0
Allergic rhinitis	3	+	+	+	+	0	0	0	0	+	+	+	+
Pharyngitis	4	+	+	+	+	0	0	+	0	+	+	+	±
Bronchitis	5	0	+	+	+	0	+	0	+	±	+	+	0
Bronchitis	6	+	+	+	0	0	0	0	0	+	+	+	0
Bronchitis	7	+	+	+	+	+	0	+	+	+	+	+	0
Bronchitis	5**	0	+	+	+	0	+	0	ND	+	+	+	0
Bronchitis	6**	+	+	+	0	0	0	0	ND	+	+	+	0
Bronchitis	7**	+	+	+	+	+	0	±	ND	+	+	+	0

* Patient 1, same individual as normal pt. # 1 (Table I)
Specimen A taken 12/11/62
Specimen B taken 2/2/63

** Sputum

Preliminary characterization of some properties of the virus neutralizing substance in normal nasal secretions, such as resistance to heating at 56°C, freezing, dialysis and lyophilization and its destruction by boiling and trypsin digestion for one hour, suggested it to be a protein. For this reason immunoelectrophoresis experiments were performed. In Table 7 are listed the protein components found in normal nasal secretions. All specimens contained albumin, an alpha globulin and a beta globulin. One specimen (Subject #8) contained a trace amount of γ-2 globulin. γ-2 globulins were found in secretions taken from 4 patients with rhinitis, as indicated in Table IX. Albumin was found in all these specimens but the alpha globulin precipitin arc was absent from specimens 1A and 1B. Purulent nasal secretions from other patients with acute respiratory disease, not included in the tables because antiviral screening was not performed, routinely showed the γ-2 globulin arc, and often several beta globulins. The beta globulin of normal secretions was identified as a γ-1A by its typical immunoelectrophoretic migration and was confirmed by immunoprecipitation with a specific goat anti-human γ-1A antiserum.

Since normal nasal secretions contained γ-1A globulins predominantly and γ-2 globulins infrequently, it seemed likely that the antiviral substance in these secretions was related to the γ-1A globulin content. A number of experiments were performed in which the γ-1A globulin was removed by absorption of the secretions with specific anti γ-1A antiserum. Absorptions were performed by mixing equal volumes of secretion and antiserum, incubating 24-48 hours in the cold and removing the precipitate by centrifugation (2000 rpm x 20 minutes). For control, an aliquot of secretion was mixed with an equal volume of saline. Since the goat antiserum was toxic to rhesus kidney cell cultures, the neutralization tests were modified by washing the serum-virus mixture from each tissue culture tube after allowing one hour for free virus to adsorb to the monolayer.

Table X shows the results of several such absorption experiments. Four fold or greater reductions in antiviral antibody titer were noted against Coxsackie A9, Poliovirus type 1, Parainfluenza type 3 and Influenza $A_2/62$ in secretions containing little or no detectable γ-2 globulin. Immunoelectrophoretic examination revealed complete removal of the γ-1A globulins following absorption of these specimens. Nasal secretions from two subjects with acute respiratory disease, however, did not lose their anti influenza A_2 activity following removal of γ-1A globulin. Absorption of these secretions with anti γ-2 antiserum resulted in loss of this activity.

In one experiment, Coxsackie A9 antibody, titer 1:4, was removed from a normal nasal secretion by both γ-1A and γ-2 absorption. Immunoelectrophoresis of these absorbed specimens demonstrated a marked reduction in γ-1A globulin following γ-2 absorption, suggesting some cross reactivity of this antiserum.

h. Gel filtration and ultracentrifugation of serum. Following gel filtration with Sephadex G-200, serum is fractionated into three major protein peaks corresponding broadly with the 19S, 7S and 4.5S fractions obtained by ultracentrifugation. The γ-1M globulin is found in the 19S peak, the γ-2 globulin in the 7S peak, and the γ-1A globulin intermediate between the 19S and 7 S peaks The 4.5S peak is primarily albumin.

Table X Effect of γ-1A Globulin on Antiviral Activity of Nasal Secretions

Specimen	Virus Tested	Neutralizing Activity: Control	γ-1A Absorbed	γ-2 Absorbed
Normal NW (CP)	Cox A9	1:8	1:1	-
Normal NW (CP)	Polio 1	$\geq$1:16	1:4*	-
Normal NW (MSA)	Parainf 3	1:1-1:2	<1:1	-
Sputum (23)	Parainf 3	1:2	<1:1	-
Sputum (22)	Infl A_2/62	1:16	<1:1	-
ARD (-5)	Infl A_2/57	1:4	1:4-1:8	<1
ARD (#140)	Infl A_2/57	1:4-1:8	1:4-1:8	<1

* γ-1A antiserum had Polio 1 antibody at dilution of 1:4

As shown in Table XI, neutralizing antibodies to poliomyelitis type 1, Cox A9, and parainfluenza 3 viruses from sera of two normal individuals resided in the 19S and 7S γ-2 globulin regions and in the intermediate γ-1A region. Neutralizing antibody to influenza A_2 virus, on the other hand, was confined largely to the 7S region with some activity overlapping into the γ-1A region.

Table XI Viral Neutralizing Activity of Immunoglobulins of Human Serum Following Sephadex G-200 Gel Filtration

	Virus Tested	Immunoglobulin: 19S, γ-1M	γ-1A	7S, γ-2
Patient #4	Polio type 1	+	+	+
	Influenza A_2	0	+	+
	Parainfluenza 3	0	+	+
	Coxsackie A9	+	+	+
Patient #12	Polio type 1	+	+	+
	Influenza A_2	0	+	+
	Parainfluenza 3	+	+	+
	Polio type 3	0	+	+

The ultracentrifugal data supported the gel filtration studies. Neutralizing antibody to poliomyelitis type 1 virus was found in the 19S and 7S regions. Neutralizing antibody to influenza A_2 virus was found largely in the 7S fractions.

i. Gel Filtration and Ultracentrifugation of Nasal Secretions. The results of typical sephadex fractionation of nasal secretions from two normal individuals are shown in Table XII. The unfractionated specimen of #4 contained a neutralizing antibody titer of 1:32 to poliomyelitis type 1 virus. By immunoelectrophoresis with the horse anti-human serum, two lines were obtained, an α and a γ-1A globulin line.

Following fractionation and concentration into 9 pools, antibody activity to polio 1 virus was detectable in a titer of 1:16 in pool #4 and present in

Table XII Viral Neutralizing Activity of Sephadex G-200 Fractions of Normal Nasal Secretions

	Cumulative eluate vol. (ml)	Neutralizing Antibody	Protein Components
Whole Nasal Secretion #4	-	1:32 (Polio 1)	α, γ-1A
Pool # 1	18	0	0
2	38	0	0
3	64	0	0
4	86	16	γ-1A
5	106	1	0
6	112	0	0
7	138	0	α
8	152	0	0
9	170	0	0
MSA 3/64 Whole Nasal Secretion	-	1:4 (Polio 3)	alb, α, γ-1A, γ-2±
Pool #1	18	0	0
2	36	0	0
3	54	±	0
4	72	±	γ-1A±
5	90	+	γ-1A
6	108	+	γ-1A
7	126	+	alb±, γ-1A±, γ-2
8	144	+	alb, γ-2±
9	162	0	alb, α
10	180	0	0

the undiluted specimen in pool #5. Immunoelectrophoretic analysis confirmed the presence of the γ-1A globulin in pool #4. Pool #7, which contained the α globulin, had no neutralizing activity. Specimen MSA 3/64 was a normal secretion which contained γ-1A globulin and a trace amount of γ-2 globulin; this specimen neutralized Poliovirus type 3 at a dilution of 1:4. Antiviral activity was demonstrable in pools 3 through 6 which contained γ-1A globulin and pools 7 and 8 in which γ-2 globulin was detected.

Sephadex fractionation of nasal secretions obtained from 3 individuals with colds are shown in Table XIII. The untreated specimen contained a neutralizing antibody titer of 1:8 against poliomyelitis type 2 virus, and by immunoelectrophoresis the presence of albumin, α, γ-1A and γ-2 globulins was revealed.

Following fractionation and concentration into 7 pools, antibody activity to polio 2 was detectable in a titer of 1:2 in pool #4 which by immunoelectrophoresis showed the presence of the γ-1A line and an α line. Antibody activity was also present in pool #6 in the undiluted specimen and in pool #7 in a titer of 1:4. Both of these latter pools contained albumin and γ-2 globulin.

Specimen #137 showed a similar concentration of antibody (against Polio type 1 virus) and γ-1A and γ-2 globulins. Specimen ARD-1 which had a very low anti-influenza titer (1:2) had antibody in the sephadex pool #7 which contained γ-1A and γ-2 globulin.

j. Significance of Observations. The present findings suggest that the antiviral substance in normal nasal secretions is a γ-1A globulin, closely related or identical to serum γ-1A globulin. In some normal secretions γ-2 globulin may contribute to the antiviral act vity. During acute inflammatory conditions γ-2 globulin is almost always pre‹ent in nasal secretions and in this situation it appears to be the predominant antiviral antibody. The studies did not include assay for interferon-like substances which have recently been described in pharyngeal washings obtained early in the course of influenza.

The presence of several serum proteins in nasal secretions obtained during respiratory infection has been previously described by Anderson, Riff and Jackson. Their failure to detect γ-1A globulins in normal nasal secretions may have been due to their use of a less potent anti-human antiserum than that used in the current studies. Remington and Vosti have recently described the occurrence of albumin and B_2A (γ-1A) globulins in normal nasal secretions. In their studies, persons with a high serum titer of hemagglutinins (HA) for tetanus toxoid and streptococcal M protein were found to have HA activity in nasal secretions. These investigators found reaginic activity in nasal secretions of allergic individuals, a fact previously reported by Sampter and Becker.

The data obtained by gel filtration of human sera indicate that γ-1A globulins are associated with inhibitory activity against many different viruses. Neutralizing activity associated with γ-1M globulins was less commonly found and may have been related to the time interval following

Table XIII Viral Neutralizing Activity of Sephadex G-200 Fractions of Nasal Secretions Obtained from Patients with Acute Respiratory Disease

	Cumulative eluate vol. (ml)	Neutralizing Antibody	Protein Components
Whole Nasal Secretion #2	-	1:8(Polio 2)	alb, α, B, γ-1A, γ-2
Pool # 1	22	0	0
2	36	0	0
3	60	0	0
4	79	2	α, γ-1A
5	97	0	γ-1A
6	115	1	alb, γ-2
7	154	4	alb, γ-2
#137 NW 2/19	-	1:8(Polio 1)	
1	17	0	0
2	34	0	0
3	52	0	0
4	70	0	0
5	88	+	α, γ-1A
6	106	+	alb$\pm$, α, γ-1A
7	124	+	alb, α, γ-1A, γ-2
8	142	+	alb, γ-2
9	160	+	alb, 0
10	178	0	alb, 0
11	196	0	0
12	214	0	0
ARD-1		1:2(Flu A_2/57)	
1	13	-	0
2	30	0	0
3	47	0	0
4	64	0	0
5	80	0	0
6	96	0	γ-1A
7	113	+	γ-1A, γ-2$\pm$
8	130	0	alb, γ-1A, γ-2
9	147	0	alb, 0
10	164	0	0
11	180	0	0
12	196	0	0
13	212	0	0

viral infection. This fact is well known for bacterial agglutinins and has recently been documented for Poliovirus neutralizing antibody following oral

immunization. A number of investigators have studied the γ-1A globulins of serum and have demonstrated antibacterial, antiviral and skin sensitizing properties.

The mechanism by which the γ-1A globulins appear in normal respiratory secretions remains to be established. Local production of antibody may be responsible; evidence to prove this is not available, however. Moreover, the influenza immunization studies of Francis and Mulder in humans, and those of Fazekas de St. Groth in mice have shown a close correlation of serum antibody with antiviral activity of nasal or bronchial secretions.

Recently, the quantitative studies of Chodirker and Tomasi have demonstrated that γ-1A globulin is normally present in several body secretions in relatively greater concentration than in serum. They have postulated a selective process of concentration and transport of γ-1A globulins from the serum into these fluids. If this is indeed the case, the mucoantibody in normal respiratory secretions can be considered to be derived directly from humoral antibody. Thus, this mechanism of selective transport of γ-1A globulin may explain the clinical experiences in which immunity to respiratory illness is closely correlated with serum antibody.

5. Physico Chemical Properties of Humoral Antibodies to Arboviruses. The preceding annual report described preliminary observations on the physicochemical characterization of antibody produced in the guinea pig following experimental infection with Japanese encephalitis (JE) virus. These studies were undertaken to ascertain whether physicochemical differences might account for the temporal dissociation of neutralizing (N), complement-fixing (CF) and hemagglutination-inhibiting (HI) responses commonly seen following natural infection with this virus. This work has been continued and extended to include studies of experimental infection in the guinea pig with RSSE virus. In experiments with JE virus, guinea pigs were infected by each of four routes by single and multiple dosages. Details of route, dose, and number of inoculations are summarized in Table XIV.

Serum samples were obtained before inoculation and on days 3, 7, 10, 14, 21, 28 and 31. Tests for circulating virus were performed on samples of blood obtained on the 3rd and 7th day post-inoculation. Virus was detected in 29 of 30 guinea pigs on the 3rd day but in none of the 7th day bloods.

a. Effect of Route and Frequency of Inoculation on the Time of Appearance of Antibody. Antibody developed equally well in all of the animals infected by each of the four routes. However, antibody could be detected earlier in the group inoculated by the intracerebral (i.c.) route. Thus, in 10 of 14 animals infected by the i.c. route, CF and HI antibody were regularly found on the 7th day, whereas animals in the other three groups showed this antibody infrequently before the 10th day. Neutralizing antibody was produced with about the same frequency on day 7 regardless of route of inoculation.

b. Effect of Route and Frequency of Inoculation on Magnitude of the Antibody Response. No difference in the magnitude of the antibody response could be attributed to either route or to number of inoculations (Tables XV, XVI).

Table XIV Guinea Pig Antibody in Response to Infection with JE Virus

Group	Route, dose of virus (LD_{50}) and frequency of inoculation	No. of animals infected	No. of animals with viremia Day 3		No. tested	No. of animals responding with antibody Day 3	Day 7	Day 10	Day 14
1	i.c. $10^{7\pm}$ x 1 and x 3	7	7	CF	7	0	4	7	7
				HI	7	0	6	7	7
				N	6	0	5	-	6
2	i.p. $10^{8\pm}$ x 1 and x 3	8	7	CF	8	0	2	8	8
				HI	8	0	1	7	8
				N	7	0	5	-	7
3	i.d. $10^{6\pm}$ x 1 and x 3	7	7	CF	7	0	0	7	7
				HI	7	0	0	7	7
				N	7	0	5	-	7
4	s.c. $10^{7\pm}$ x 1 and x 3	8	8	CF	8	0	0	8	8
				HI	8	0	1	6	8
				N	8	0	2	8	8

Table XV Frequency Distribution of Maximal Antibody Titer

Group	CF Titer					HI Titer						N Titer				
	No. tested	64	128	256	512	No. tested	40	80	160	320	640	No. tested	125-250	250-625	625-1250	1250-3125
i.c.	7	1	2	3	1	7	1	3	1	1	1	6	2	4	0	0
i.p.	8	0	1	4	3	8	0	3	4	1	0	7	3	1	3	0
i.d.	7	0	2	3	2	7	3	3	1	0	0	7	4	1	1	1
s.c.	8	0	1	2	5	8	1	2	1	4	0	8	7	0	1	0

Table XVI Cumulative Frequency of Maximal Antibody Titer by Day

Group		No. tested	Day 3	7	10	14	21	28
i.c.	CF	7	0	0	2	3	7	7
	HI	7	0	0	2	3	7	7
	N	6	0	0	-	0	-	6
i.p.	CF	8	0	0	0	3	8	8
	HI	8	0	0	0	0	7	8
	N	7	0	0	-	0	-	7
i.d.	CF	7	0	0	0	2	7	7
	HI	7	0	0	0	2	7	7
	N	7	0	0	-	0	-	7
s.c.	CF	8	0	0	0	3	8	8
	HI	8	0	0	0	2	8	8
	N	8	0	0	-	1	-	7

The titers of antibody obtained were essentially similar and independent of dose and route. While it appeared superficially that route of inoculation influenced the titer, these were seldom more than 2-4 fold differences and probably of no real significance.

c. Characterization of Antibodies. Because of the relative homogeneity of response, only selected serum samples have been subjected to complete characterization. This analysis has consisted of sedimentation characteristics as determined by sucrose gradient centrifugation, gel filtration with sephadex G-200, chromatographic properties on DEAE cellulose, and zone electrophoresis using geon-pevikon; CF, HI and N antibody determinations were performed on the fractions. Sera selected were from animals immunized by the i.c. and i.p. schedules and were obtained at 7, 10, 13, 31 and 110 days following initial inoculation. Wherever possible more than one method was used on the same serum.

d. Complement-fixing antibody. The earliest CF antibody detectable either at 7 or 10 days was invariably of the 7S class because it was only detectable in the upper portion of the sucrose gradient (Table XVII), and in the second protein peak on gel filtration. In sera on days 14 or 31, CF activity continued to reside in the 7S region in the sucrose gradient (Table XVII). A 110 day post-inoculation serum studied by gel filtration showed CF activity within the second peak, and by DEAE cellulose chromatography within the first peak, the areas in which 7S gamma globulin resides.

Table XVII Results of Fractionation of Viral Antibodies Produced Following Infection of Guinea Pigs with JE Virus

Animal No.	Interval after initial injection (days)		Untreated serum titer	Sucrose density gradient titer* Tube No. 1	2	3	4	5	6	7	8	9	10
917	7	CF	8	-	-	-	-	-	2	4	-	-	-
		HI	10	-	U	2	-	-	-	U	-	-	-
		N	10	-	U	U	-	-	-	-	-	-	-
	14	CF	64	-	-	-	-	2	8	4	-	-	-
		HI	10	-	-	-	-	U	8	4	U	-	-
		N	10	-	U	U	-	-	4	2	-	-	-
	31	CF	128	-	-	-	-	2	16	8	-	-	-
		HI	40	-	-	-	-	-	4	32	8	U	-
		N	250	-	-	-	-	≥8	≥8	≥8	4	U	-
902	7	HI	10	-	-	U	-	-	U	-	-	-	-
	10	CF	64	-	-	-	-	-	8	2	-	-	-
		HI	20	-	U	U	-	U	2	U	U	-	-
		N	35	-	-	U	2	-	U	-	-	-	-
	31	CF	32	-	-	-	-	-	4	-	-	-	-
		HI	20	-	-	-	-	-	4	U	U	-	-
		N	250	-	-	-	-	-	4	≥8	U	-	-
923	7	CF	8	-	-	-	-	-	4	U	-	-	-
		HI	10	-	U	2	-	-	-	U	-	-	-
		N	15	-	-	U	-	-	-	-	-	-	-
	10	CF	128	-	-	-	-	16	16	-	-	-	-
		HI	40	-	4	16	2	2	16	16	U	-	-
		N		-	4	4	-	2	4	2	-	-	-
	31	CF	512	-	-	-	-	16	32	16	-	-	-
		HI	320	-	-	-	U	8	32	32	16	2	-
		N	800	-	-	-	2	≥8	≥8	≥8	2	U	-

* U = undiluted. Nos. refer to titer.

e. Hemagglutination-inhibition Antibody. The earliest detectable HI antibody at 7 days consisted of both 19S and 7S antibody. Activity was found predominantly in the lower region of the sucrose gradient but also some in the upper portion. By 10 days the bulk of activity was found in the upper portion of the gradient with very little remaining in the 19S region. By 14 days all HI activity was of the 7S type. By gel filtration of a 10 day serum, HI activity was found in two regions, a small amount of activity in the first peak, where 19S gamma globulins are found, and the bulk of activity in the second peak, where the 7S are located. Gel filtration and DEAE cellulose analysis of the 110 day serum again showed the HI activity to reside in the 7S region.

f. Neutralizing Antibody. The earliest detectable N antibody was solely of the 19S variety. It was found in the lower region of the sucrose gradient on the 7 day serum. By 10 days 7S activity was noted along with 19S. Even as late as 14 days some 19S activity was observed. The gel filtration studies on the 10 day serum revealed antibody in the 19S and 7S regions. Gel filtration of the 110 day serum showed all neutralizing activity to be of the 7S type. DEAE chromatography of the 110 day serum revealed most of the antibody activity to be in the 7S region but also some activity extended into other regions. Thus it appears that the earliest HI and N antibody produced in the guinea pig to infection with JE virus is made up entirely or in large measure of a 19S gamma globulin. This appearance of 7S activity is seen by the 14th day in the case of the HI response and somewhat later in the case of N response. In contrast, the earliest CF antibody is made up exclusively of 7S gamma globulin and continues to remain so in later bleedings.

Parallel experiments performed in guinea pigs infected with RSSE virus have given similar results as these in the JE infected animals. Studies are now in progress to determine the passive-protecting value of various globulins isolated from various antisera.

6. Evaluation of Attenuated Dengue Virus Vaccine (Type 1).

a. Studies of attenuated dengue virus, type 1, in volunteer troops of the XVIII Airborne Corps and the Special Warfare Center, Fort Bragg, in 1963 established the following:

(1) The strain (MD-1) could be administered to susceptible troops over a wide dose range (1-10,000 mouse IC LD_{50}) without inducing morbidity of any consequence. In 2 different experiments utilizing approximately 200 men, no time lost from duty was observed in a 3 week post immunization interval. Oral temperatures of over 100°F were rarely observed, and were of less than 24 hours duration. Mild, morbilliform eruptions on the face, neck, upper trunk, and rarely on the arms was seen in 13% of those inoculated. It persisted 2-4 days; its occurrance was unrelated to the dose of attenuated virus administered.

(2) Evaluation of the neutralizing antibody status of vaccinees to Types 1 and 2 dengue virus 30 days after inoculation showed the response to be monotypic (to the type 1 vaccine virus). Further, it was shown in both experiments that 1 mouse infective dose was equivalent to 1 human infective dose.

(3) Limited studies in human volunteers showed that 60 days post immunization, persons were solidly resistant to challenge with 100 human infective doses of fully virulent type 1 dengue virus.

b. Recognition of epidemic dengue in Puerto Rico during late August of 1963 provided an opportunity to evaluate this attenuated dengue virus vaccine in the face of spreading disease. Field evaluation of dengue virus vaccine was not anticipated before the opportunity arose in Puerto Rico; therefore the standard controlled experiment was adapted to local circumstances as knowledge of the nature, occurrence and distribution of disease became apparent. The basic problem was to enumerate the occurrence of dengue-like illness in immunized persons and in the preselected control population. While this problem did not change during the interval, the methods for identifying diseased subjects were modified to meet the specific administrative problems presented by the various study groups.

c. Dengue Virus Vaccine. The vaccine was mouse adapted dengue type 1 virus in 33rd passage, derived by Dr. C. L. Wisseman Jr. through limiting dilution passage of virus originally evaluated for virulence in human volunteers by Dr. Albert B. Sabin. A single lot of vaccine virus was prepared. It was used for earlier evaluations in Army volunteers and in the current field test of effectiveness against epidemic disease. Based upon earlier observations in volunteers, the dose for the present study was adjusted to contain an estimated 100 human infective doses of virus per 0.5 ml. The diluent for the vaccine was sucrose phosphate-glutamate solution containing 2.5% human albumin. The same material without virus was administered to control subjects. Both vaccine and placebo were administered subcutaneously by jet injector developed by the Medical Department of the U. S. Army.

d. Subjects. Male students of the 10th, 11th and 12th grades were obtained from schools in Fajardo, Naguabo, Humacao and Yabucoa. Male civilian employees of Roosevelt Roads Naval Station and certain employees of Humacao were also included in the study. Alternate individuals identified by number received vaccine; the others, a placebo injection. The numbers of individuals in the study at the commencement and termination, and the dates of immunization are summarized in Table XVIII.

e. Plan for Follow-up. The reliability of the study depended upon prompt and accurate identification of dengue-like illnesses in the subjects. This was not always easy to do, and in the attempt to obtain the best follow-up procedure, the following methods were evolved. Initially for the student groups, the identification of illnesses depended upon reported absences from classes. This method was reliable for the duration of the study for Colegio San Antonio and Naguabo High School. However, in order to obtain a similar efficiency for other schools, it was necessary to supplement routine attendance reports by a daily count. This was done initially by homeroom teachers, and later by students solicited for this purpose. For the adult groups at Roosevelt Roads and Humacao, payroll records identified absences for illness. Three times during the study period the student subjects of the Fajardo, Humacao and Yabucoa schools were required to summarize histories of recent illness on a questionnaire.

Table XVIII Composition of the Study Group
Dengue Vaccine Evaluation, Puerto Rico, 1963-1964

Dates Immunized 1963	Study Group	Number Persons				Bled at outset study
		Immunized		Controls		
		Original	Final	Original	Final	
9/13	Fajardo High School	91	87	90	84	19
10/11	Naguabo High School	41	40	42	41	14
9/18	Humacao High School	168	157	162	155	58
9/27	Yabucoa High School	76	71	73	72	29
10/1	Colegio San Antonio	47	45	47	46	14
9/16	Civil Servants, Roosevelt Roads	98	94	97	87	38
10/17	Civil Servants, Humacao	40	34	41	36	37
	Totals	561	528	552	521	209

This was a check list of symptoms associated with febrile infectious disease and included those cardinal to dengue (see below). Individuals identified as ill, either by absence or through questionnaire, were interviewed by at least one physician. The interview and examination were done as often as possible at home for those persons identified as ill by absence; individuals recording symptoms suggestive of dengue on questionnaires were interviewed at work or in school. These interviews were standardized by the examining physicians and were based upon accumulating experience with the local disease pattern. They attained their final form for all study groups by mid-November, and at that time those interviewed earlier were re-interviewed. All interviews were summarized on a standard individual record form. At the termination of the investigations all individuals in the study group were interviewed for history of illness.

f. Diagnosis. Diagnoses of recorded illnesses were made upon completion of the study by review of individual records by at least four of the seven regularly interviewing physicians. Three categories of illness were recognized: "Dengue", "possible dengue" and "other illness". (Table XIX) The last category was used to classify illness which did not meet the criteria outlined in the table for dengue or possible dengue.

g. Etiologic Investigations. Because the specific etiology of the epidemic was unknown at the outset of these studies, the plan included efforts to recover and identify viruses, and to measure potential serologic responses of persons in the study group. However, it was not regularly possible to obtain clinical specimens from them during the first day of illness. Accordingly, to identify agents being transmitted in the various communities, other persons with dengue in the first day were investigated virologically. This phase of the investigation is incomplete. Serological responses in the study group will be made to both prototype and newly recovered viruses using sera obtained upon termination of the field study.

Table XIX Signs and Symptoms Used for Clinical Diagnosis of Dengue Puerto Rico, 1963-1964

1. Subjective fever and/or chills.
2. Headache.
3. Muscle, bone or joint pain.
4. Eye pain.
5. Macular or maculopapular eruption, with or without petechiae.
6. Anorrhexia.
7. Nausea and/or vomiting.
8. Weakness and/or dizziness.
9. Alteration of taste.
10. Biphasic pattern of illness.
11. Lymphadenopathy (objective).

For a diagnosis of "Dengue", a patient was required to have 4 of the first 5 signs or symptoms, or 3 of the 5 plus one other.

For a diagnosis of "possible dengue", 2 of the first 5.

h. The occurrence of disease considered to be dengue in the immunized and control groups is summarized by study groups in Table XX. There was no significant difference in the occurrence of disease in vaccinated and control groups during the first 2 weeks following inoculation. However, after the second week less dengue was recognized in the immunized than the control groups, the overall ratio being 1:2.1. On the other hand there was no significant difference between vaccinees and controls for the diagnosis of possible dengue. The occurrence of illnesses other than dengue, whether determined after absence from school and work or in the final interview was similar in controls (121) and vaccinees (113).

i. Evaluation of Data. Data obtained in any field trial of vaccine effectiveness is difficult to evaluate. In the present study these difficulties were enhanced by the variability of clinical expression of dengue, and the number of specific viruses that can cause dengue-like illness. Thus, during the epidemic in Puerto Rico, the complete clinical picture as previously described was not seen with regularity. Prolonged prostration and convalescence were rarely observed. Rash was not a prominent symptom. It was fleeting and highly variable in its distribution and hemorrhagic manifestations rarely occurred. Indeed, the disease and the reaction of the patients to it was less severe than that anticipated from earlier descriptions. This made definitive clinical diagnosis difficult and was the reason that the specific criteria for diagnosis in this epidemic were established.

Subsequent laboratory studies have shown the virus responsible for epidemic dengue in Puerto Rico to be dengue virus, type 3 (see section 7. following). Preliminary analysis of attack rates of subjects based upon site of residence suggests that the protective ratio of 2:1 shown for the study as a whole may be

Table XX Effect of Immunization upon the Incidence of Clinical Dengue

Study Group	Dengue				Possible Dengue			
	No. Cases Observed Post Immunization				No. Cases Observed Post Immunization			
	1st & 2nd weeks[1]		3rd - 18th week		1st & 2nd weeks[2]		3rd - 18th week	
	Vac.	Cont.	Vac.	Cont.	Vac.	Cont.	Vac.	Cont.
Fajardo High School	7	5	13	39	1	3	9	7
Naguabo High School	1	2	4	9	1	1	3	1
Humacao High School	12	8	18	26	8	6	8	10
Yabucoa High School	7	10	8	15	5	4	3	5
Colegio San Antonio	3	1	2	6	1	0	1	1
Civil Servants, Roosevelt Roads	9	1	13	24	0	1	3	4
Civil Servants, Humacao	5	4	4	7	1	0	0	2
	44	31	62	126	17	15	27	30

[1] Includes 14 persons with history of dengue prior to immunization.

[2] Includes 6 persons with possible dengue prior to immunization.

even greater. When attack rates among students residing in urban areas are compared with those in suburban or rural areas, it is seen that 68% of the cases occurred in 36% of the studied students, and in this group, the protective ratio was 3 to 1. Further, in a sample of approximately 150 students studied serologically thus far, it appears as though the incidence of HI antibody responses of vaccinees and controls to type 3 virus is similar; the attack rates being approximately 50%. It can be concluded that the test vaccine reduced significantly the occurrence of clinical dengue; it did not apparently prevent infection, however, since the antibody conversions were similar in both vaccinees and controls. Thus the vaccine effect seems to be the result of conversion of potentially overt to subclinical infections.

7. Recovery of Dengue Viruses Using the Cell Culture Interference Test.

a. Initial efforts to recover dengue viruses from acute phase blood specimens obtained from patients in the Puerto Rico epidemic (see section 6.) were made in Puerto Rico using the newborn mouse as the laboratory host. After clinical materials from 52 patients had been so inoculated without obtaining transmissible agents, efforts were redirected to use of other methods for attempts at isolation of dengue viruses. For this purpose primary emphasis was placed upon evaluation of a cell culture interference test similar to that used for the recovery and identification of rubella virus (see section 3, this report). Halstead (SEATO Laboratory, Bangkok) had adapted this technique for propagation of dengue viruses having shown that continuous African green monkey kidney cells (BSC-1) were resistant to superinfection with poliovirus II 8-14 days following inoculation with mouse adapted virus. Confirmation of this fact was obtained in this laboratory, and techniques were modified to provide a useful system for the rapid recovery and identification of dengue viruses from field collected material.

b. The principal modification was the substitution of primary vervet kidney (GMK) monolayers for the continuous line of cells. GMK cultures propagated with 5-10% calf serum in Eagles basic medium (BME) were maintained with BME containing 2% chicken serum, and appropriate antibiotics. Cultures inoculated with materials suspected of containing dengue viruses were incubated at 36°C for 9-13 days prior to challenge with 1000 TCD_{50} of poliovirus II. Cultures containing dengue viruses were uniformly resistant to infection. Media over infected cultures required replacement every 2-3 days of incubation.

c. Seventeen of the first 29 acute phase blood specimens obtained in Puerto Rico have yielded transmissible interfering agents; 9 have been identified conclusively as dengue virus type 3, using hyperimmune mouse ascitic fluid (see section 8, this report, for the preparation of these reagents). The remaining strains from Puerto Rico, and at least one other from the Caribbean, possess properties similar to the identified viruses. The cell culture system is presently being standardized for measurement of neutralizing antibodies to the dengue viruses, and for the recovery of other arboviruses not currently known to produce cytopathic effects in cell cultures.

8. Preparation of Hyper Immune Ascitic Fluids in Albino Mice. A practical and reproducible procedure for stimulation of antibody bearing ascitic fluid in mice has been developed. By this procedure, serologic reagents can be prepared which are highly specific, and without tissue reacting components. The method

involves sensitizing the peritoneal cavity with a small amount of Freund's complete adjuvant at the time immunization is commenced. Later, after the animal is immune, introduction of additional vaccine adjuvant mixtures results in the continued production of ascitic fluid containing high titered antibody.

a. The ideal schedule for production of ascitic fluid is summarized in Table XXI. By 7 to 10 days after the last intraperitoneal injection mice have accumulated 7-10cc of ascitic fluid, and continue to accumulate fluid after abdominal paracentesis is performed, and may be tapped every seven to ten days thereafter. The yields from individual mice vary as shown in Table XXII, but the total quantity of fluid after removal of clot is ten times larger than comparable yields of serum. This value may be increased

Table XXI Schedule for Immunization of Mice with Freund's Complete Adjuvant and Mouse Brain Vaccine

Day	Vaccine		Emulsion of equal parts of vaccine and adjuvant		Adjuvant	
	Route	Volume	Route	Volume	Route	Volume
1	SC-IM	0.4-0.1			IP	0.25
3	SC-IM	0.4-0.1				
24	SC-IM	0.4-0.1				
27			IP	0.5		
30			IP	0.5		

Table XXII Yields of Ascitic Fluid in ml from Individual Mice Immunized with Dengue III Virus

7.8	20.0	12.5	7.6	4.2	
16.2	2.7	12.2	6.0	5.0	Total = 142.8
8.2	5.2	0.2	11.0	5.0	After
3.4	4.2	7.5	0.4	3.5	clot = 100 ml

with additional tappings at weekly intervals. Female mice approximately four months of age or in excess of 30 grams are most desirable for this type of work. Younger mice have a tendency to form abdominal adhesions or "wooden" abdomens rather than fluid. Fluid is allowed to clot at room temperature, stored overnight at 4°C, and centrifuged at 1000 X G for 20 minutes at 4°C. Centrifugation at higher speeds or at room temperature may cause the gel-clot to dissipate, with the result that clots re-form in the storage bottles. While centrifugation tends to condense the clot, it may extend up through the fluid to the surface rather than settle to the bottom. It is most convenient to block the movement of the clot with several pipettes while decanting the

fluid into another centrifuge tube for further centrifugation if desired.

b. CF antibodies in ascitic fluid obtained from the first paracentesis usually titered a 2-fold dilution lower by CF than the material obtained from the second and third taps performed at 7 to 10 day intervals (Table XXIII). It was found that these latter taps were performed during the peak of the secondary response when material taken from a group of mice every few days formed an antibody response curve that contained a plateau of approximately two weeks in length before the titers declined. Two or three taps during this interval provides adequate quantities of high-titering reagent.

Table XXIII Complement-fixation Titers of Various Arbovirus Immune Ascitic Fluids Harvested on Three Different Occasions from Each Group

Ascitic Fluid	First Tap	Second Tap	Third Tap
Anopheles A	512	1024	1024
Naples	512	1024	1024
Sicilian	256	1024	512
Dengue I	256	512	512
Dengue II	256	512	512
Dengue III	256	512	512
Dengue IV	128	512	256
Dengue V	512	2048	1024
M 291	1024	-	-
M 400	128	256	128
M 303	256	512	256
M 515	256	256	256
M 517	128	256	256
M 273	256	256	512

Summary and Conclusions:

1. A variant A_2 influenza virus was found to be responsible for epidemic disease in eastern United States during early 1963. The variant differs from classical A_2 virus in the absence of one or more antigens, and can be readily distinguished from the prototype by antigenic analysis. This distinction is best made by cell culture neutralization tests. Investigations of the immune status of recruits, summer 1963, showed that in areas where disease rates were high, incoming recruits possessed more antibody, more frequently than those entering military service from non-epidemic areas. The significance of these findings is discussed.

2. Intensive study of alveolar gas exchange in military recruits with mild to severe respiratory infections showed that diffusion of CO was impaired only in persons with obvious extensive pulmonary disease. No significant changes were observed in less severe infections.

3. Continued investigations of the biological and epidemiological properties of rubella virus have been made using the cell culture-interference test for its detection. These studies confirm the unique nature of rubella virus, its epidemic dissemination among young adults, and establish the difficulties likely to be encountered in any searches for effective immunizing agents.

4. Active antiviral substances have been demonstrated and identified in nasal secretions from normal and diseased persons. In the normal, they appear to be γ-1A globulins; following infection of the mucous membrane, classical γ-2 globulins appear. Both substances appear to be specific for various viruses, and each apparently plays a different role at the mucous membrane surface in the modification of respiratory infection. The significance of the findings is discussed.

5. The CF, HI and neutralizing antibodies produced by guinea pigs following experimental infection with Japanese encephalitis virus have been found to possess different biophysical properties. These differences are shown to correlate well with the time of synthesis of specific immunoglobulins following infection.

6. A field investigation of an attenuated dengue virus (type I) vaccine was made in volunteer students during the 1963 epidemic. The controlled study showed a specific reduction of type III disease by 50 to 67%.

7. A new, more sensitive method for recovery of dengue virus is described. The technique, adapted from that used for recovery of rubella virus, employs a cell culture interference system, and another animal virus. The application of the method to identify the etiology of epidemic dengue in Puerto Rico is presented.

8. An improved method for producing antibodies in high titer in large volume is described. It resulted from studies which showed that ascitic fluids of immune mice contain antibodies, and which established the immunization schedule producing the greatest volume of ascitic fluids.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-4(R2)

ACCESSION NUMBER 36181

PROJECT, TASK, OR SUBTASK NO. 3A012501A8180122

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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49

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576-2146 or Interdepartmental Code 198, Ext 2146 See Continuation Sheet 49

6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Rickettsial infections (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME' (U)Complement fixation tests employing purified suspensions of the Gilliam, Karp and Kato strains of R. tsutsugamushi and immune guinea pig sera have confirmed the antigenic individuality of these strains. Cases of scrub typhus from Malaya, where a multiplicity of heterogenetic strains are known to exist, can be diagnosed serologically with these three complement-fixing antigens or with a group-reactive antigenic fraction recovered from the Gilliam strain. The potential use of the fraction as a vaccine is being evaluated. Studies to detect rickettsial antigens in the urine during the acute phase of disease in man and animals using a red cell agglutination-inhibition test have shown that antigens are not excreted regularly in the urine in sufficient concentration for this serological system to be of value in early diagnosis. Coincident quantitative data have been collected on the dynamics and interaction among the tick vectors, vertebrate hosts and R. rickettsii in a natural environment. Cotton rats were experimentally infected to assess their role as a vertebrate reservoir of R. rickettsii. A transmissible agent possibly belonging to the psitticosis-lymphogranuloma venereum group was isolated from a wild cottontail rabbit.

9. KEY WORDS
Rickettsia, scrub typhus, antibody, Rocky Mountain spotted fever, ecology.

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

DA FORM 1 June 63 1309R PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____

ACCESSION NO. 38181	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	3A012501A818 / 01 / 22
14. DATE OF REPORT (30-33)	0664
15. SECURITY OF WORK (34)	4
16. TYPE OF REPORT	3 / 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	010608
18. OSD CLASSIFICATION (65-66)	AR
19. R&D CATEGORY (67)	1

Card "D"

20. CONTRACT NUMBER	DA
21. GRANT NUMBER	DA / G
22. ESTIMATED COMPLET. DATES	1CONT / 2 / 3 / 4 / 5

Card "E"

23. PRIORITY (11-14)	1
24. PROGRAM ELEMENT (15-26)	6.11.25.01.1
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27. FUNDING

a. Est. Total Cost (11-15)	
b. % Spent Intern. (16-18)	1
" " Extern. (19-21)	2
c. Total Obligation (22-26)	
d. Progmd. Cur. FY (27-33)	
e. " " " +1 (34-40)	
f. " " " +2 (41-47)	
g. " " " +3 (48-54)	
h. " " " +4 (55-61)	
i. " " " +5 (62-68)	
j. " " " +6 (69-75)	
k. Total Man Years of Effort (76-78)	

DA FORM 1 June 63 1309R — Previous Editions are Obsolete — Page 2 of ____

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REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. 3A012501A818 Title: COMMUNICABLE DISEASES AND IMMUNOLOGY

Task No. 01 Title: Communicable Diseases

Subtask No. 22 Title: Rickettsial infections

Description:

Investigations were undertaken (1) to characterize the complement-fixing reactivity of whole rickettsial antigens and a group-reactive fraction prepared from Rickettsia tsutsugamushi; (2) to detect rickettsial antigens in the urine during the acute phase of disease in man and animals as a possible means of early diagnosis; (3) to collect coincident quantitative data on the vector ticks, vertebrate hosts, and Rickettsia rickettsii in order to assess the relative importance of the different factors responsible for the perpetuation of an endemic focus of Rocky Mountain spotted fever and to recognize the conditions associated with a high risk of occurrence of disease in man; (4) to study the course of experimental infection of cotton rats with Rickettsia rickettsii in order to evaluate its importance as a vertebrate reservoir; (5) to identify a transmissible agent isolated from a wild cottontail rabbit; and (6) to establish a diagnosis of Rocky Mountain spotted fever for suspect cases submitted from military and civilian hospitals.

Progress:

1. Complement-fixing Reactivity of Antigens Prepared from Rickettsia tsutsugamushi.

Suspensions of the Gilliam, Karp and Kato strains of Rickettsia tsutsugamushi were partially purified as described previously utilizing a cation exchange resin, Amberlite XE-97, and 1% bovine serum albumin in a series of low and high speed centrifugations. The rickettsial suspensions, relatively free of yolk sac tissue, were highly infectious for mice and evoked high levels of antibody in guinea pigs, mice and rabbits When used as complement-fixing antigens they were far superior to ether-extracted soluble antigens. The Gilliam, Karp and Kato strains have been employed by the Japanese workers as representative prototypes of three antigenic categories into which it has been possible to classify strains of scrub typhus rickettsiae recovered in Japan. Furthermore, the Japanese investigators report that these purified antigens can be used for the serological diagnosis of scrub typhus and the detection of past or inapparent infections.

In order to evaluate the antigenic dissimilarity of the three strains under study, grid-type complement fixation tests were carried out in which the purified suspensions were tested against homologous and heterologous immune guinea pig sera. The results of a typical test are presented in Table I.

TABLE I

COMPLEMENT-FIXING REACTIVITY OF THREE STRAINS OF R. TSUTSUGAMUSHI AGAINST GUINEA PIG ANTISERA

ANTIGENS	GUINEA PIG ANTISERA		
Purified Suspensions	Gilliam	Karp	Kato
Gilliam	40 * / 2560	10 / 40	10 / 80
Karp	<10 / <40	20 / 2560	10 / 160
Kato	<10 / <40	10 / 80	20 / 2560

* Number above the line indicates the reciprocal of the highest dilution of antigen fixing complement in grid-type complement fixation tests. The value below the line is the reciprocal of the highest dilution of serum.

The purified antigens exhibited a high degree of strain specific reactivity. The titer of antibodies detected by the homologous antigens was at least 16-fold greater than with the heterologous antigens. The results suggest that the Gilliam strain contains minor antigenic components in common with the Karp and Kato strains. Although Karp and Kato suspensions usually showed a certain amount of reciprocal reactivity, they did not fix complement in the presence of Gilliam immune guinea pig sera. Occasionally, Karp antigens, prepared and tested the same day showed only homologous reactivity. The degree of heterologous reactivity varied somewhat in different preparations of the three antigens and was observed to increase after repeated freezing and thawing, or prolonged storage at 4°C.

The usefulness of the purified complement-fixing antigens for the serological diagnosis of human R. tsutsugamushi infections was evaluated. Serial serum specimens were available from patients who were experimentally infected with either the Karp or the Gilliam strain in the course of vaccine-chemoprophylaxis trials in Malaya or as a result of known accidental exposure to these agents in the laboratory. High titers of complement-fixing antibodies which persisted over a year were detected with antigens corresponding to the infecting strain. Antibodies to heterologous strains were present at lower levels for a period of only one or two months after infection.

In addition, acute and convalescent sera from scrub typhus patients naturally infected in Malaya, an area where a multiplicity of strains is known to exist, were tested in complement fixation with purified suspensions of Gilliam, Karp and Kato. The reciprocal of the highest dilution of serum showing significant fixation of complement is given in Table II. Only 4 of the patients had developed OXK agglutinins, and none of the sera at a 1:10 dilution fixed complement in the presence of ether-extracted soluble antigens prepared from Gilliam- and Karp-infected yolk sacs. Diagnostic rises in antibody were detected with one or more of the purified antigens in 12 of the 13 cases. ST-14, the last patient shown, already had a high titer by the ninth day, when the first serum was drawn. Six of the cases showed a 4-fold or greater rise with all three antigens, 4 patients had 4-fold rises with two strains, and in the remaining 2 cases, a diagnostic rise was demonstrated only with the Gilliam strain. In the late specimens from 6 of the 13 cases, the antibody titers detected with one of the strains of R. tsutsugamushi were at least 4-fold greater than with the other antigens. Predominant serological responses in 4 patients were to Gilliam, in 1 patient to Karp, and in another to Kato. The levels of antibody in the convalescent serum of the other 7 patients were not sufficiently distinctive to permit this differentiation and these responses have been designated polytypic

One of the objectives of this investigation was to obtain a group-reactive antigen from R. tsutsugamushi; i.e., a fraction common to strains with dissimilar antigenic composition Disruption of the organisms with chemicals, such as ether or sodium desoxycholate proved to be of little value since there was concurrent destruction of complement-fixing antigen. Mechanical disruption by ultrasonic vibration was more effective. The complement-fixing reactivity of purified suspensions of the three strains before and after sonication is depicted in Figure 1. After 3 minutes sonic vibration, the Gilliam preparation had broader reactivity with Karp and Kato antisera than the intact organisms. On the other hand, sonication of Karp and Kato suspensions for periods up to 10 minutes failed to yield an antigen capable of reacting with the Gilliam antiserum. There was, however, some increase in the reciprocal activity between the Karp and Kato antigens and antisera Separation of the fraction responsible for the group-reactivity from the sonicated Gilliam suspensions was not accomplished by high speed centrifugation; a portion of the group antigen was sedimented with the remaining intact organisms When continuous sucrose density gradients were used, the group-reactive fraction was recovered in the uppermost less dense portion of the gradient. Intact organisms which fixed complement only in the presence of Gilliam immune serum formed an opaque band in the lower more dense sucrose solution.

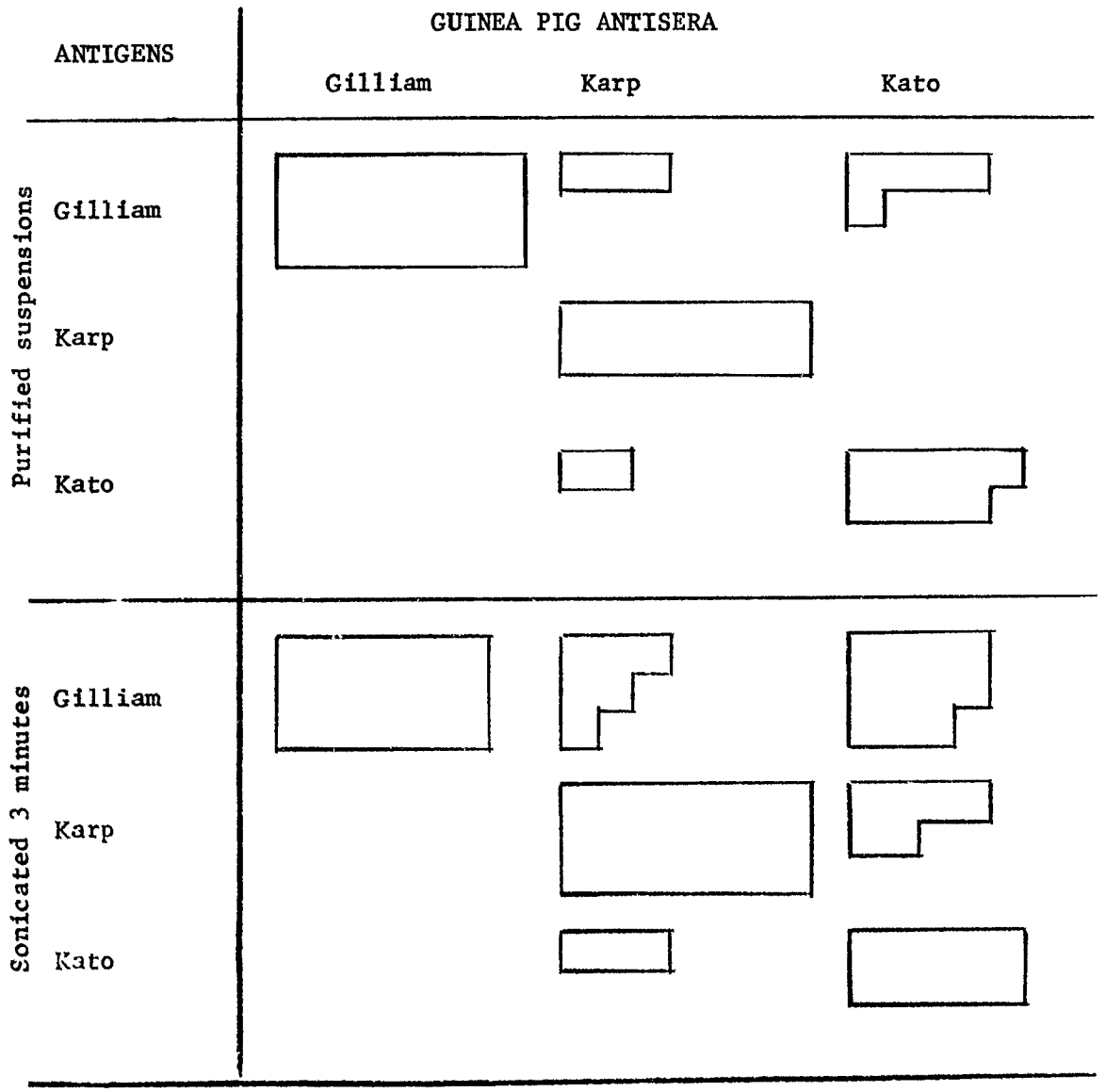
TABLE II

RESULTS OF COMPLEMENT FIXATION TESTS ON SCRUB TYPHUS PATIENTS

Patient	Day of disease	Antibody titer against purified suspensions			Predominant serologic response
		Gilliam	Karp	Kato	
ST-1	1	10	<10	<10	Polytypic
	31	80	160	40	
ST-2	3	160	40	40	Gilliam
	15	640	160	160	
ST-3	3	20	20	20	Polytypic
	13	160	160	160	
ST-4	3	20	20	20	Gilliam
	26	320	40	80	
ST-5	5	<10	<10	<10	Polytypic
	20	160	<20	80	
ST-6	5	40	20	20	Polytypic
	12	160	40	80	
ST-7	5	20	20	20	Karp
	40	40	320	80	
ST-8	6	<10	<10	10	Polytypic
	21	640	1280	≥1280	
ST-9	7	10	10	10	Polytypic
	26	80	80	80	
ST-10	4	10	10	10	Kato
	78	40	40	640	
ST-11	5	10	10	20	Polytypic
	11	40	20	20	
ST-12	6	20	20	20	Gilliam
	12	320	40	40	
ST-14	9	≥1280	80	80	Gilliam
	22	2560	40	80	

FIGURE 1

COMPLEMENT-FIXING REACTIVITY OF THREE STRAINS OF R. TSUTSUGAMUSHI BEFORE AND AFTER SONICATION*



* Blocks enclose limits of significant fixation of complement.

The group-reactive fraction is currently being evaluated for its suitability as a diagnostic antigen. A comparison of the results of complement fixation tests on the acute and convalescent sera from 6 scrub typhus patients employing the group-reactive antigen and the intact Gilliam organisms is presented in Table III.

TABLE III

COMPARISON OF COMPLEMENT-FIXING ANTIBODY TITERS OF SCRUB TYPHUS PATIENTS' SERA USING AS ANTIGENS THE GROUP-REACTIVE FRACTION OBTAINED FROM GILLIAM RICKETTSIAE AND THE UNTREATED PURIFIED GILLIAM SUSPENSION

Patient	Day of disease	Antibody titer with Purified Gilliam suspension	Antibody titer with Group-reactive fraction
ST-1	1	10	10
	31	80	80
ST-3	3	20	40
	13	160	320
ST-4	3	20	20
	26	320	320
ST-7	5	20	20
	40	40	160
ST-8	6	<10	20
	21	640	320
ST-11	5	10	20
	11	40	80

Diagnostic rises were shown in 5 cases with the intact Gilliam suspension. In ST-7 only a 2-fold rise in titer was found in the convalescent serum. In contrast, all 6 paired sera demonstrated 4-fold or greater increases in antibody against the group-reactive antigen prepared from the Gilliam strain.

2. Urinary Excretion of Rickettsial Antigens.

During the past year, studies of the urinary excretion of rickettsial antigens in guinea pigs have been extended and urine from patients infected with R. rickettsii and R. tsutsugamushi have been similarly examined. A hemagglutination-inhibition technique, described previously (Annual Progress Report, WRAIR, July 62-June 63, pp228-229) was employed for antigen detection.

Guinea pig studies.

Scrub typhus: Antigen related to the OXK lipopolysaccharide of Proteus mirabilis could not be demonstrated in 24 hour unconcentrated urine specimens from 8 guinea pigs infected with the Gilliam strain of R. tsutsugamushi.

Spotted fever: When either the Proteus OX19 or spotted fever group ESS test systems were employed to study 24 hour unconcentrated urine specimens from 8 R. sibericus-infected guinea pigs, much inhibitory activity in preinoculation control urines was observed. Therefore, contrary to preliminary findings reported last year, it was impossible to distinguish specific from nonspecific inhibitory activity in postinoculation urines. Efforts to circumvent this difficulty failed.

Murine typhus: Nonspecific (preinoculation) inhibitory activity was not encountered when urine was examined for typhus group ESS antigen except very rarely. Postinoculation inhibitory activity was found in 21 of 98 unconcentrated urine specimens collected from 6 guinea pigs during the first two weeks of infection. However, the presence of this activity could be confirmed upon repeat testing in only 4 specimens. Twenty-four hour urine specimens from 3 additional guinea pigs, collected during the first week after inoculation, were concentrated tenfold. Inhibitory activity was found in only 2 specimens from the same animal.

All animals reacted clinically by the third postinoculation day following inoculation of rickettsiae in a manner consistent with multiplication of each agent. As expected, none developed measurable serum agglutinins against the antigens being sought in the urine. It is concluded that rickettsial antigens, for which satisfactory sensitive test systems are available, were excreted either not at all, or in quantities too small to be detected in unconcentrated or tenfold concentrated urine.

Human rickettsial diseases.

Spotted fever: Twenty-six morning urine specimens, between 50 and 200 ml in volume, collected from seven patients with proven infection with R. rickettsii, were available for study. Unfortunately, only 6 specimens 5 from one patient, were collected prior to the 9th day of disease, and significant agglutinin titers to one or more of the OX19, OX2 and spotted fever ESS antigens were present in the serum at the time urine was collected in four of the seven patients. Most urine specimens were concentrated tenfold. None contained inhibitory activity in HAI systems designed to test for Proteus OX19, Proteus OX2, or spotted fever group ESS antigens. Forty-two urine specimens, collected from 8 other febrile, but nonrickettsial disease patients, as well as urine specimens from 55 healthy adults, all similarly prepared, were also free of inhibitory

activity in the same test systems. All specimens were then re-examined for antigen by incubating human type O, Rh negative, red blood cells in the prepared urine specimens, then washing the cells free of urine, and exposing them to appropriate human serum containing antibody. By this method, one urine specimen, collected on the 9th day of disease, was shown to contain antigen related to spotted fever group ESS.

Scrub typhus: One to two liter volumes of urine were collected daily from one patient with scrub typhus, beginning at the fourth day of disease. After 100-fold concentration these specimens were examined for antigen by both methods employed in the study of urines from spotted fever cases. The patient's own convalescent serum, which contained Proteus OXK agglutinins after the 14th day of disease, was used as a source of antibody. No antigen was detected.

The scope of this investigation of guinea pig and human urine, in terms of numbers of individuals studied for each rickettsial disease, is limited. Nevertheless, on the basis of experience thus far gained, it appears doubtful that this approach to antigen detection will yield a practical procedure for the acute phase laboratory diagnosis of rickettsial disease. Further study will be confined to examination of urine from more patients with scrub typhus.

3. Factors Affecting the Maintenance, Distribution and Dispersion of Rickettsia rickettsii in Nature.

During the past year the principal effort has been concerned with field and laboratory studies designed to provide coincident quantitative information about the populations of adult and subadult vector ticks, their vertebrate hosts and Rickettsia rickettsii in an endemic focus of Rocky Mountain spotted fever. Correlative analysis of these data should reveal the interaction that occurs among the different components of the infection cycle and show how they are influenced by environmental conditions. These investigations have been possible only through collaboration with members of the Bureau of Insect and Rodent Control, Virginia State Department of Health, the Department of Biology, Old Dominion College, Norfolk, and the Section of Biostatistics, Division of Preventive Medicine, WRAIR. The ultimate aim in attaining a better understanding of the ecology of Rocky Mountain spotted fever is to be able to assess the relative importance of the different factors responsible for the perpetuation of an endemic focus and to recognize the conditions associated with a high risk of occurrence of disease in man.

The area selected for the field investigations is situated on a 400 acre farm near Montpelier in Hanover County, Virginia. This site is in the piedmont province and it is from this physiographic region of Virginia that the highest number of cases of Rocky Mountain spotted fever are reported annually. One of the inhabitants of the farm had severe Rocky Mountain spotted fever in 1962.

Because of limitations imposed by available personnel and funds, it was initially decided to study principally the small mammals and the adult and subadult Dermacentor variabilis, and to expand the investigation to include medium-sized mammals and birds when possible. Accordingly, a study area of 40 acres comprised of approximately equal proportions of old fields and forest was marked off. It was subdivided into 400 plots each 0.1 acre in size. A small mammal live-trap was located in the center of each plot and regular estimates of the populations of the resident small mammals were made by standard capture-mark-release methods. At the time of capture, subadult ticks, if present, were removed, identified and tested for rickettsiae by inoculation of guinea pigs. A specimen of blood was obtained by the orbital bleeding technique and the serum examined for spotted fever antibodies. Within each of the 400 plots, two 0.001 acre zones were randomly located and used for the collection of adult ticks by systematic flagging. One of the zones was designated for population studies and ticks obtained in it were returned to the zone of origin after being identified and marked with paint. The color of paint was changed each month and indices of the population of active adult ticks were calculated from the numbers of marked and unmarked ticks taken. Ticks collected in the other zone were used to determine rickettsial infectivity rates. A complete survey of the types, density and height of the vegetation in the study area was made and meteorological data was obtained from a nearby weather station.

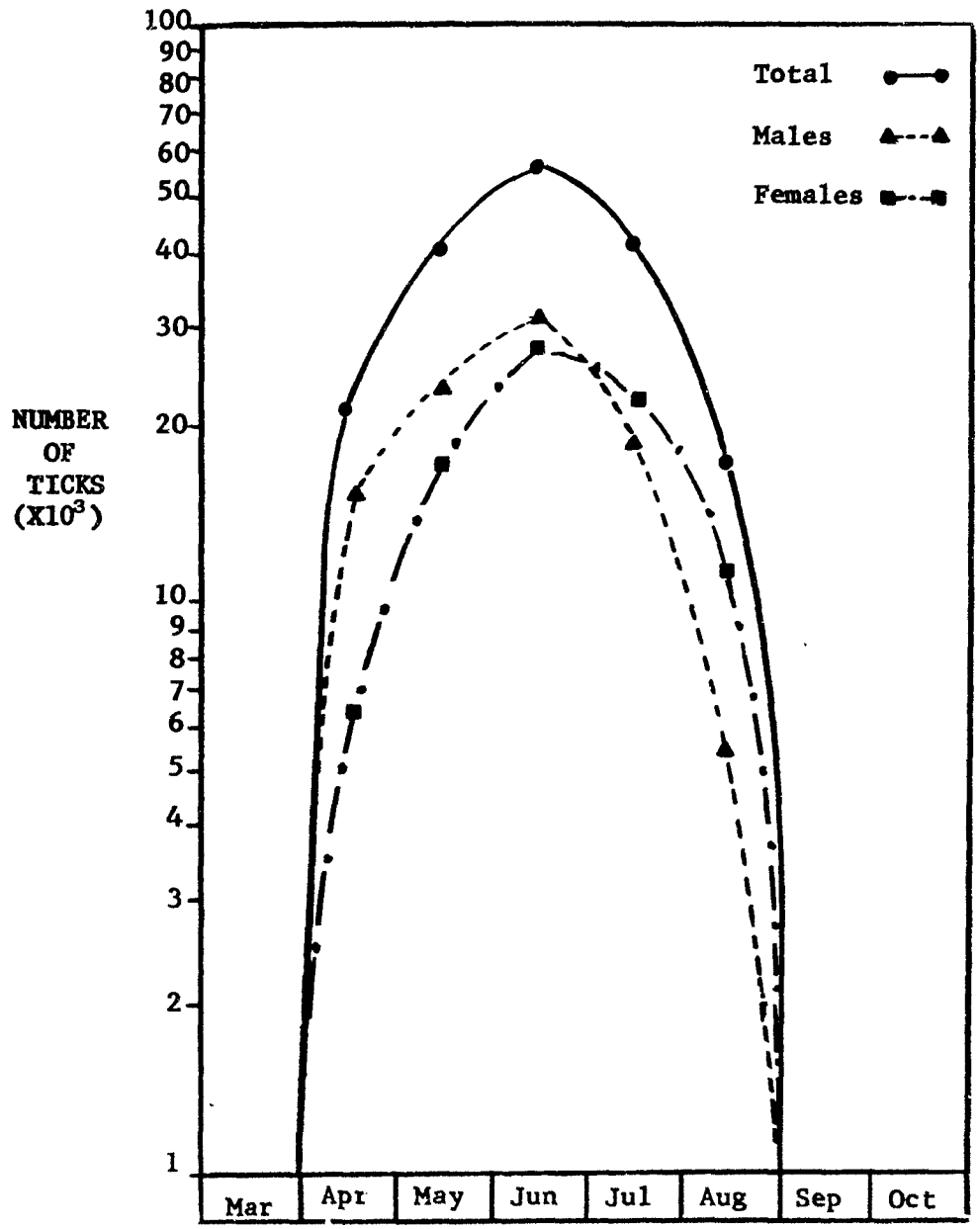
The investigative plan was put into operation in March last year and has been continued up to the present. A massive amount of data has been collected. Much of the tabulation and correlative and statistical analyses have not yet been completed. However, sufficient information is available for presentation which should prove the feasibility of this type of investigative approach.

The changes that were observed in adult D. variabilis population are shown in Figure 2. The estimates are expressed as monthly population indices of active male and female ticks. In the current tick season an improved sampling method will be used to obtain absolute population values for semimonthly periods. Activity of both the males and females was first observed at the end of March and was no longer detectable after August. The rate of increase of males was greater than the females early in the season. In mid-April there were 15,000 males and 6,400 females. By mid-June both stages were present in about equal numbers when the maximum of 56,000 mature stages were active in the 40 acre study area. Following the June peak the females remained active in greater numbers than did the males and in mid-August there were 11,000 females as compared to 5,400 males

A different cycle of activity of the subadult D. variabilis was found. The semimonthly rates of infestation of the immature stages of the dog tick on the three species of small rodents pres-

FIGURE 2

POPULATION INDICES OF ACTIVE ADULT DERMACENTOR VARIABILIS
MONTPELIER, 1963



ent in the largest numbers in the study area are graphically represented in Figure 3. The bimodal character of the activity of both subadult forms is evident. As might be expected, changes in the larvae population preceded those observed in the nymphs. Larvae were first seen on the rodents early in March, increased rapidly and reached peak abundance during the same month. Thereafter, the infestation rate declined and none were found in July and early August. Larvae reappeared in the later part of August, increased to moderate levels in September and early October, and then disappeared. The maximum peak activity of the nymphs occurred in May, with a second peak observed in October.

A total of 1,224 ticks were collected in the study area during the period 1 April-15 October 1963 to determine the rates of infection with R. rickettsii. The numbers of the different species of ticks and their developmental stages are given in Table IV. Approximately 75% of the ticks survived the rigors of collection, shipment to WRAIR and preliminary incubation at 37° C for 2-3 days. Pools comprised of approximately 25 ticks were prepared as the ticks were received and these were inoculated into guinea pigs. Of the 36 pools examined, 18 were infected with spotted fever rickettsiae. Aliquots of each of the ticks had been stored at the time of the initial processing to permit subsequent identification of the infected individuals in each pool. The number of infected arthropods found in a single pool varied from 1 to 6; six of the pools containing 3 or more infected ticks. In two instances reinoculation of aliquots of individual ticks of positive pools failed to reveal the infected tick. R. rickettsii has been recovered from all stages of D. variabilis: 30 of 611 adults or about 5% harbored spotted fever rickettsiae, 6 of 163 or about 4% of the nymphs were infected, and rickettsiae were recovered from only 1 of the 62 larvae tested. None of the Amblyomma, Haemophysalis or Ixodes ticks examined were infected.

The serological status of the small animals trapped in the study area during the period 19 March 1963 to 17 January 1964 is summarized in Table V. Due to death of animals in the traps and some inadvertent losses during removal of ticks and bleeding, it was not possible to carry out complement fixation tests for spotted fever antibodies on all of the 263 animals captured. Animals whose sera had anticomplementary activity were also excluded from this tabulation. Among the 206 animals examined were included 12 different species. Serological evidence of R. rickettsii infection was found in 57 animals belonging to 4 species, i.e., Peromyscus, Reithrodontomys, Blarina, and Microtus, in order of decreasing incidence. Antibodies were present in the sera of 32 animals at the time of first capture and conversions from serological negative to positive at the time of a subsequent recapture were observed in 21 Peromyscus and 4 Reithrodontomys.

FIGURE 3

RATE OF INFESTATION OF MICROTUS, PEROMYSCUS AND REITHRODONTOMYS WITH DERMACENTOR VARIABILIS SUBADULTS MONTPELIER, 1963

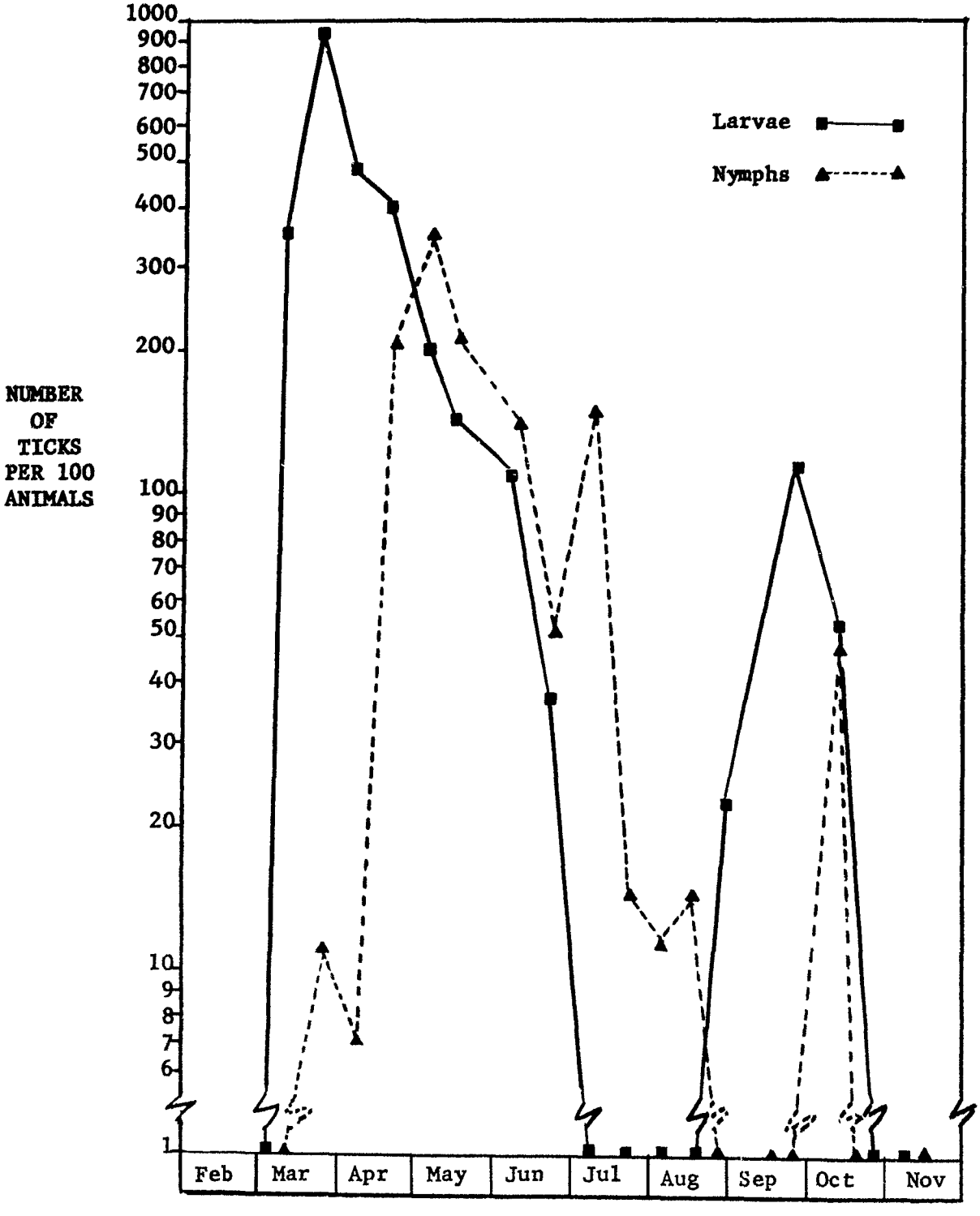


TABLE IV

TICKS COLLECTED IN MONTPELIER STUDY PLOT FOR EVALUATION OF *RICKETTSIA RICKETTSII* INFECTIVITY, 1 APRIL--15 OCTOBER 1963

Disposition of Ticks Received	*Dermacentor variabilis*			*Amblyomma americanum*			*Haemaphysalis leporis-palustris*			*Ixodes cookei*			TOTALS
	L*	N**	A***	L	N	A	L	N	A	L	N	A	
Living-Processed for isolation attempts	62	163	611	-	12	14	13	1	2	-	4	-	882
Dead-Discarded	186	18	87	-	17	12	10	-	2	-	10	-	342
TOTALS	248	181	698		29	26	23	1	4		14		1224

* Larva
** Nymph
*** Adult

TABLE V

SEROLOGIC STATUS OF SMALL ANIMALS TRAPPED IN MONTPELIER STUDY AREA
19 March 1963 - 17 January 1964

ANIMALS WITH SPOTTED FEVER CF ANTIBODIES

Species	Individuals Tested	At First Capture	After Recapture	Total	Percent Positive
Peromyscus	94	25	21	46	49
Microtus	56	4	0	4	7
Blarina	16	2	0	2	13
Reithrodontomys	15	1	4	5	33
Mus	5	0	0	0	--
Glaucomys	5	0	0	0	--
Didelphis	5	0	0	0	--
Tamias	4	0	0	0	--
Pitymys	2	0	0	0	--
Sylvilagus	2	0	0	0	--
Procyon	1	0	0	0	--
Sorex	1	0	0	0	--
TOTALS	206	32	25	57	28

The course of R. rickettsii infection in the Peromyscus population is shown in Table VI. The figures in the table denote the serological status of the rodents trapped during each month. The number of animals which had reverted to a negative serology after having been previously positive is inclosed in brackets under the CF negative heading. No evidence of prior infection was found in wood mice trapped during March. Thereafter, the incidence of serological positives progressively increased reaching a maximum of 83% in June. A decrease was observed during the period August through November with a slight secondary rise evident during October. Only 6% of the wood mice trapped in November had demonstrable antibodies and no positive reactors were captured in January. It is of interest that this pattern of infection in the wood mice parallels the curves of the subadult D. variabilis infestation rates.

TABLE VI

ROCKY MOUNTAIN SPOTTED FEVER INFECTION OF PEROMYSCUS IN MONTPELIER STUDY AREA (1963)

Trapping Period	NUMBERS OF ANIMALS Tested	CF*Negative	CF Positive	Percent Positive
MARCH	24	24	--	0
APRIL	19	11	8	42
MAY	30	7	23	76
JUNE	18	3	15	83
JULY	12	3	9	75
AUGUST	12	4(1)**	8	67
SEPTEMBER	7	6(3)	1	14
OCTOBER	11	8(4)	3	27
NOVEMBER	17	16(4)	1	6
DECEMBER	--	--	--	--
JANUARY	13	13(2)	0	0

* Complement fixation test for Rocky Mountain spotted fever antibodies.

** Numbers in brackets represent animals having detectable complement-fixing antibodies at times of previous trapping.

4. Experimental Infection of Cotton Rats with Rickettsia rickettsii.

Ecologic studies on Rocky Mountain spotted fever have implicated certain common wild mammals in the biologic survival of Rickettsia rickettsii in nature. Little is known, however, concerning the role of such animals as hosts for rickettsiae, as reservoirs of infection, and as a medium for transferring R. rickettsii from infected to uninfected ticks. In order to assess the significance of a mammalian species as a reservoir for an infectious agent transmitted by hematophagous arthropods, it is necessary to study experimental infections under controlled laboratory conditions. Therefore, the present investigation was undertaken to study R. rickettsii infection in the cotton rat, Sigmodon hispidus ssp. This rodent was selected because (1) it is a native wild mammal in the southeastern United States, (2) it is an important host for the immature stages of Dermacentor variabilis, (3) a strain of spotted fever rickettsiae was recovered from this species trapped in southern Virginia (see Annual Progress Report, WRAIR, 1 July 1960-30 June 1961, p 298), and (4) there are technical advantages with respect to availability, storage of large numbers and ease of handling.

The Sheila Smith strain of R. rickettsii was employed. This strain, recovered from a 4-year-old girl with a severe case of Rocky Mountain spotted fever acquired in the Bitter Root Valley of Western Montana, grows moderately well in the yolk sac of embryonated eggs, and is highly virulent for guinea pigs, i.e., it causes high fever, scrotal reaction accompanied with necrosis of the scrotal skin, ears and foot pads and finally death. A 20% suspension of Sheila Smith-infected yolk sac tissue comprised the seed material.

Titrations of the infected yolk sac suspension in cotton rats and guinea pigs yielded the following results: (1) None of the cotton rats died or showed any overt clinical signs or symptoms. They were bled 28 days postinoculation and the sera were tested for the presence of spotted fever complement-fixing antibodies. The 50% infectious dose for cotton rats was $10^{-6.4}$. All of the cotton rats which were infected, as exemplified by the presence of complement-fixing antibodies, had titers of 1:320 or greater. The cotton rats received 0.25 ml intraperitoneally. (2) The guinea pigs, inoculated intraperitoneally with 1.0 ml amounts, responded with all of the manifestations typical of infection with a highly virulent strain of R. rickettsii, viz, fever, scrotal reaction and death. The 50% lethal dose for guinea pigs was $10^{-7.1}$. None of the surviving guinea pigs had demonstrable antibodies; thus, the LD_{50} and ID_{50} for this animal were identical.

Intracardial and intracerebral inoculation of cotton rats produced no deleterious effect, but, as was found following the intraperitoneal route, the animals did develop antibodies. Thus, by these methods, no means of challenging cotton rats suspected of

having been infected with R. rickettsii was found.

Rickettsemia studies: In order to evaluate the importance of a mammalian species to the perpetuation of spotted fever in nature, it is necessary to know the length of time that R. rickettsii circulate in the blood, and thus may serve as a donor of rickettsiae to ticks. A group of cotton rats were inoculated intraperitoneally with 25,000 cotton rat ID_{50}s. Blood was obtained from 2 different members of the group after 1, 6, 12, 18, 24, 36 and 48 hours, and daily through the 14th day. Each blood was inoculated into embryonated eggs and the yolk sac of any embryo found dead after the 2nd day was smeared and stained by Macchiavello's stain and by immunofluorescence. Rickettsiae were considered to be present when observed by either of the staining methods. Table VII shows that rickettsiae were present in the blood only at the 6th, 18th and 24th hour of infection. Bloods taken after the first day were negative for R. rickettsii.

TABLE VII

CIRCULATION OF RICKETTSIAE IN BLOOD OF COTTON RATS FOLLOWING PRIMARY INFECTION WITH R. RICKETTSII (SHEILA SMITH STRAIN)

Cotton Rat Number	Time of Rickettsemia (hours) 1	6	12	18	24
A210	0*				
A213	0				
A223		0			
A224		+**			
A254			0		
A230			0		
A236				+	
A238				+	
A197					0
A199					+

* 0 = No rickettsiae detected in the blood.
** + = Rickettsiae present in the blood.

Experimentally infecting cotton rats in the laboratory by intraperitoneal injection of yolk sac material does not simulate the circumstances of natural infection, but the results do provide information about the relative efficiency of this species as a reservoir of the disease. The short period of rickettsemia manifested by the cotton rat minimizes the importance of this vertebrate host in the perpetuation of R. rickettsii in nature. In contrast, it has been reported that rickettsiae could be demonstrated in the blood of the meadow vole (Microtus pennsylvanicus) for about 1 week after infection. Also, experimental infection of opossums with R. rickettsii was

accompanied by a rickettsemia of 3 to 4 weeks' duration (see Annual Progress Report, WRAIR, 1 July 1961-30 June 1962, p 201).

Occurrence and persistence of rickettsiae in various tissues: Persistence of an infectious agent in tissues may constitute a potential reservoir of infection, as exemplified by the recrudescence of R. prowazeki infection in cases of Brill-Zinsser disease many years after the initial disease. Studies of various laboratory animals infected with epidemic typhus, murine typhus and scrub typhus rickettsiae have shown that the organisms may persist in the tissues for relatively long periods of time. The only reports found on the persistence of spotted fever rickettsiae in animal tissues was the recovery of the agent from the brain of a white rat after 24 and 30 days, and from a guinea pig brain 20 and 45 days after inoculation. In this study, cotton rats were inoculated intraperitoneally with 25,000 ID_{50}s. Two of the animals were sacrificed daily for 21 days and on the 28th day postinoculation, and the brain, kidney, liver, lungs and spleen were removed aseptically. The presence of rickettsiae was determined by (1) examining Geimsa and immunofluorescent stained smears prepared from the cut surface of each tissue, and (2) inoculation of suspensions of the individual tissues into the yolk sacs of embryonated eggs. The results are presented in Table VIII.

TABLE VIII

PRESENCE OF R. RICKETTSII IN VARIOUS TISSUES OF COTTON RATS FOLLOWING PRIMARY INFECTION WITH SHEILA SMITH STRAIN

	Days Postinoculation																			
Tissues	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	28
Brain	0	0	0	0	0	0	+	+	+	0	+	+	+	+	0	+	+	+	0	0	0	0
Kidney	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	0	0	0
Liver	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	+	0	0	0
Lungs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0
Spleen	+	+	+	+	+	+	+	+	+	+	+	+	0	+	0	+	+	+	+	0	0	0
Peritoneum	0	+	+	+	+	+	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0

0 = No rickettsiae in tissue of either of 2 animals sacrificed.
+ = Rickettsiae detected in the tissue of 1 or both animals sacrificed.

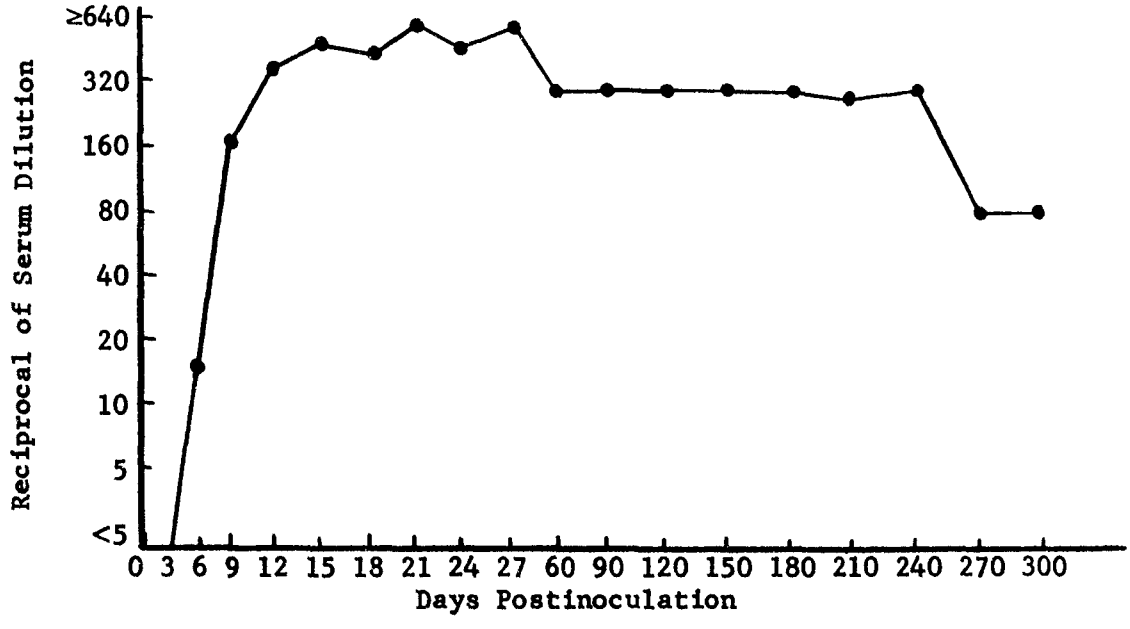
The organisms appeared in the kidney, liver, lungs and spleen on the first day after infection and persisted in these organs for almost 3 weeks. Rickettsiae were not detected in the brain until the 7th day postinoculation. From the rickettsemia studies, it was shown that under the conditions of these experiments, the cotton rat

circulated rickettsiae for only a short period of time; yet, the organisms are present in the various tissues for 18-19 days. It is conceivable that this may represent a latent spotted fever infection and, under some physiological alteration due to environmental factors or stress, the infection may become activated so that the animal is infectious for ticks feeding on it.

Rate of development and persistence of spotted fever antibodies: Fifteen cotton rats, inoculated intraperitoneally with 25,000 ID_{50}s, were bled from the ophthalmic venous plexus every 3 days for 27 days, and once a month thereafter. Each serum was tested for the presence of spotted fever complement-fixing antibodies. The mean titers obtained are shown in Figure 4.

FIGURE 4

MEAN COMPLEMENT-FIXING ANTIBODY TITERS* OF 15 COTTON RATS FOLLOWING INFECTION WITH R. RICKETTSII (SHEILA SMITH STRAIN)



* Sera tested against 2-4 units of spotted fever group-reactive antigen.

All of the animals had detectable antibodies ranging from 1:5 to 1:40 on the 6th day after inoculation. On the 12th day, 8 rats had titers ≥1:640, and the lowest titer obtained at that time was 1:160. The titers ranged from 1:160 to 1:640 for 8 months and then dropped about a 4-fold dilution in the 9th and 10th months, when the experiment was terminated. The results of this study indicate that infection of cotton rats with a highly virulent strain of spotted fever rickettsiae results in a fairly rapid antibody response which attains relatively high titers and probably persists throughout their natural

life. This is somewhat contrary to the findings obtained from animals which have been naturally infected. In the ecological studies conducted jointly by the Virginia State Health Department and the Department of Rickettsial Diseases, WRAIR, 4 of 31 cotton rats trapped in southeastern Virginia had titers of 1:10 to 1:20. Similarly, trap-release studies indicate that the natural infection of meadow voles and wood mice elicits only a low antibody response (1:4-1:32), which persists for a short time. The virulence of the infecting strain may be a significant factor. Based upon the clinical response observed in guinea pigs, as well as the levels of antibodies attained, it is generally accepted that strains of R. rickettsii recovered from D. variabilis, the American dog tick found in the east, are consistently less virulent, or avirulent, when compared with most of the strains isolated from D. andersoni, the wood tick found in the Rocky Mountain area. Likewise, the strains recently recovered from animals trapped in Virginia are far less virulent than those isolated from wild animals in Montana. The Sheila Smith strain used in the present study is a highly virulent western strain and, thus, it is not surprising that the cotton rats developed high levels of antibody.

Susceptibility of "immune" animals to reinfection: It is presumed that once an animal is infected, it is immune and can no longer play a role in the maintenance of rickettsial infection. The following studies were performed to determine if previously infected animals could be reinfected. A group of cotton rats which had been initially infected 7 months before were inoculated intraperitoneally with 25,000 ID_{50}s. At the time of reinoculation, they had complement-fixing antibodies ranging in titers from 1:80 to ≥1:160. The procedures for detecting the presence of rickettsiae in the blood and tissues were the same as those previously described. Table IX shows that rickettsiae were detected in the yolk sacs of eggs inoculated with the blood of the 2 rats bled 12 hours after reinoculation.

The brain, kidney, liver, lungs and spleen of each of 2 "immune" animals were harvested 2, 6, 10 and 14 days after reinoculation. Rickettsiae were present in the kidney on days 2, 6 and 10, and in the liver and lungs only on days 14 and 6, respectively. Organisms were not detected in the brain and spleen or in smears of the peritoneum. Because of lack of sufficient numbers of immune cotton rats, the experiment could not be extended beyond 14 days. Each animal was also bled for serum at the time it was sacrificed for autopsy. The antibody levels remained the same in those rats sacrificed on the 2nd and 6th day, while there was a 2-fold increase in titer in those harvested at 10 and 14 days. The significant finding in this study was that in the presence of relatively high levels of antibodies, the cotton rats did circulate rickettsiae in their blood following reinfection. If the results of these studies can be applied to the field, it could be assumed that animals which had experienced one infection could again become donors

TABLE IX

PRESENCE OF RICKETTSIAE IN BLOOD OF COTTON RATS FOLLOWING REINFECTION WITH R. RICKETTSII (SHEILA SMITH STRAIN)

Cotton Rat Number	Complement-fixing Antibody Titer	Time of Rickettsemia (hours) 1	6	12	18	24
T16	≥1:160	0*				
T31	≥1:80	0				
A32	≥1:80		0			
A117	≥1:160		0			
A109	≥1:160			+**		
A48	≥1:80			+		
A52	≥1:160				0	
A156	≥1:80				0	
A59	1:160					0
A62	1:160					0

* 0 = No rickettsiae detected in the blood.
** + = Rickettsiae detected in the blood.

of rickettsiae to ticks. Therefore, it is possible that some species of animals could serve as reservoirs throughout their life as long as they are infected periodically by the feeding of infected ticks.

Serial passage of R. rickettsii in cotton rats: It is well known that the biological properties of some strains of rickettsiae change by continuous passage in animals or embryonated eggs. Serial passage, at 4 day intervals, of spleen and liver suspensions of R. rickettsii-infected cotton rats indicated (1) the organisms can be maintained by serial passage in cotton rats at least for 15 passages, (2) the virulence of the strain with respect to guinea pigs was not altered, and (3) the strain remained nonpathogenic for cotton rats.

5. Recovery of a Transmissible Agent from a Wild Cottontail Rabbit.

In the course of studies to determine the role of wild animals in the ecology of Rocky Mountain spotted fever (Annual Progress Report, WRAIR, 1 July 1961-30 June 1962, p 199), 2 nonrickettsial, nonbacterial transmissible agents were recovered; one from the tissues of a white-footed mouse (Peromyscus leucopus) and one from a cottontail rabbit (Sylvilagus floridanus). The characterization of the Peromyscus virus was reported in the Annual Progress Report, WRAIR, 1 July 1962-30 June 1963, p 416. The isolation and preliminary studies on the characterization and identification of the agent recovered from the cottontail rabbit are the subject of this report. A 20% suspension of liver and spleen of a wild rabbit trapped on the eastern shore of Virginia in April 1962 was inoculated into the yolk

sac of six 7-day-old embryonated eggs and intraperitoneally into 3 guinea pigs. One of the embryos of the inoculated eggs was dead on the 10th day. From the yolk sac of the dead embryo, a transmissible agent was recovered which readily propagated in embryonated eggs and a variety of cell cultures. The agent has been maintained in eggs by passage of yolk sac tissue or allantoic-amniotic fluids. The allantoic-amniotic fluids had an infectious titer of $10^{-4.5}$ in embryonated eggs inoculated via the yolk sac route. No microorganism could be seen in Macchiavello stained smears of infected yolk sac tissue observed at 980 X magnification. No pocks were produced on the chorioallantoic membrane. None of the guinea pigs inoculated with the original rabbit tissue suspension developed any signs of illness.

The agent produced a cytopathic change in cultures of human diploid cells, primary rhesus monkey kidney and African green monkey kidney, and hamster kidney after 4-7 days. No degenerative changes were observed in a continuous line of rat fibroblasts, but fluids removed from the cultures killed chick embryos in 5 to 6 days. Cytoplasmic inclusions which stained red or blue with Macchiavello stain were observed in some of the cells. These inclusions somewhat resembled the inclusions seen in cells infected with the P4 strain of ornithosis, but no elementary bodies could be seen.

The rabbit agent produced fatal disease in 4-8 days in suckling Swiss mice following intracerebral inoculation, but failed to produce any signs of overt illness in weanling or adult mice. Guinea pigs, laboratory rabbits, and wild cottontail rabbits inoculated either intraperitoneally, intracerebrally or intranasally for production of antiserum did not show any evidence of illness.

The agent was stable for at least 24 hours at 4° C, for 6 hours but not for 24 hours at 22-24° C, for ½ hour but not for 2 hours at 37° C, and was completely inactivated when exposed to 56° C for ½ hour. It was inactivated by 20% ethyl ether after 18 hours at 4° C.

Attempts to identify the new agent or relate it to a number of known viruses were made by complement fixation tests. Complement was not fixed by hyperimmune guinea pig serum prepared against the rabbit agent in the presence of any of the viral antigens listed in Table X.

Table XI presents the results of complement fixation tests on sera from 8 species of wild animals, laboratory rabbits and 131 different human beings. The rabbit agent antigen was prepared from vervet monkey kidney cell cultures. As shown, only 8 of 25 wild cottontail rabbits had antibodies to the agent.

TABLE X

VIRAL ANTIGENS WHICH FAILED TO FIX COMPLEMENT IN THE PRESENCE OF THE RABBIT AGENT HYPERIMMUNE GUINEA PIG ANTISERUM

Enteroviruses	Myxoviruses
ECHO 4, 9, 16, 20	Influenza A, B
Coxsackie A-4, -8, -10, -24	Parainfluenza 1, 2, 3, 4
Coxsackie B-1, -3, -5	Mumps
Polio 1, 2, 3	Simian virus 5
Reo 3	

Other Viruses

Vaccinia	Fibroma virus (Patuxent strain)
Mouse hepatitis (Manaker)	Herpes simplex
Respiratory syncytial (CCA)	Adenovirus (Group)
Lymphocytic choriomeningitis	K virus (Kilham)

TABLE XI

PRESENCE OF COMPLEMENT-FIXING ANTIBODIES TO THE RABBIT AGENT IN SERA OF ANIMALS AND MAN

Species	Number Tested	Number Positive	Number with serum titer* of 10	20	40	80
Rabbit, wild cottontail	25	8	2	4	1	1
Rabbit, laboratory	46	0				
Deer	10	0				
Fox, gray	10	0				
Fox, red	10	0				
Opossum	10	0				
Raccoon	10	0				
Cotton rat	3	0				
Squirrel	3	0				
Man	131	0				

* Reciprocal of dilution.

Amniotic-allantoic fluid from the 18th passage in embryonated eggs was used for filtration through gradocol membranes to determine the size of the rabbit agent. The organism passed through a membrane of average pore size of 300 mμ, but did not pass through the 220 mμ membrane. Thus, the size of the new agent is in the range of the larger viruses.

Antibiotic sensitivity tests were performed in embryonated eggs. Penicillin (50,000 units/egg), dihydrostreptomycin (10 mg/egg), gantricin (5 mg/egg) or chloramphenicol (1 mg/egg) did not prolong the life or prevent death of embryos infected with 100 egg LD_{50}s.

Only recently has it been possible to obtain psittacosis antigen, but preliminary complement fixation tests suggest that the new agent is related to the psittacosis-lymphogranuloma venereum group of agents. Studies are now in progress to verify these conclusions.

Several months after the original isolation of the rabbit agent, the remaining portion of rabbit tissue which had been stored at -70° C since autopsy, was thawed for reisolation purposes. A transmissible agent was again recovered which was indistinguishable from the original isolation.

6. Cases of Rocky Mountain Spotted Fever Diagnosed by the Department of Rickettsial Diseases.

At the Annual Meeting of the Commission on Rickettsial Diseases, AFEB, held at WRAIR 6-7 March 1964, the Army Preventive Medicine Officer stated that only 4 cases of Rocky Mountain spotted fever had been reported in Army personnel in 1963; 2 cases occurred in active duty personnel and the other 2 were in dependents.

Isolation of R. rickettsii from clinical specimens and results of complement fixation tests on sera sent to the Department of Rickettsial Diseases indicate that the incidence of spotted fever infection was greater than reported. Table XII is a summary of all of the cases, both military personnel and civilians, from whom specimens were submitted for laboratory confirmation of Rocky Mountain spotted fever infection.

Only one of the cases in this tabulation was included in the Preventive Medicine report to the Rickettsial Diseases Commission, viz. a fatal case from Ft. Belvoir, Virginia. All of the cases from the military hospitals were dependents, except for 2 in military personnel. Four of the civilian patients were in hospitals in Washington, D.C. and the other 2 were in the Medical College of Virginia Hospital in Richmond.

Two of the patients succumbed to their R. rickettsii infection. The first fatal case, a 42-year-old female Army dependent, occurred at DeWitt Army Hospital, Ft. Belvoir, Virginia in April 1963.

TABLE XII

SOURCE OF SUSPECTED CASES OF ROCKY MOUNTAIN SPOTTED FEVER

Specimens Submitted From	Number of Patients	RMSF Confirmed*
DeWitt Army Hosp, Ft. Belvoir, Va.	7	1
Walson Army Hosp, Ft. Dix, N.J. or 1st Army Med. Lab.	7	4
Walter Reed General Hosp.	3	1
Lackland Air Force Base	1	1
Veteran's Administration Hosp.	4	3
Civilians	14	o

* Diagnosis was established by recovery of R. rickettsii from clinical specimens or by serological tests.

Blood drawn on the 4th day of illness was sent tc the Department of Rickettsial Diseases. A strain of R. rickettsii was recovered in embryonated eggs from the blood clot and guinea pigs developed spotted fever complement-fixing antibodies. The patient died on the 5th day of disease. There was a history of tick-bite about one week prior to onset of illness. The other fatal case was an 8-year-old male Army dependent who was transferred from Kennard Army Hospital, Ft. Lee, Virginia to Walter Reed General Hospital on the 11th day of illness (15 Oct 63). He died shortly after admission. R. rickettsii was isolated in guinea pigs and embryonated eggs from a suspension of brain, liver, spleen, lung and kidney removed at autopsy.

Summary and Conclusions:

1. Partially purified suspensions of whole rickettsiae have been prepared from yolk sacs infected with the Gilliam, Karp and Kato strains of R. tsutsugamushi. When these suspensions were used as antigens in complement fixation tests with homologous and heterologous immune guinea pig sera strain-specific reactivity predominated. Cross-reactivity between heterologous reagents indicated that minor components of both the Karp and Kato strains are included in the antigenic make-up of the Gilliam strain. The Karp and Kato antigens, however, showed some degree of reciprocal reactivity but did not fix complement in the presence of Gilliam immune sera. These antigens were also highly satisfactory for the serological diagnosis of human cases of scrub typhus that occurred in Malaya where a variety of different strains of R. tsutsugamushi have been recovered. The

ability of the purified suspensions to detect significant levels of antibody in convalescent human sera suggests that the antigenic spectrum provided by the Gilliam, Karp and Kato strains may be sufficiently broad to encompass the antigenic diversity that exists among the members of the scrub typhus group. Further studies must be undertaken to confirm these initial findings. Group-reactive components were recovered by density gradient centrifugation of suspensions of the Gilliam strain disrupted by ultrasonic vibration. Although the homologous reactivity of the fraction in complement fixation tests with immune guinea pig sera was essentially the same as the suspensions of intact organisms, the heterologous reactivity had been substantially increased. The results of tests on sera from 6 scrub typhus patients indicate the potential usefulness of the group-reactive fraction as a diagnostic antigen. Further biological and biochemical characterization of the group-reactive fraction will include an evaluation of its ability to evoke antibodies in a variety of laboratory animals, as well as of its effectiveness in affording protection against disease.

2. The excretion of antigen in the urine of guinea pigs and patients during the acute phase of scrub typhus, murine typhus and spotted fever infection has been investigated as a possible approach to the early laboratory diagnosis of these diseases. Antigen was rarely found, even in concentrated urine specimens. On the basis of this experience, little diagnostic value is seen in this procedure.

3. Quantitative data has been obtained on the populations of subadult and adult Dermacentor variabilis, their small mammalian hosts, and Rickettsia rickettsii in an endemic area and these data can be interrelated with respect to time, place and circumstance. The experimental design employed in which multiple scientific disciplines are directed toward the common purpose of understanding the kinetics of Rickettsia rickettsii in nature has much promise of providing knowledge about the ecology of Rocky Mountain spotted fever that has not been obtainable heretofore. Furthermore, it can serve as a model for the investigation of other tick-borne and mite-borne diseases.

4. The experimental infection of cotton rats with a highly virulent strain of R. rickettsii was undertaken to evaluate the role that this native wild mammal might play in the ecology of Rocky Mountain spotted fever. Infection with massive doses of the rickettsiae did not produce any clinical symptoms in the animals, but did evoke high levels of complement-fixing antibodies which persisted for at least 10 months. Rickettsiae were detected in the blood only during the first day after infection, but the organisms persisted in various tissues for almost 3 weeks. Cotton rats which had been previously infected and had existing high levels of antibody were susceptible to reinfection. They again circulated rickettsiae in their blood for a short time during the first day

after reinoculation. The short period of rickettsemia experienced by the cotton rats would tend to minimize the role of this mammal in the maintenance of R. rickettsii in nature. Yet, ticks could become infected if they fed during the rickettsemic period. The fact that the animals circulated rickettsiae again following reinfection enhances their importance, since they have the potential to function repeatedly as a reservoir.

5. An unidentified transmissible agent was recovered from the visceral tissues of a wild cottontail rabbit during the course of studies to evaluate the role of wild mammals in the ecology of Rocky Mountain spotted fever. Serological tests failed to relate the new agent with a number of myxoviruses, enteroviruses, or a variety of other viruses. However, on the basis of preliminary tests it appears to possess certain properties characteristic of the psittacosis-lymphogranuloma group of agents. The rabbit agent grew readily in embryonated eggs, produced lethal infection in suckling mice and caused degenerative changes in a variety of cell cultures. Antibodies against the new agent were found in 8 of 25 wild rabbit sera in titers ranging from 1:10 to 1:80, but were not found in the sera of 8 other species of wild animals, laboratory rabbits or in 131 human sera.

6. Sera or tissues from 36 individuals were submitted for laboratory confirmation of Rocky Mountain spotted fever infection. Diagnosis of the disease was established in 7 of 18 patients from military hospitals, 3 of 4 in Veteran's Administration hospitals, and 6 of 14 civilians. R. rickettsii was recovered from the tissues of 2 fatal cases which occurred in Army dependents. An apparent discrepancy has been disclosed between the number of cases occurring in military personnel and their dependents and those reported at the Meeting of the Commission on Rickettsial Diseases by Preventive Medicine Officers.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER 36182

PROJECT, TASK, OR SUBTASK NO. 3A012501A8180123

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The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY

Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY

NA

4. CONTRACTOR AND/OR GOV'T LABORATORY

A Walter Reed Army Inst of Rsch
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723-1000, Ext. 3552 49

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6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Bacterial diseases (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME'

(U) Etiology of chronic and recurrent diseases such as rheumatic fever, rheumatoid arthritis, Reiter's syndrome and endocarditis. Presence and persistence of antibiotic-resistant, granule-like variants of bacteria have been demonstrated.

Interrelationships of P. pestis, flea vectors, rodent reservoirs, micro and macro climate with studies of pathogenesis, physiological, immunological and ecological aspects in an attempt to elucidate mechanisms involved in persistence of enzootic plague and development of epidemics.

Development and evaluation of a holding medium for collection and shipment of clinical specimens from epidemics. Recovery of N. meningitidis from nasopharyngeal swabs, and effects of storage temperatures on viability are noted.

Studies on the etiology of acute gastroenteritis from a bacteriological, virological and parasitological approach are in progress.

Studies of the pathogenesis, physiological, immunological, epidemiological and ecological aspects of sulfa resistant meningococcal infections with current emphasis on technics, epidemiology, mechanisms of resistance and prophylactic methods.

9. KEY WORDS

Bacteria, immunology, sulfa, plague, rheumatic fever, Shigella Salmonella, meningitis, epidemiology, ecology

10. SUPPORTING PROJECTS

Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES

☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES

☒ NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36182	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	11–22: 3A012501A818 · 23 24: 01 · 25 26: 23 · 27 28: · 29:
14. DATE OF REPORT (30–33)	30–33: 0664
15. SECURITY OF WORK (34)	34: 4
16. TYPE OF REPORT	35: 3 · 36–47: · 48 49 50 51: · 52–55: 1263
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19. R&D CATEGORY (67)	67: 1

Card "D"

20. CONTRACT NUMBER	11 12: DA · 13 14: · 15–17: · 18–21: · 22–26: · 27:
21. GRANT NUMBER	28 29: DA · 30–33: · 34 35: · 36–38: · 39 40: · 41–45: G · 46:
22. ESTIMATED COMPLET. DATES	47–51: 1CONT · 52–56: 2 · 57–61: 3 · 62–66: 4 · 67–71: 5

Card "E"

23. PRIORITY (11–14) 24. PROGRAM ELEMENT (15–26)	11–14: 1 · 15–26: 6•11•25•01•1
25. CMR&D CODES	27–29: N/A · 30–32: · 33–35:
26. CDOG REFERENCE a. Paragraph No. (36–44) b. Functional Group (45)	36–39: 1412 · 40: · 41 42: a · 43 44: · 45: 6

Card "F"

27. FUNDING

a. Est. Total Cost (11–15)	11–15: · 16–18: 1 · 19–21: 2 · 22–26:
b. % Spent Intern. (16–18) " " Extern. (19–21)	
c. Total Obligation (22–26)	
d. Progrmd. Cur. FY (27–33)	27 28: · 29–33: · 34 35: · 36–40:
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f. " " " +2 (41–47)	41 42: · 43–47: · 48 49: · 50–54:
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k. Total Man Years of Effort (76–78)	

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ANNUAL PROGRESS REPORT

Project No. 3A012501A818 Title: Communicable Diseases and Immunology

Task No. 01 Title: Communicable Diseases

Subtask No. 23 Title: Bacterial diseases

Description: Investigations are related to studies on the etiology, pathogenesis, physiological, immunological and ecological aspects of diseases of microbiological origin which are current or potential problems to the military forces. Current emphasis is on the study of such diseases as rheumatic fever, pyelonephritis, plague, sulfa-resistant meningococcal infections and acute gastroenteritis.

Progress:

1. Significance of Mycoplasma (PPLO), Bacterial L-forms and Transitional Forms in Human Disease.

   a. Mycoplasma Contamination of Tissue Cultures.

Studies on the source of Mycoplasma contamination of tissue cultures (Annual Progress Report, WRAIR, 1963) were continued. It was found that during trypsinization of Mycoplasma-infected tissue cells grown in monolayers in bottles, aerosols were produced within the bottles by the trituration procedure. Furthermore, it was found that an aerosol droplet containing one Mycoplasma cell was sufficient to initiate infection of a Mycoplasma-free tissue culture. The mechanism of spread of Mycoplasma contamination in tissue culture laboratories was demonstrated in the following manner. A burette was used to add sterile medium to freshly trypsinized Mycoplasma-infected tissue cultures. Immediately thereafter Mycoplasma-free tissue cultures were fed from the same burette. From 50 to 75 per cent of the latter tissue cultures became infected via aerosols contaminating the burette from which the fresh medium was dispensed.

All the Mycoplasma isolates from the one tissue culture laboratory studied were serologically similar to each other and to a newly reported human oral strain of Mycoplasma. Thus it appeared that the primary origin of Mycoplasma contamination within this laboratory was a Mycoplasma from the mouth of a technician, and the secondary source was widespread cross-contamination via infected aerosols. Culture checks of 2,500 bottles of tissue cells showed that the rate of contamination from the primary source (mouth of the technician) was one bottle contaminated per every 500 trypsinized.

b. Experimental Pyelonephritis in Mice.

The role of transitional or L forms of bacteria in facilitating persistence of an organism in an antibiotic-treated host has been documented in both man and experimental animals. There have been no studies in man and only a few investigations in experimental animals, however, dealing with the capacity of these organisms per se to cause histopathologic change.

The effect of a stable L form of Proteus mirabilis on rabbit kidney cells in tissue culture was studied. Moderate to marked CPE was produced in tissue cells inoculated with 24 hr. broth cultures of Proteus L-9, whereas control cells with broth alone remained healthy. With evidence that Proteus L-9 could cause CPE in tissue culture, production of histopathologic change was sought in the intact animal. The test system used to produce experimental pyelonephritis was the laboratory mouse inoculated with Proteus L forms. The reasons for the choice of this test system were: (1) chronic pyelonephritis is a disease in which persistence of L forms has been postulated; (2) experimental pyelonephritis has been produced in other rodents; (3) the parent bacillary form of Proteus is known to be pathogenic for the genitourinary system; and (4) the renal medulla provides an environment with the high osmolarity (1200 milliosmoles/ml) necessary for osmotic stabilization of some transitional and L-forms. Two conventional methods for predisposing the kidneys of experimental animals to pyelonephritis (unilateral ureteral ligation and unilateral renal massage) were initially used prior to IV injection of Proteus L-forms. Since animals with ligated ureters did not withstand IV injections of L-9, and renal massage did not seem to predispose to renal infection by the IV route, it was later elected to ligate the ureter and make intrarenal and intrapelvic injections of Proteus L-9 concentrates in the homolateral kidney. When this was done it was found that the incidence of recovery of L-forms from the pelvis of the kidney was 33 per cent on day 1, rose to 100 per cent by day 4, and was 33 per cent on day 6. No organisms were recovered from animals sacrificed on days 8, 10 and 14. The results of culturing kidney macerates revealed the same pattern, but with recoveries only on days 2, 3 and 4. This is the first instance reported on recovery of stable L-forms from a host experimentally inoculated with L forms.

It appeared that in vivo multiplication of these organisms could take place in renal pelvic urine. Growth in the kidney parenchyma appeared to be slower or, in this study, may have reflected contamination of the renal macerate from pelvic urine. In mice in which the ureteral ligations were cut relieving the hydronephrosis one or two days prior to culturing the pelvic urine or kidney macerate, no organisms were recovered from either source. Persistence, in vivo, of the L-form of Proteus would, therefore, appear to be tenuous and dependent on sequestration of urine in the renal pelvis or upon increased susceptibility of the renal parenchyma during hydronephrosis.

Further studies on a larger scale are in progress to better define the histolopathologic changes resulting from experimental L-form infection. Investigations are also planned to elucidate the observation made during the above studies that IV injections of Proteus mirabilis L forms in concentrations of 7.8×10^7 colony forming units/ml were uniformly lethal within 30 minutes of injection, while at equivalent concentrations the parent organism was well tolerated.

c. Studies on the Etiology of Rheumatic Fever.

Investigation of the morphology and cultural characteristics of laboratory-made group A Streptococcus L forms was undertaken. Basic studies of this type were expected to facilitate recognition and cultivation of similar variants from the blood or tissues of rheumatic fever patients. Various solid and liquid media were tested for cultivation of streptococcal L forms, and the medium of choice was found to be brain heart infusion agar or broth containing 10 per cent horse serum, 2 per cent sodium chloride, 0.5 per cent yeast extract and 1000 units per ml. of penicillin. Growth in broth was enhanced by the addition of 0.1 per cent agar. Studies are now in progress on optimal methods for freezing and lyophilizing the L forms, so that cultures and specimens may be safely preserved for later investigation. Experiments have been initiated on the growth of streptococcal L forms in tissue culture. Primary cell strains of rabbit kidney tissue grown in Leighton tubes with coverslips were inoculated with streptococcal L forms harvested from agar cultures. Pink-staining intracytoplasmic inclusions were found in the test preparations stained with Giemsa but not in control cells. Studies are in progress on the serial propagation of the inclusion-forming agent and on reisolation and reversion of the agent in cell-free culture media.

d. Type Culture Collection of PPLO and L Forms.

Acquisitions to the collection of Mycoplasma and L form species, a a collaborative project by WRAIR and the American Type Culture Collection, brought the total number of available reference strains to 30.

During the past year viability tests were carried out on a number of strains lyophilized in 1960-1961. It was found that storage of lyophilized vials at + 18°C for 4 years resulted in a marked loss of viability, whereas storage at + 4°C retarded loss of viability but was still not satisfactory for long-term preservation of Mycoplasma and L forms. Cultures stored at -50°C, -60°C and -196°C for 4 years consistently retained excellent viability. Three cultures stored at -50°C for 10 years likewise retained excellent viability. From now on all lyophilized reference strains will be stored at WRAIR and ATCC at -60°C and a seed stock at -196°C. Investigations are in progress on improved methodology

for suspending, freezing, drying and storage of particularly delicate strains, such as Mycoplasma pneumoniae and Streptococcus L forms. Mycoplasma and L form strains in the ATCC collection have been distributed to various investigators throughout the country and have been found satisfactory as reference strains.

e. Serology of PPLO.

Production of specific hyperimmune rabbit serum to Mycoplasma and L form strains in the WRAIR collection was initiated. All strains were first purified by repeated single colony isolations and millipore filtration. Strains were then adapted to growth in rabbit serum for production of antigens. Immune serum was checked for titer and specificity by the macro- and micro-complement fixation tests and by the fluorescent antibody test. During these studies it was found that Mycoplasma hominis, Type 2, strain Campo and Mycoplasma arthritidis, strain H606 cross reacted to high titers by all tests employed indicating a close serologic relationship. Similar reports on the close relationship and possible identity of M. hominis, Type 2 and M. arthritidis have since appeared in England.

2. Plague Studies.

A project concerned with testing the feasibility of incorporating serological tests on rodent sera into the routine methods employed in plague surveys has been in progress for several years. This work has been hampered by lack of adequate material from known plague foci. During the present reporting period, testing of large numbers of rodent sera from known plague foci in Iran, New Mexico, Utah and Vietnam was accomplished. The complement fixation and hemagglutination tests employing a specific Pasteurella pestis antigen (Fraction I) were used to evaluate these rodent sera. Details of these two test procedures have been reported in preceding annual reports.

a. A circumscribed, uncontrolled focus of sylvatic plague in Indian Farm Canyon, Utah, has been under study for several years. Plague appears to be confined to Peromyscus spp., which are the predominant rodents in the area, comprising about 80 per cent of all trapped animals. P. pestis was isolated from Peromyscus spp., or their flea ectoparasites in March, April, May and December of 1961 and in February 1962 (see Marchette, et al., Zoonoses Research 1:341, 1962). These P. pestis strains were shown to have the specific Fraction 1 antigen when tested by the Ouchterlony technic. A collection of sera made on a chronological basis was available for testing to see if plague foci could be detected by serological methods.

Table I lists the results obtained when sera were tested for complement fixing (CF) antibody to Fraction 1 antigen. Table II shows the results obtained when the same sera were tested for hemagglutinating HA antibody. The lowest serum concentration tested was 1:8 in the case of the CF test and 1:24 in the HA. Since nonspecific reactions are unknown with these tests all positive reactions are listed. Table III presents the information on those animals demonstrating both CF and HA antibody. Eight sera could not be compared due to anticomplementary activity. It can be seen that HA antibody persisted through the entire survey period while CF antibody "clusters" in close conjunction to actual isolations of P. pestis.

b. A total of 91 rodent sera from Meriones libycus, M. persicus, M. tristrami, M. vinogradovi and Microtus migratorius were collected in a Kurdistan plague focus by Dr. M. A. Eftekhari of the Pasteur Institute, Tehran, and sent to WRAIR for testing, as were representative strains of P. pestis isolated from the focus. The Iranian strains of P. pestis possessed the Fraction 1 antigen. Table IV shows the results of the serological tests on these sera. Positive serological reactions were obtained only with M. persicus, a rodent highly resistant to plague. The fact that no CF antibodies are evident could indicate that these sera were collected in an inactive plague foci. This conclusion is confirmed by the fact that no P. pestis isolations had been made from this plague foci for some time.

c. Rats and their flea ectoparasites are the major source of plague infection for human beings. From 409 Rattus norvegicus sera (collected in Vietnam foci) tested, 75 positive CF reactions and 26 positive HA reactions were obtained (See SEASIA Reports, Ann. Rpt., WRAIR, 1963-64 for details of results). Although no chronological information as to isolations of P. pestis from R. norvegicus was available, a good percentage of the rats trapped in the vicinity of plague infected human beings demonstrated plague antibody. The P. pestis isolated from human beings in the various test areas had Fraction 1 antigen.

d. An interesting focus in the State of New Mexico has been under study by WRAIR for several years. The focus is located on the Red Bluff ranch area, some 30 miles north and east of Roswell. A SAC missile site and Walker AFB are in close proximity to this focus. Two airmen were infected with plague in 1960 when a fulminating epizootic was in progress. Plague was detected in the rodent and lagomorph population although no serological tests were made by the USPHS who investigated the outbreak (Kartman, L., 1960, Zoonoses Research 1:1). Since the original outbreak, annual trapping by WRAIR personnel has revealed that aberrant P. pestis strains have persisted in rabbits and rabbit fleas of this area and that the presence of these and/or other P. pestis strains is indicated by persistent serological

TABLE I

Serum Complement Fixing Antibody Titers to *Pasteurella pestis* Fraction 1 Antigen - Indian Farm Canyon, Utah - 1961-62

Month	Serum Titer					Total Positive	Total Tested	Per Cent Positive
	1:8	1:16	1:32	1:64	1:256			
III						0	17	0*
IV						0	9	0*
V			1			1	20	5*
VI		1				1	11	9
VII		1			1	2	28	7
VIII	3		1			4	41	10
IX	2		1			3	63	5
X	3	1				4	57	8
XI						0	56	0
XII				1		1	28	4*
I						0	19	0
II	1					1	29	4*
III	4	1	1			6	23	26
IV	8	1		1		10	63	16
V	2	1				3	47	6
VI		1				1	23	4
VII						0	78	0
VIII						0	87	0
IX						0	57	0
X						0	67	0
XI						0	81	0
Totals	23	7	4	2	1	37	904	4

*P. pestis isolated

TABLE II

Serum Hemagglutinating Antibody Titers to Pasteurella pestis Fraction 1 Antigen
Indian Farm Canyon, Utah - 1961-1962

Months	Antibody Titer Expressed as Reciproccal of Dilution									Total Pos.	Total Tested	% Pos.	Remarks
	24	48	96	192	384	768	1,536	6,144	24,576				
III	2	1				1				4	17	24	P. pestis isolated
IV										0	9	0	P. pestis isolated
V	1									1	20	5	P. pestis isolated
VI				1						1	11	9	
VII		1	1	1		1		1		5	28	18	
VIII										0	41	0	
IX		2			1					3	63	5	
X	2		1	1						4	57	8	
XI		1	1	1						3	56	5	
XII								1		1	28	4	P. pestis isolated
I				1						1	19	5	
II	3						1			4	29	14	P. pestis isolated
III	2			1						3	23	13	
IV	1	1		1						3	63	5	
V	1	2								3	47	6	
VI	2									2	23	9	
VII	3	1		6			1	1		12	78	15	
VIII		3	1							4	87	5	
IX			1						1	2	57	4	
X		2								2	67	3	
XI	1									1	81	1	
Totals	18	14	5	13	1	2	2	3	1	59	904	6.5	

TABLE III

Peromyscus maniculatus Trapped in an Enzootic Plague Area Demonstrating both Complement Fixing and Hemagglutinating Antibodies to *Pasteurella pestis* (Fraction 1 Antigen)*

Month Trapped	Rodent Number	Titer		Remarks
		CF**	HA***	
VI/61	259	1:64	1:192	
VII/61	299	1:256	1:6,144	
XII/61	643	1:64	1:6,144	This rodent was infected with *P. pestis* and was infested with 12 *P. pestis* infected fleas.
III/62	717	1:8	1:24	
III/62	724	1:64	1?192	

* Eight rodents demonstrating hemagglutinating antibody could not be compared due to anticomplementary sera.

** Complement fixing antibody.

*** Hemagglutinating antibody.

TABLE IV

Pasteurella pestis Fraction 1 Antibody Titers in Sera of *Meriones persicus*. Collected in the Kurdistan Plague Focus of Iran

M. persicus Serum No.	Titer	
	CF*	HA**
3	-	1:48
12	-	1:24
20	-	1:1,536
23	-	1:24
30	-	1:24
43	-	1:24
50	-	1:1,536
51	-	1:384
53	-	1:24
57	-	1:384

findings in the rodent population. The results are shown in Table V.

In the winter of 1963, a fatal case of plague occurred in an adult male Indian in New Mexico. He had apparently contracted the infection while hunting rabbits near his home in Arizona and had skinned and fed several of these rabbits to his two dogs some days prior to the onset of his fatal infection. Mr. Bryan Miller, New Mexico Department of Health, obtained sera from these two dogs and forwarded them to WRAIR for serological testing for plague antibody. One dog had an HA titer of 1:5,120; the other dog, an HA titer of 1:2,560. This is the second known instance in which family dogs have appeared to have acquired the infection at the same time as their masters. Dogs and possibly other carnivores must be considered in any epidemiological investigations of sylvatic plague.

Two strains of ferrous dependent P. pestis (Ann. Rept., WRAIR, 1962) were isolated in the Red Bluff Ranch focus. One strain was isolated from Sylvalagus tissue in the spring of 1962 and one strain from Sylvalagus fleas in the spring of 1963. These strains are avirulent unless FeSO4 is directly incoporated into the inoculum. It is of interest that although ferrous ions are required to initiate a fatal plague infection with these strains, virulence of the individual strains is proportional to the number of clones in the inoculum containing VW antigen. The differential plating medium of Higuchi, et al. (J. Bact., 77:317, 1959) was used to detect VW clones in the inoculum. In one instance, one strain (10% VW+ : 90% VW-) had an LD50 of 1,450 bacilli when tested with FeSO4 in white mice. In a second experiment (40% VW+ : 60% VW-) the LD50 was 300 organisms. A test of the efficacy of two vaccines (EV living attenuated P. pestis and Fraction 1 antigen) in protecting white mice against these ferrous dependent P. pestis demonstrated that both vaccines were highly effective. Vaccine scores of less than 10 (Ann. Rept., WRAIR, 1962) were obtained with both vaccines.

Conclusions which can be drawn from the above investigations, based primarily on the chronological Utah observations are:

(1) The P. pestis isolations were seasonal being obtained in 5 of the 21 months survey period.

(2) The P. pestis isolated possessed the specific, capsular antigen of P. pestis, Fraction 1.

(3) Many rodents in the area demonstrated antibody to the Fraction 1 antigen, either by complement fixation or hemagglutination tests.

TABLE V

Persistence of Pasteurella pestis Fraction 1 Antibody and Aberrant P. pestis (Ferrous Dependent) in a New Mexico Focus of Sylvatic Plague

No. Tested	Survey Period	Serological Findings		Remarks
		CF*	HA**	
	Winter, 1959/60	***		Plague epizootic****
46	Fall, 1961			
	Lepus californicus	-	1:3840	P. pestis not isolated
	Onychomys leucogaster	-	1:120	
	Dipodomys ordii	-	1:30	
10	Spring, 1962			
	Sylvalagus audubonii	1:8	1:240	Ferrous dependent P. pestis isolated
	Dipodomys ordii	1:120	-	
103	Spring, 1963			
	Dipodomys ordii	-	1:240	Ferrous dependent P. pestis isolated

* Complement fixation

** Hemagglutination

*** Serology not performed by USPHS

**** See Kartman, L., 1960, Zoonoses Research 1:1

(4) The hemagglutinating antibody appeared to be quite persistent and was detectable in animal sera in 20 of 21 months of the survey.

(5) The complement fixing antibodies were not persistent, and were only detected in 2 of the 21 months of the survey, being closely associated, in time, to actual isolations of plague bacilli.

These conclusions are supported by results of studies on animal sera from plague foci in New Mexico, Iran and Vietnam. In addition, from the many thousands of animal sera examined, positive serological tests have not been found except from animal sera collected in proven plague endemic foci. It can be further theorized that these two serological procedures used in a systematic and chronological study of rodent sera from a plague foci, would provide adequate information on the efficacy of instituted control measures.

e. In previous reports, usefulness of Stuart's transport medium for the shipping of specimens for the isolations of P. pestis has been discussed. Stewart's medium and a modification thereof (Barlow, et al., Jap. J. Med. Sci. & Biol. 8:171) are very useful for transportation of specimens that are free of enteric saprophytes. A new medium, which prevents overgrowth by saprophytic contaminants (Cary, et al., Bacter. Proc. 1964:56) has been evaluated and shown to maintain P. pestis in a viable state for over 7 months' time at room temperature and should prove to be an excellent medium for field survey use, since many specimens of rodent origin are likely to be grossly contaminated.

3. Gastroenteritis Studies.

During the period of 1 July 1963 through 30 June 1964 the Escherichia coli typing service received a total of 840 cultures from 327 infants for serological identification of enteropathogenic E. coli, representing an increase of 706 cultures or an 84 per cent increase over last year. Thirty-one or 7.5 per cent were enteropathogenic E. coli representing 7 serotypes, and 8 or 2.4 per cent were suspect pathogens. Included were 9 E. coli 0125, 7 E. coli 055, 4 E. coli 0111, 4 E. coli 0012, 2 each of E. coli 0126, 0119, 0128 and 073 and 1 each of E. coli 0127 and 074. Ninety-three per cent of the cultures were received from Walter Reed General Hospital.

Bacteriological, virological and parasitological studies of acute gastroenteritis in Puerto Rican infants were initiated in 1958 (Ann. Rpts., WRAIR, 1958 to date). The present report describes the characterization of 3 of these viruses (PR20, PR22 and PR28) which have been submitted to the Panel for Picornaviruses.

PR20, PR22 and PR28 were isolated in rhesus monkey kidney tissue cultures from female infants, 12 months old or less. All infants had diarrhea, nausea and fever prior to admission. Of these viruses only PR22 produced an antibody titer (1:160) in the infant from whom it was isolated although antibody to all three were detected in normal adult sera from Puerto Rican and Washington, D. C. residents. The infant with PR20 also harbored an E. coli 07 and the infant with PR22 harbored an E. coli 016 and a Pseudomonas spp. while the third infant had no other enteric bacteria or parasites. Since none of the viruses would plaque by the method of Hsuing and Melnick (J. Immunol. 78:137, 1957) using tris buffer overlay or that of Gochenour and Baron (Proc. Soc. Exptl. Biol. and Med. 102:732, 1959) the viruses were purified by three consecutive terminal passages. PR22 and PR28 produce polio-like CPE in 3-6 days achieving a $TCID_{50}$ titer of $10^{7.5}$ while PR20 achieves a $TCID_{50}$ titer of $10^{7.0}$/ml. in 2-4 days. PR28 might still be a mixture since it produces CPE with some characteristics of both polio and adenoviruses, and efforts are in progress to repurify this virus by cesium chloride density gradient centrifugation. Complement fixation tests for adenovirus was negative for all three viruses. Employing the colloidium film technic, monkey kidney tissue cultures showing all stages of CPE of the three viruses, were stained with hematoxylin-eosin and the histological appearance was found to be compatible with that of enteroviruses. Susceptibility of various tissue culture lines to these viruses is shown in Table VI. These viruses produced no observable illness or death in suckling mice (i.c. and i.p.), adult mice (i.c.), hamsters (i.c.), rabbits (i.c.), guinea pigs (i.c. and embryonated eggs (yolk sac).

Neutralization and reciprocal neutralization tests against polio virus 1, 2 and 3 and ECHO virus types 1-28 and 12 candidate enteroviruses (Frater, Bastianni, Pett, Vanscoy, 1923/60, Taylor, JV 10, JV 6, 4707's and Caldwell) were negative.

PR22 and PR28 do not hemadsorb or hemagglutinate human O, guinea pig, rhesus monkey or rat red blood cells at 4, 25 or 37°C, but PR20 hemagglutinates human and rhesus monkey rbc's at 4°C to a titer of 256 and 64 respectively. The viruses are stable to 20 per cent ether and sodium deoxycholate indicating an absence of essential lipids, unaffected by heating at 50°C for 1 hr. in the presence of M $MgCl_2$, and are stable in a 1:10 dilution of Eagles' Basal Medium at pH 3.0 for 3 hrs. at 25°C. All three viruses demonstrated a red staining reaction with acridine orange indicating that they possess a ribonucleic acid core. Calculations of average pore diameter of Swinny Millipore filters based on Elford's factors (Proc. Roy. Soc. B. 112:354, 1933) show the viruses to be 16.5-25 mu.which is well within the range of enteroviruses.

TABLE VI

Tissue Culture Susceptibility of Three Puerto Rican Viral Agents from Infants with Gastroenteritis

	PR20	PR22	PR28
Tissue			
Rhesus monkey kidney	+	+	+
KB	+	+	+
M.A.F.	+	+	+
HeLa	-	+	-
H. Ep. #2	-	+	-
Hamster kidney	-	-	-
Chang liver	+	+	-
Detroit #6	+	+	+
Henle	+	+	+

The indirect hemagglutination test has proved to be a more sensitive and reliable method for determining infection with Shigellae than the bacterial agglutination test. Neter (Amer. J. Pub. Health 52:61, 1962) concluded that it is of epidemiological significance that many individuals have mild or even subclinical infections during epidemics. He also observed (N. Y. Acad. Sci. 66:141, 1956) that titers of hemagglutinins against Shigella antigens were much higher than titers against heterologous antigens (e.g. E. coli). The present study was undertaken to determine titer levels of hemagglutination antibodies in normal adult sera collected from widely separated geographic areas. It was hoped that this investigation would further establish a baseline for determination of significant titer levels.

Sera for the survey were collected from normal adults from five geographic areas, namely, New York, Massachusetts, California, Colorado, and Texas. Heated NaOH lipopolysaccharid extracts of Shigella flexneri 1a and 4b were used to sensitize human O Rh negative cells. Sh. flexneri 1a gives cross reactions to a high titer with all Sh. flexneri antisera except 4b, and was used as a broad spectrum antigen while Sh. flexneri 4b which reacts weakly with only two other strains, e.g. Sh. flexneri 4a and 2a, was used as an indicator of prior infection to this specific strain. Since there is disagreement concerning significant titer levels, (Neter, Amer. J. Pub. Health 52:61, 1962 and Gottoff, Amer. J. Hyg. 78: 261, 1963), a level of 128 or greater was chosen as significant of infection.

Of a total of 707 serum samples from six geographic areas tested, 62 or 8.8 per cent had hemagglutinin titer levels of 128 or higher to Sh. flexneri 4b and 360 or 50.9 per cent had similar titers to the broad spectrum antigen of Sh. flexneri 1a (Table VII). Although not shown in the table, the number of serological reactors to Sh. flexneri 4b declines as titers increase while Sh. flexneri 1a reactors reach a peak at 128. There were also considerably higher serum antibody titers present in most instances to the broad spectrum Sh. flexneri 1a antigen.

The low titers of Mexican sera can be explained by the fact that half of these sera were from infants under one year of age. Thirty-four per cent of these infants had no hemagglutinins against Sh. flexneri 4b and 17 per cent had none against Sh. flexneri 1a.

This survey has demonstrated that exposure to Sh. flexneri antigens is a commonplace event. With the exception of the Mexican sera, all other areas had few individuals in which no detectable normal antibody was present. In addition, it is indicated that there were considerably lower serum antibody titers, in most instances, to the specific Sh. flexneri 4b antigen.

TABLE VII

Shigella flexneri Hemagglutinin Titers* from Six Geographic Areas

Geographic Area	Shigella flexneri 1a					Shigella flexneri 4b				
	Total Sera	Titers				Total Sera	Titers			
		<128		>64			<128		>64	
		No.	%	No.	%		No.	%	No.	%
California										
Urban	44	23	52.2	21	47.8	44	41	93.1	3	6.9
Rural	37	26	70.2	11	29.8	37	37	100.0	0	0
Massachusetts										
Urban	55	37	67.2	18	32.8	55	55	100.0	0	0
Rural	22	16	72.7	6	27.3	22	19	86.3	3	13.7
New York	121	59	48.7	62	51.3	121	118	97.5	3	2.5
Mexico	94	71	75.5	23	24.5	94	78	82.9	16	17.1
Texas	96	42	43.7	54	56.3	96	73	76.0	23	24.0
Colorado	238	86	36.1	152	63.9	238	224	94.1	14	5.9
Totals	707	360	50.9	347	49.1	707	645	91.2	62	8.8

* Titer exposed as reciprocal of serum dilution

Although it is well known that a number of Shigellae have antigens with E. coli, Gillem (J. Bact. 84:896, 1962) demonstrated that the mere presence of an E. coli strain in the adult intestine does not stimulate production against itself and, therefore, E. coli should not provide a source of cross reaction normal antibodies to like Shigellae antigens. The presence of a high percentage of individuals with significant titer in areas such as Mexico, Texas, Colorado, New York and urban California might well reflect the true epidemiologic status of frank illness as well as subclinical infection. From the data presented here, and the previous work of Gottoff and Neter, it is apparent that exposure to Shigella flexneri antigens is a relatively commonplace event.

4. Meningococcal meningitis.

a. Epidemiological investigations. In the spring of 1963, because of the increased incidence of meningococcic meningitis among recruits at Fort Ord, California, and the failure to control this disease by sulfadiazine prophylaxis, WRAIR was assigned the task of investigating this problem. As a result, numerous field investigations have been made in an attempt to clarify the situation.

In the initial exploratory study, the team arrived at Fort Ord 24 hours after the completion of a postwide sulfadiazine prophylaxis program. This precluded any attempt to assay the effectiveness of the program. However, nasopharyngeal cultures were obtained from a sample of recruits drawn from a company in the third week of training. These cultures were obtained 36 hours following their last dose of sulfadiazine. They showed that 14 per cent of the men were still harboring meningococci in the nasopharynx and follow-up cultures revealed that this percentage increased over the next few days.

A sample of men, newly arrived on the post, were given sulfadiazine in the same doses as given in the prophylaxis program. These men were bled at intervals following administration of the drug and biochemical studies of their sera were made by the Division of Chemistry, WRAIR. These showed that the blood levels attained from this regime were sufficiently high that under usual circumstances could be expected to free the nasopharynx of 95 to 99 per cent of the men harboring the meningococcus.

Most of the strains isolated from healthy recruits were found to be resistant to 0.1 mgm per cent or greater of sulfadiazine.* This finding was to be expected since all recruits had recently had a course of the drug.

*Subsequent field trials demonstrated that strains at this level or more were resistant to sulfadiazine prophylaxis while below this level the strains were sensitive.

During June 1963, a study of the effect of prophylaxis on the prevalence of meningococci in recruits was made. Two companies in their sixth week of training were isolated while on bivouac. Both companies were cultured at the beginning of isolation and in each over 50 per cent were found to be harboring meningococci (see Table VIII). The men of one company were given 2 doses of 2 grams sulfadiazine with a 12-hour interval. Satisfactory blood levels of drug were attained but the prevalence dropped only to 30 per cent and within a few days was again up over 50 per cent. The other company was given no sulfadiazine. Here, the prevalence remained steady between 50 and 60 per cent throughout observation.

Cross-sectional studies of the post population were made in June, July and September. The findings are summarized in Tables IX, X and XI. The findings of these studies were similar to those made during World War II with the exception that the type of organism found was different and a large proportion of the meningococci were resistant to 0.1 mgm per cent or greater of sulfadiazine. The following inferences appear justified in the light of these studies:

(1) In the Armed Forces meningococcus meningitis is a disease of recruits and dependent children. In recruits it is a disease of basic training.

(2) Crowding appears to be a factor although no direct evidence was obtained on this point. Troops quartered in the new type barracks appear to be equally attacked as men in the old type barracks.

(3) The prevalence of nasopharyngeal infection among newly arrived recruits is low. It increases steadily as the recruits pass through basic and advanced training and reaches over 50 per cent by the 7th or 8th week.

(4) The occurrence of clinical cases does not parallel the prevalence rate among recruits. Clinical cases are most likely to occur during the basic training period and the highest prevalence of meningococci is during advanced training. It would appear that the clinical cases are more likely to occur at the time the recruit acquires his first infection during Army life.

(5) Dependents, permanent party and training cadres have a much lower prevalence than is found in companies of recruits after they have been in one or more weeks in training.

(6) There is no evidence to indicate close contacts of clinical cases are at any greater risk of contracting meningitis than the rest of the recruit population.

TABLE VIII

Results of Sulfadiazine Prophylaxis

Fort Ord, June, 1963

Group	Strength	Sample Size	Prevalence of Nasopharyngeal Infection		
			Prior to Sulfa	2 Days Post Sulfa	5 Days Post Sulfa
Sulfa Company	264	50	52%	32%	52%
Control Company	262	50	58%	56%	53%

TABLE IX

Prevalence of Meningococcus - Nasopharyngeal Culture Surveys - Fort Ord, 1963

	June 1963	July 1963	September 1963
New Recruits	1%	8%	9%
Pretraining Week		8%	
1st Week		3%	12%
2nd Week		14%	15%
3rd Week		19%	8%
4th Week	13%	16%	24%
5th Week	32%	22%	18%
6th Week	56%	8%	6%
7th Week	57%	22%	16%
8th Week		43%	35%
12th Week	64%		35%
15th Week		55%	
17th Week			47%
Training Cadre	15%	9%	8%
Dependents	4%		
Hospital Staff	10%		
Reception Center	7%		

TABLE X

Distribution of Strains by Groups
Nasopharyngeal Culture Surveys
Fort Ord, 1963

Group	June	July	September
A	1	3	0
B	159	116	100
C	4	20	23

TABLE XI

Percent of Cultures Resistant to 0.1 mgm % Sulfadiazine
Nasopharyngeal Culture Surveys
Fort Ord, 1963

	Percent Resistant		
	June	July	September
New Recruits	0	15	0
Pretraining Week		0	
1st Week		0	67
2nd Week		15	87
3rd Week		56	25
4th Week	67	100	58
5th Week	100	100	22
6th Week	94	100	33
7th Week	100	82	89
8th Week		100	83
12th Week	not done		88
15th Week		96	
17th Week			96

(7) Although the meningococci isolated from clinical cases were resistant to sulfadiazine, the medical staff of the Fort Ord hospital found this resistance not great enough to interfere with the therapeutic use of sulfadiazine and penicillin.

(8) The predominating strain among both cases and healthy soldiers was Group B. Group A which was responsible for almost all of the clinical cases seen in World War II was encountered only rarely. Group C was found in varying proportions ranging from 3 to 20 per cent of identifiable strains.

(9) The majority of the Group B strains were resistant to 0.1 mgm per cent or more of sulfadiazine. Most of the Group C and A strains encountered were sensitive at 0.1 mgm per cent.

Since it is not possible to culture all military personnel at Fort Ord due to laboratory limitations, the prevalence of the meningococcus in the nasopharynx must be determined by sampling. Cross-sectional studies are affected by sampling errors and these lead to difficulty in interpretation. Accordingly it was decided to make a longitudinal study of the spread of meningococcus through two companies of recruits by culturing a sample of the men twice a week during their course of basic training. For this purpose, two newly formed companies, each from a different training brigade, were chosen and observed from October to mid-December 1963. Neither of these two companies had received any sulfadiazine prophylaxis and none was given until the end of the 7th week of training. Thus the course of events was studied in the absence of any sulfadiazine.

Although both companies were provided 72 square feet per man in their sleeping quarters, they occupied different type barracks. One company (D-5-3) was billeted in the new company type barracks. This company comprised 220 men in 5 platoons. The other company (C-4-1) was quartered in the old cantonment type barracks and had 270 men in 6 platoons. Nasopharyngeal cultures were obtained of all men, recruits and cadre, during the pretraining week. Thereafter swabs were taken from half the recruits in each company twice a week during basic training. Selection of the men to be cultured was determined by the terminal digit of the serial number. In one company, those with odd digits were cultured while in the other the sample consisted of men with even digits. The bacteriologic methods are discussed elsewhere in this report.

During the first two weeks of the study the sample was surveyed by means of a questionnaire for information regarding possible sources or means of spread which might result in infection after arrival on post. No positive findings resulted. However, it did serve to point out that new recruits mix freely with other personnel on post and this mixing proceeds at a more rapid rate than is generally recognized.

TABLE XII

Prevalence (%) of Nasopharyngeal Infection
Longitudinal Study - Fort Ord, 1963

	C-4-1	D-5-3
Pretraining Week	5.2	7.6
1st Week	19.3	20.0
2nd Week	34.1	18.1
3rd Week	36.3	18.1
4th Week	43.0	22.8
5th Week	48.9	37.2
6th Week	53.4	44.8
7th Week	63.7	47.6
	Sulfa Prophylaxis	
8th Week	66.0	41.9

TABLE XIII

Prevalence (%) of Group B Sulfadiazine Resistant Meningococci - Longitudinal Study

Fort Ord, 1963

	C-4-1	D-5-3
Pretraining Week	2.2	1.0
1st Week	14.1	1.9
2nd Week	28.9	1.9
3rd Week	34.1	3.8
4th Week	41.5	6.7
5th Week	47.4	21.0
6th Week	50.4	28.6
7th Week	60.7	32.4
	Sulfa Prophylaxis	
8th Week	65.2	41.0

TABLE XIV

Prevalence (%) of Group C Sulfadiazine Sensitive Meningococci - Longitudinal Study

Fort Ord, 1963

	C-4-1	D-5-3
Pretraining Week	0	2.9
1st Week	3.0	10.5
2nd Week	2.2	14.3
3rd Week	0.7	10.5
4th Week	0.7	13.3
5th Week	0	16.2
6th Week	0	12.4
7th Week	0.7	15.2
	Sulfa Prophylaxis	
8th Week	0	0

TABLE XV

Gains and Losses of Infection in Recruits by Culture Period - Longitudinal Study

Fort Ord, 1963

Week of Training	Sulfadiazine Resistant Group B			Sulfadiazine Sensitive Group C		
	Loss	Gain	Difference	Loss	Gain	Difference
0	-	4	+ 4	-	3	+ 3
1	3	10	+ 7	1	8	+ 7
	0	10	+ 10	8	5	- 3
2	2	9	+ 7	3	4	+ 1
	2	13	+ 11	5	10	+ 5
3	4	6	+ 2	7	2	- 5
	2	9	+ 7	0	4	+ 4
4	5	6	+ 1	4	3	- 1
	3	13	+ 10	0	4	+ 4
5	3	19	+ 16	5	5	0
	3	10	+ 7	5	1	- 4
6	6	14	+ 8	5	3	- 2
	8	7	- 1	0	5	+ 5
7	2	16	+ 14	2	4	+ 2
	7	12	+ 5	6	1	- 5
	Sulfa Prophylaxis					
8	4	13	+ 9	11	0	- 11

The percentage of men harboring meningococci in the nasopharynx during each week of basic training is shown for each company in Table XII.

The experience of the two companies did not run parallel and the difference appeared to be due to the group of meningococci involved (Tables XIII and XIV). The recruits of Company C-4-1 were initially infected almost entirely with Group B meningococci. In this company, the percentage of men with positive cultures rose steadily week by week until at the end of 7 weeks 64 per cent of the recruits were infected. Almost all of these Group B strains were resistant to 0.1 mgm per cent or greater of sulfadiazine and tended to remain in the nasopharynx. Very few of those who acquired their infection during observation tended to lose it and the prevalence rates shown in the tables are essentially a cumulation of the number of men infected. On the other hand, in Company D-5-3 both Group B and C organisms were present in the initial cultures. During the first four weeks the C strains tended to spread. These strains were sensitive to sulfadiazine and tended to disappear from the nasopharynx at approximately the same rate as new infections were acquired. Thus from the second through the fourth week, the prevalence stayed at about 20 per cent. During the fifth week, the Group B strains began to spread and since they remained longer in the nasopharynx, the prevalence of infection rose rapidly reaching 41 per cent in the eighth week. The balance of the gains and losses in the prevalence rate is shown in Table XV.

b. Laboratory investigations. A large part of the Department of Bacteriology's time and effort in the study of meningococcal disease during the past year can be directly related to field support of the above epidemiologic investigations. It became evident, early in these field studies, that technics and methodology for collection, isolation, identification and determination of sulfadiazine resistance studies on Neisseria meningitis strains, from the large numbers of nasopharyngeal surveys and meningitis cases, would have to be developed and evaluated. The "time-honored" technics, while adequate and reliable, are costly in relation to the amount of professional and technical help, materials and time required to process clinical specimens, particularly in those instances where microbiological support of epidemiologic investigations is needed.

(1) Recovery of N. meningitidis from nasopharyngeal specimens.

The vast majority of the epidemiological investigations reported above were supported by standard microbiological technics for the collection of specimens, isolation and identification of the meningococcal isolates. Personnel and time limitations, of necessity, imposed sampling restrictions on these studies. Thayer and Martin (3rd Interscience Conference on Antimicrobial Agents and Chemotherapy, October, 1963 and Pub. Hlth. Reports 79:49, 1964) reported the development of a new medium for the cultivation of Neisseria gonorrhoeae and

N. meningitidis. While this medium was designed and field tested primarily for the isolation of N. gonorrhoeae from clinical materials, initial field trials by us demonstrated its value for the recovery of N. meningitidis from nasopharyngeal specimens. The medium as initially used consisted of a Mueller-Hinton based chocolate agar containing 25 units/ml. of polymyxin B and 10 mcg/ml. of ristocetin. Later studies indicated that for examination of nasopharyngeal specimens, the addition of blood was not required.

In a typical comparison between Mueller-Hinton chocolate agar and the Thayer Martin (TM) modification, duplicate nasopharyngeal swabs were inoculated on both media and examined for the presence of meningococci after 24 hours' incubation in a candle jar at 37°C. Of 281 duplicate swabs compared in this way, a total of 132 isolations of N. meningitidis were obtained on the Mueller-Hinton chocolate medium while 148 isolations were obtained on TM medium. There were 24 positive TM plates which failed to yield meningococci on the chocolate agar and eight positive chocolate plates which were missed by the TM medium. A total isolation rate with both plates yielded a carrier rate of 55.5 per cent. Mueller-Hinton chocolate agar alone gave a carrier rate of 47 per cent as opposed to a rate of 52.6 per cent with the TM medium.

This comparison of results does not present a complete picture of the advantages and superiority of the TM medium over that of the standard chocolate agar. Bacteria commonly inhabiting the nasopharyngeal region such as Hemophilus sp., viridans type Streptococci and saprophytic Neisseria are almost totally inhibited, permitting serological grouping from the primary isolation plate without the necesssity for further isolation and purification procedures. The only contaminants noted in several thousand specimens procured to date have been a number of yeasts, an occasional Proteus, and in two instances a pigmented saprophytic Neisseria. These contaminants have not presented a serious problem to a trained bacteriological technician. For example, the time involved in screening a series of 100 nasopharyngeal cultures on standard chocolate agar by a well trained bacteriologist varies from one and one-half hours to two hours, while a good bacteriological technician can screen the same set on TM medium in about 15 minutes. Suspensions for lyophile preservation and carbohydrate fermentation can be made directly from this original plate since in most all instances the antibiotics in the TM medium are bactericidal to the usual contaminants.

(2) Determination of resistance to sulfa drugs.

A medium in which sulfa-drug sensitivities are to be performed must be free of certain proteins, peptides and other materials which will bind, inactivate or otherwise compete with the sulfa

compounds. Use of lysed horse erythrocytes in complex media to overcome the effect of these inhibitory materials was suggested by Harper and Cawston (J. Path. and Bact. 57:59-66, 1945) Development of a medium for the isolation of N. meningitidis and N. gonorrhea by Mueller and Hinton (Proc. Soc. Exp. Biol. Med. 48:330, 1941), and the subsequent recommendation and nearly universal use of this medium as a substrate for sulfa resistant studies was challenged by Jewell (Am. J. Med. Tech., Nov.-Dec., 1958). Initially all in vitro determinations of sulfa resistance were performed by the basic technic of Harper and Cawston, using Mueller-Hinton base in which was incorporated 0.1, 1.0, 2.0, 3.0 and 5.0 mg per cent of sulfadiazine. Since the preparation of media containing lysed horse erythrocytes is impractical for large scale sensitivity testing, comparisons were made using Mueller-Hinton medium with and without lysed horse cells for the purpose of performing sulfa-sensitivity tests on meningococci. Thirty-eight (38) recently isolated strains of meningococci of varying resistance levels to sulfadiazine were tested in duplicate on Mueller-Hinton medium with and without incorporated lysed horse cells. There were no significant differences between the two base media and all subsequent resistant studies have been performed on plain Mueller-Hinton base medium with incorporated sulfadiazine.

It became apparent early in the course of these studies that the concentration of the inoculum placed on the sulfa plates had a marked effect on the sensitvity results. In order to evaluate this effect on the apparent sensitivity of strains to sulfa drugs, a series of experiments was performed in which attempts were made to standardize the inoculum spectrophotometrically. The turbidometric measurements did not coincide well with viable counts, apparently due to significant differences in die-off rates between strains of the meningococci. Therefore, tenfold serial dilutions were made and aliquots of each dilution plated on agar plates containing various concentrations of sulfa drugs. Several resistant strains of N. meningitidis were examined in this way. The results from 52 strains are shown in Table XVI, A, B and C. Table XVI-A lists 26 strains resistant to 0.1 mg% and sensitive to 1.0 mg% of sulfadiazine at a dilution of 10^{-5}. It can be seen that if resistance was determined at a dilution of 10^{-1}, the apparent resistance of these strains would appear to be greater than 5.0 mg%. Similar results are shown in Table XVI-B and C, where 13 strains each, selected at resistance levels of between 1.0 and 2.0 mg% (Table XVI-B) and between 2.0 and 3.0 mg% (Table XVI-C), were examined.

Severe restrictions on the numbers of strains which could be examined were imposed by the tedious hand labor involved in the placing of calibrated inocula on the sulfa plates. The Lidwell phage applicator was modified to permit the simultaneous application of 13 strains to a test plate versus a maximum of 8 permitted by the hand technic. Inoculum dilutions (10^{-5}) were prepared by diluting a stock suspension

TABLE XVI

Relationship of Inoculum Concentration to Sulfadiazine Sensitivity

Mg% of Sulfadiazine	Inoculum expressed as the reciprocal of log dilutions														
	A					B					C				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
0.1			6	17	26										
1.0		4	10	9				2	11	13					
2.0		7	10					8	2				1	8	13
3.0		5					2	3					5	5	
4.0		1					1						4		
5.0	26	9				13	10				13	13	3		

of meningococci (turbidity approximating that of a #4 MacFarlane barium sulfate standard) through two 5.0 ml trypticase soy broth-saline blanks and one 0.5 ml blank in the teflon well of the Lidwell phage applicator, using a 0.05 ml Microtiter diluting loop instead of pipettes. Use of this phage applicator thus permitted an increase in the range of sulfa-concentrations and the numbers of strains which could be examined, since as many as 200 plates could be inoculated in the same amount of time required to inoculate 7 by the standard hand technic. Results are identical to those attained with the original method of Harper and Cawston if no more than 13 evenly spaced inocula per 90 mm petri plate are tested at one time. Some initial trials using 27 inocula per plate (as is done in the phage typing of staphylococci), led to erroneous results since resistant strains elaborated a diffusable sulfa-competing component which permitted adjacent sensitive strains to multiply at sulfa concentrations normally inhibitive.

(3) Determination of carbohydrate fermentation.

Since some serological cross reactions between N. meningitidis and other members of the genus Neisseria, as well as with certain yeasts, are not uncommon, it is necessary to examine some biochemical characteristics in order to make definitive identifications. The minimum biochemical criterion in this instance is a determination of an isolate's carbohydrate fermentation characteristics, using dextrose, maltose and sucrose as the differential substrates. With the successful adaptation of the Lidwell phage applicator to the in vitro determination of sulfadiazine resistance, investigations were initiated to adapt this technic to the determination fermentation patterns. Numerous authors over the past 20 years have used solid fermentation media of various compositions for the biochemical characterization of many bacterial genera. Several of these media were tried and evaluated. The simplest medium yielding results comparable to the standard tube fermentation tests consisted of Mueller-Hinton base medium supplemented with a 2 mg per cent Phenol Red, 2 per cent rabbit serum and 1 per cent carbohydrate. The dilute inoculum used for the sulfa sensitivity tests, however, did not provide sufficient growth to give a clear-cut reading of the indicator change. Consequently, sulfa plates were first stamped with the diluted inocula then one 0.05 ml Microtiter loop from the original undiluted suspension was added to the remaining broth in the phage applicator inoculum well and the carbohydrate plates then stamped. This increased inoculum provided clear-cut evidence of fermentation activity after 24 hours' incubation in a candle jar at 37°C. All strains tested correlated perfectly with the standard tube fermentation method.

Under field conditions an occasional contaminant, present in concentrations too low to be evident on the sulfadiazine media, is noted in the more concentrated inoculum on the carbohydrate plates. These contaminants negate the validity of fermentation reactions and

necessitate reisolation and repeat identification procedures. Incorporation of polymyxin B and ristocetin in the base fermentation medium was shown not to interfere with the fermentation reactions of the meningococcus and, except for a rarely occurring yeast contamination, no further problems of this kind have appeared.

(4) Sensitivity of N. meningitidis to various drugs.

Twelve sulfa compounds (Gantrisin, Kynex, sulfadiazine, sulfaguanidine, sulfamerizine, sulfapyridine, sulfasuxidine, sulfathalidine, sulfathiazole, thiosulfil, Gantanol and Madribon) were examined in vitro for their inhibitory effect on the meningococcus. Of these 12 sulfa compounds only thiosulfil and Gantrisin were equivalent to sulfadiazine in their in vitro activity. Since Gantrisin produces maximum blood levels much more rapidly than sulfadiazine, it was used in an in vivo comparison with sulfadiazine in one field study noted above (Section a.). It was not effective against the resistant meningococci in the nasopharynx. Since several members of the Meningococcal Subcommittee of the Armed Forces Epidemiological Board (AFEB) reported that Gantrisin was not an effective prophylactic against nasopharyngeal meningococcal infection, the following experience is worthy of note. During the course of the above epidemiological investigations, three of our laboratory workers became infected with sensitive N. meningitidis, Group B strains. These people were all treated with a 12 gm. therapeutic regimen of Gantrisin. The meningococci were eradicated and the individuals have remained free of infection while under observation. The length of the observation period to date has been 7 months, 2 months and 1 week.

(5) Growth of N. meningitidis in tissue culture.

Studies were initiated to determine whether N. meningitidis would grow in monolayer tissue cell cultures and whether or not such a system might prove to be a useful tool in antimicrobial drug studies or in immunological assay procedures. Of the two cell lines examined so far, Hela and Rhesus monkey kidney, the monkey kidney cell line appears to be best suited for further investigation. Hela cell cultures were abandoned due to the rapid destruction of the tissue sheets by the meningococci.

Methods used are as follows: 1. The Rhesus monkey kidney leighton tubes were washed five times with warm bovine amniotic fluid (BAF) in order to eliminate the antibiotics. 2. 0.1 ml. of a thin suspension of a sulfa-resistant strain of N. meningitidis, Group B (about 10^2 organisms) was added to 0.9 ml. of BAF. 3. The tubes were allowed to incubate and were removed at hourly intervals (1-12 hrs.). 4. The tissue was washed five times with BAF to remove the extracellular growth and finally all tissues were fixed with 10 per cent

formalin for 15 minutes, stained with Wayson's Stain for 8 seconds, mounted in permount and viewed microscopically.

Data show that after three hours of incubation at 37°C, there is evidence of phagocytosis and/or intracellular growth. Increase in intracellular counts is directly proportional to the incubation time. Repeated observations have shown that the meningococci appear only in the cytoplasma and are surrounded by a thin delineating halo which is capsular-like in appearance.

(6) Antibody response to N. meningitidis invasion of the nasopharynx.

Sera were obtained from 71 recruits during their first week of training and again at the termination of their eighth week in an attempt to determine whether some evidence of immune response to non-clinical infection with N. meningitidis could be demonstrated. Recruits were followed with biweekly nasopharyngeal cultures throughout the eight-week period. A centrifuge agglutination technic (Mayer and Dowling, J. Immun. 51:349, 1945) was used since it has been demonstrated that this procedure yields greater specificity for meningococcal group antibody than other reported agglutination procedures. Table XVII shows the numbers of individuals demonstrating evidence of immunological response to the invasion of the nasopharynx by Groups B and C N. meningitidis. Fifty-four per cent of the individuals from whom meningococci were recovered at some time during the study demonstrated antibody response while only 17 per cent of those who were never shown to be infected had a response. The results suggest that there is an immune response to subclinical infection with meningococci; however, the significance of the response as an expression of immunity to overt disease is not known. Further studies using other serological technics with purified antigen preparations and serially collected sera must be performed in order to characterize the nature of this response and its relationship and duration to the frequency and duration of exposure to the invading meningococci.

(7) A rapid meningococcal surveillance technic.

The increasing spread and/or development of sulfa-resistant N. meningitidis throughout Army recruit training posts has made it difficult for Post Surgeons to make a decision as to when or if a sulfa prophylactic program should be instituted. Evidence has been accumulating which indicates that the use of sulfa prophylaxis on a partially resistant population might well result in the development of a predominately resistant one. A decision of the Army Surgeon General in late March of 1964 to institute an Army-wide continuing surveillance program placed a rather formidable burden on the medical laboratory system.

TABLE XVII

Antibody Response to Nasopharyngeal Infection

Infected with	No. with titer rise to			
	B	C	B&C	None
B (31)	3	4	6	18
C (14)	0	8	0	6
B&C (14)	0	5	6	3
Neg (12)	1	1	0	10

A possible solution to this problem was formulated in late November 1963, at Fort Ord, California. It was noted at this time that all meningococcal strains examined which were resistant to 0.1 mg% of sulfadiazine were also resistant to 0.5 mg%. Two pilot studies were carried out in an effort to determine if it would be possible to differentiate, on original isolation media, individuals carrying sulfa-resistant organisms versus those carrying sensitive organisms. Duplicate nasopharyngeal swabs were collected from 56 recruits in one of the study companies (D-5-3 in 4 a. above). One swab was inoculated to a plain Thayer Martin plate and the other to Thayer Martin medium containing 0.5 mg% of sulfadiazine. After 24 hrs.' incubation at 37°C, the plates were inspected for meningococci and isolates picked from the plain TM media for identification and sulfa resistance studies. In every instance but one, when a carrier harbored sensitive meningococci, the TM + sulfa plate was negative for meningococci. The one discrepancy consisted of a single colony growing in an area of heavy mucous inoculum, while on the control TM plate the meningococcal colonies were present in numbers too numerous to count. In this series of 56 recruits, there were 12 carriers of sensitive strains and 16 carriers of resistant strains. These results were confirmed on a much larger scale at Fort Knox, Kentucky in February 1964.

A bacteriologist, experienced in recognition of meningococcal colonies on this media, could within 24 hours determine the total carrier rate simultaneously with the resistance carrier rate in a specified study group with an accuracy of greater than 90 per cent by mere inspection of the two plates. Definitive information, such as serologic group, carbohydrate fermentation and degree of resistance, would have to be determined later but the information needed to make a decision for the institution of a prophylactic program would be immediately available.

Whether or not this technic will prove adequate in all situations and on all recruit posts is not known. Surveillance programs now being conducted by the six Army Area Medical laboratories should provide this information.

5. Diagnostic methodology.

a. During a recent study of Neisseria meningitidis carriers a new transport medium (Cary, et al., Bact. Proc., 1964) was evaluated and modified for use as a method for collection and shipment of oropharyngeal swabs from remote areas to a central laboratory. One hundred and forty-four (144) oropharyngeal swabs were collected in duplicate at a military recruit installation. One set was placed in transport medium and the duplicate swabs streaked immediately on the selective medium of Thayer and Martin (Public Health Reports, 79:49-57, 1964). The transport vials were shipped by air to our laboratory and subcultured. In addition to N. meningitidis on the oropharyngeal swabs, large numbers of

Enterobacteriaceae, Pseudomonas sp. and yeasts were present. There was no overgrowth by these contaminating organisms in the transport medium and N. meningitidis has been recovered in significant numbers after storage at room temperature for as long as two and three days. The vast majority of strains maintained their serological specificity during storage and transport in this holding medium.

In case of more remote areas there may be a three- to four-day interval before specimens reach a central laboratory. Consequently, a second series of oropharyngeal swabs was collected in duplicate and stored in transport medium at room temperature (approximately 20°C) before subculture. After three days' storage, 50 per cent of the original positives were recovered. The effect of various temperatures of storage on N. meningitidis was studied using 50 freshly isolated strains. The temperatures studied so far were 6°C, 20°C, 26°C and 35°C. Subcultures were made at 24 hr., 48 hr., three and four days. The results are summarized in Table XVIII. Further studies on the effect of storage temperatures in the range of 4°C to 26°C are in progress.

TABLE XVIII

Survival of Neisseria meningitidis Strains in a New Transport Medium

	Storage temperatures			
Subculture	6°C	20°C	26°C	35°C
24 hr	100 %	100 %	85 %	65 %
48 hr	100 %	100 %	30 %	0
3 day	85 %	90 %	30 %	
4 day	70 %	55 %	0	

b. Various workers in studies on the preservation of staphylococcal bacteriophages by lyophilization have reported a drop of 1 or 2 Log dilutions from the original titer for certain phages. Recently Clark, et al. (Appl. Micro. 10:463, 1962) reported on attempts to freeze a number of bacteriophages for gram negative bacteria and those specific for staphylococci. All bacteriophages studied were cooled to 4°C, slowly frozen to -25°C, and then exposed to a dry ice-ethylene glycol slush at -78°C and stored in a liquid nitrogen refrigerator. When tested there was little, if any, change in titer. In preliminary trials in our laboratory,

staphylococcal phage 29 (which drops 1 log dilution in titer with standard lyophilization procedures) was selected for preliminary study. Vials stored at 8°C were slowly frozen to -50°C and one set placed in a dry ice-aceton slush -70°C, then lyophilized. The duplicate set was left at -50°C. Lyophilized and frozen phage (rapidly thawed) were then titered. The RTD of the original stock liquid phage was 10^{-4} with a phage count of 8.5×10^{10}. Following lyophilization, it produced semiconfluent lysis at 10^{-4} and the count had dropped to 2.28×10^{10}. The frozen phage, thawed and titered, gave confluent lysis at 10^{-4}, and the count was 6.05×10^{10}. Further studies on methods to preserve the initial titers of staphylococcal bacteriophages are being carried out.

Summary and Conclusions:

1. Studies on the etiology of the chronic diseases rheumatic fever and pyelonephritis were actively pursued. The mechanism of widespread Mycoplasma contamination of tissue cultures was elaborated. Expansion of the type culture collection of Mycoplasma and viability studies on stored cultures is reported.

2. Epidemiologic investigations of plague foci in several areas of the world by cultural and serological methods indicate the value of immunologic technics for the study of both "urban" and "Sylvatic" plague foci.

3. Characterization of three viral agents recovered from infants with diarrhea and the results of a serologic survey for shigella antibodies in normal adults from six geographic areas are reported.

4. Extensive studies on the etiology and epidemiology of meningococcal meningitis with particular emphasis on the prophylactic sulfa-resistant strains are described. The development and testing of rapid, simplified, and reliable microbiological technics for the isolation and characterization of Neisseria meningitidis strains from the nasopharynx are described.

5. Investigations on the use of a newly designed holding medium for collection and shipment of nasopharyngeal culture swabs were initiated. The effects of temperature on viability and on N. meningitidis in this medium are described. Initial studies on lyophilization technics for the preservation of staphylococcal bacteriophages are reported.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER 36183

PROJECT, TASK, OR SUBTASK NO. 3A012501A8180124

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER
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576-3758 or Interdepartmental Code 198, Ext 3758 See Continuation Sheet 49

6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Mycotic infections (U)

7. DATE OF REPORT DAY 30 MONTH June YEAR 1964

8. RESUME'

(U) Investigations are related to the improvement of serological techniques as rapid and accurate diagnostic aids for mycotic diseases (principally histoplasmosis, coccidiomycosis, blastomycosis and dermatomycosis); ecology and epidemiology of mycotic agents of importance to the military; and fundamental explorations of the metabolic capabilities of those fungi pathogenic for humans.

Two hundred and forty-nine small animals, live trapped in the Lower Sonoran and Transitional Life Zones of New Mexico, were cultured for Coccidioides immitis and Emmonsia parva. No C. immitis was recovered but 18 per cent of the rodents were infected with E. parva. Of 14,853 histoplasmin skin tests applied to residents of Honduras 49 per cent were positive and 70 per cent or more of the population over 24 years of age gave positive reactions. Further studies using histoplasmin and coccidioidin are in progress.

Studies on use of the fluorescent antibody technique as a rapid method for identification of dermatophytes indicate that these fungi share one or more common antigens. Further developmental studies are in progress.

9. KEY WORDS

Mycotic, fungus, histoplasma, coccidiomyces, dermatophytes, immunology, skin test, fluorescent antibody, Emmonsia.

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

DA FORM 1 June 63 1309R PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____

ACCESSION NO. 36183	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	11–22: 3A012501A818 · 23 24: 01 · 25 26: 24 · 27 28: · 29:
14. DATE OF REPORT (30–33)	30–33: 0664
15. SECURITY OF WORK (34)	34: 4
16. TYPE OF REPORT	35: 3 · 36–47 48 49 50 51: · 52–55: 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56–61) b. Functional Class (62–64)	56–61: 010608 · 62–64:
18. OSD CLASSIFICATION (65–66)	65 66: AR
19. R&D CATEGORY (67)	67: 1

Card "D"

20. CONTRACT NUMBER	11 12: DA · 13 14: · 15–17: · 18–21: · 22–26: · 27:
21. GRANT NUMBER	28 29: DA · 30–33: · 34 35: · 36–38: · 39 40: · 41–45: G · 46:
22. ESTIMATED COMPLET. DATES	47–51: 1CONT · 52–56: 2 · 57–61: 3 · 62–66: 4 · 67–71: 5

Card "E"

23. PRIORITY (11–14)	11–14: 1
24. PROGRAM ELEMENT (15–26)	15–26: 6•11•25•01•1
25. CMR&D CODES	27–29: N/A · 30–32: · 33–35:
26. CDOG REFERENCE a. Paragraph No. (36–44) b. Functional Group (45)	36–39: 1412 · 40: · 41 42: a · 43 44: · 45: 6

Card "F"

27. FUNDING

a. Est. Total Cost (11–15)	11–15: · 16–18: 1 · 19–21: 2 · 22–26:
b. % Spent Intern. (16–18)	
" " Extern. (19–21)	
c. Total Obligation (22–26)	
d. Progrmd. Cur. FY (27–33)	27 28: · 29–33: · 34 35: · 36–40:
e. " " " +1 (34–40)	
f. " " " +2 (41–47)	41 42: · 43–47: · 48 49: · 50–54:
g. " " " +3 (48–54)	
h. " " " +4 (55–61)	55 56: · 57–61: · 62 63: · 64–68:
i. " " " +5 (62–68)	
j. " " " +6 (69–75)	69 70: · 71–75: · 76–78:
k. Total Man Years of Effort (76–78)	

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ACCESSION NUMBER	ARMY RESEARCH TASK REPORT
36183	Continuation Sheet

PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued:

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REPORTS. Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964.

ANNUAL PROGRESS REPORT

Project No. 3A012501A818 Title: Communicable Diseases and Immunology

Task No. 01 Title: Communicable Diseases

Subtask No. 24 Title: Mycotic infections

Description: Investigations are related to the improvement of serological techniques as rapid and accurate diagnostic aids for mycotic diseases (principally histoplasmosis, coccidiomycosis, blastomycosis and dermatomycoses); ecology and epidemiology of mycotic agents of importance to the military; and fundamental explorations of the metabolic capabilities of those fungi pathogenic for humans.

Progress: During the current reporting period the Mycology Section has been understaffed. The death of a key member of the staff in September 1963 and her prior prolonged absence on sick leave reduced the working team to three persons. The absence of the Chief of the Mycology Section from 3 January to 27 May 1964 to attend the Medical Field Service School, Course 8A-C23, has also interrupted current projects. During most of this time the Mycology Section has maintained its function as a reference laboratory but has had little time for pursuit of investigative projects.

1. Ecology of Coccidioides immitis and Emmonsia parva in New Mexico.

In 1942 Emmons and Ashburn reported the isolation of C. immitis and Haplosporangium parvum (now recognized as E. parva) from wild rodents in Arizona. So far as is known E. parva has been recovered only from animals in the arid southwest and possibly Africa. The most northerly recoveries of E. parva in the U. S. have been from St. George, Utah.

The mycelial forms of these two organisms are different but the early stages of parasitic growth are very similar and could be confusing to the pathologist. In addition, serological cross reactions have been demonstrated among Emmonsia, Coccidioides and Histoplasma. For this reason some mycologists feel that Emmonsia is genetically related to the human pathogens Histoplasma and Coccidioides.

The present investigation was conducted as a follow-up of a preliminary serological survey conducted in 1962 in collaboration with Dr. James Rust. Complement fixation tests on animal sera, collected in a study of plague ecology, indicated that naturally acquired coccidioidomycosis might be prevalent in southern New Mexico. The purpose of the present study was to investigate the incidence of naturally acquired C. immitis and E. parva infections, since serological studies suggested the presence of mycotic infections among small animals.

All animals were trapped in four geographical areas of New Mexico representing Lower Sonoran and Transitional Life Zones. A total of 249 animals distributed among the following genera were examined: Dipodomys, Citellus, Neotoma, Onychomys, Lepus, Peromyscus, Reithrodontomys, Sigmodon, Eutamias and Sylvalagus.

Animals were live trapped, sacrificed, bled and autopsied. A portion of the liver, lung and spleen was minced with scissors and plated onto Sabourauds Dextrose and Mycosel Agar and incubated at room temperature. The cultures were examined periodically for characteristic growth of C. immitis and E. parva. Cultures that could not be identified microscopically were homogenized and inoculated into white Swiss mice. These animals were sacrificed after three weeks and examined for the tissue form of the fungus. Portions of liver, lung and spleen were cultured for recovery of the organism.

As can be seen in Table I, from a total of 249 animals examined, 44 isolations (18%) of E. parva were made, while C. immitis was not recovered from any of these animals. The majority of the animals were live trapped in the Red Bluff area (a ranch 29 mi. N., 6 mi. E. of Roswell, New Mexico). The Pecos and Bottomless Lakes area accounted for fewer animals but the percentage of positive isolations (25% and 29%) was somewhat greater than the Red Bluff area. The Las Cruces area accounted for 26 animals of which only 2 were positive. Dipodomys ordii accounted for the largest number of animals trapped with 17% of the animals positive for E. parva.

This high percentage of animal infections would appear to indicate that E. parva is highly endemic in those areas investigated. At present plans are being made for another survey of these and possibly other areas in New Mexico with special emphasis on isolation of Coccidioides and Emmonsia from animals and the soil surrounding their burrows. Attempts will also be made to define the serological relationships among these fungal species, using human and animal sera obtained from these Emmonsia endemic areas.

2. Histoplasmin Sensitivity in Honduras, C. A.

Studies on histoplasmin sensitivity in Honduras have been in progress since 1962. These studies are being done in collaboration with Dr. Rigoberto Alvaredo L., Republic of Honduras. A total of 14,853 histoplasmin skin tests were done in 1963. The indigents were grouped according to age ranging from 1 year to 75+ years, in increments of four. Of the 14,853 persons tested, 7275 (49%) were histoplasmin positive.

As can be seen in Table II there appears to be a steady increase in the number of positive skin tests per age group until age 30. After that the number of positive reactors appear to stabilize and remain around 70%.

This high percentage of histoplasmin reactors indicates that Histoplasma capsulatum is very prevalent in Honduras. Further plans are in progress to expand this survey to the coast lines of Honduras using histoplasmin and coccidioidin.

3. Investigation of the Fluorescent Antibody Technique for Identification of the Dermatophytes.

Preliminary investigations have been initiated to determine whether a fluorescent antibody technique could be developed and employed as a rapid method for identifying dermatophytes.

Trichophyton rubrum, T. mentagrophytes, Microsporum canis, M. audouini, and M. gypseum were selected as antigens to be examined. They were grown in Sabourauds broth, harvested and sterilized with ethylene oxide. The suspensions were standardized to 25% light transmission at 550 mμ using the Coleman spectrophotometer (Model 14). Male rabbits were inoculated with 0.5 ml, 1.0 ml and 2.0 ml I.V. for three consecutive days, then 2 ml I.V. on alternate days for two weeks. Seven days after the final inoculation the rabbits were trial bled and CF tests run on the sera. Homologous titers of 1:256 were obtained, and all animals were bled and sera separated. Preliminary trials with the CF and FA tests indicated that these antisera react with homologous and heterologous antigens equally well. Adsorption studies are in progress to see if this nonspecificity can be eliminated. If the cross reactions can then be removed, the specific antisera would permit rapid identification of dermatophytes in clinical material, since the conventional technique of cultivating clinical material on suitable media and identification by macro and microscopic morphology often delays diagnosis for as long as a month. With a specific FA technique, positive identification could be made in a few hours.

Summary and Conclusions: Ecologic studies indicate that naturally acquired E. parva infections are very common among small animals in southern New Mexico. C. immitis was not recovered from any of the 249 animals trapped. Future plans include investigation of the serological relationship of E. parva to human pathogens such as H. capsulatum and C. immitis.

Data obtained from 14,853 skin tests applied to residents of Honduras show that histoplasmosis is highly endemic in that country.

Preliminary investigations to determine the practicability of the FA technique as a tool for identification of dermatophytes were initiated. Preliminary results have demonstrated that these fungi share one or more common antigens and adsorption studies have been initiated in an attempt to prepare specific antisera.

TABLE I. *Emmonsia parva* Isolations

Species	Geographical Area								Total Examined	Positive	
	RED BLUFF		LAS CRUCES		PECOS		BOTTOMLESS LAKE				
	No. Trapped	Pos.	No. Trapped	Pos.	No. Trapped	Pos.	No. Trapped	Pos.		No.	%
S. audubonii	9	0	3	0	2	1			14	1	7
L. californicus	6	0	1	0					7	0	0
C. spilosoma	16	0	4	0					20	0	0
C. lateralis					6	4			6	4	67
S. hispidus	22	7					1	0	23	7	30
O. leucogaster	9	2							9	2	22
N. albigula	22	6	1	0			8	2	31	8	26
D. ordii	85	13	14	2			4	1	103	16	15.5
D. spectabolis	3	3							3	3	100
P. maniculatus	9	0					4	2	13	2	15
R. megalotis	1	0	3	0					4	0	0
E. minimus					5	1			5	1	20
N. cinerea					3	0			3	0	0
Z. princeps					7	0			7	0	0
Tamias sp.					1	0			1	0	0
TOTALS	182	31 17%	26	2 7.6%	24	6 25%	17	5 29.4%	249	44	17.7%

TABLE II

Results of Histoplasmin Skin Test Survey in Honduras (1963)

Age Groups	Males			Females			Total		
	Positive		Total	Positive		Total	Positive		Total
	No.	%		No.	%		No.	%	
-1	11	4.4	250	3	1.0	286	14	2.6	536
1-4	182	16.5	1104	176	14.0	1261	358	15.1	2365
5-9	557	37.2	1498	570	34.9	1631	1127	36.0	3129
10-14	720	56.5	1274	696	52.7	1320	1416	54.6	2594
15-19	413	67.3	614	481	61.4	783	894	64.0	1397
20-24	348	78.6	433	397	68.8	577	745	73.0	1020
25-29	305	82.4	370	322	70.8	455	627	76.0	825
30-34	241	88.3	273	265	67.1	395	506	75.7	668
35-39	187	87.0	215	196	60.9	322	383	71.3	537
40-44	176	82.0	214	171	62.6	273	347	71.3	487
45-59	130	84.4	154	131	61.2	214	261	70.9	368
50-54	86	77.5	111	106	60.2	176	192	66.9	287
55-59	60	73.2	82	59	61.5	96	119	66.9	178
60-64	74	76.3	97	63	47.4	133	137	59.6	230
65-69	32	76.2	42	44	62.9	70	76	67.9	112
70-74	12	40.0	30	14	51.9	27	26	45.6	57
75+	21	80.8	26	26	70.3	37	47	74.6	63
Total	3555	52.3	6,797	3720	46.2	8,056	7275	49.0	14,853

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36184

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8180125

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
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2. FUNDING AGENCY
Army Medical R&D Command
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3. CONTRACTING AGENCY
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4. CONTRACTOR AND/OR GOV'T LABORATORY
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6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Pathogenesis of enteric disease (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME'

(U) The pathogenesis of enteric disease is studied to elucidate the mechanisms by which enteric pathogens produce symptoms. By understanding the disease process, improved procedures for prevention and treatment of diarrheal diseases will become evident. The tools of fluorescent and electron microscopy have been utilized to clarify steps in the infectious process of dysentery bacilli and from this work a series of safety tests have been proposed for living attenuated Shigella vaccines. An orally administered vaccine prepared from 1 strain of S. flexneri protects monkeys against experimental challenge. Natural mechanisms of resistance to enteric disease are also being investigated, and in this regard, the liver has been shown to be the major organ which detoxifies bacterial endotoxin in the body. Studies on the pathogenesis of Asiatic Cholera have shown that an antigenic cell-free product which causes symptoms resembling the disease in infant rabbits is released in young aerated cultures of V. cholerae in a chemically defined medium supplemented with casamino acids. A cholera vaccine prepared at WRAIR elicits an immune response in human beings when injected in one-twentieth the amount normally used for such preparations.

9. KEY WORDS
Enteric, dysentery, cholera, endotoxin, diarrhea, immunity

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36184	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	11–22: 3AO125O1A818 · 23 24: 01 · 25 26: 25 · 27 28: · 29:
14. DATE OF REPORT (30–33) 15. SECURITY OF WORK (34)	30–33: 0664 · 34: 4
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Card "D"

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21. GRANT NUMBER	28 29: DA · 30–33: · 34 35: · 36–38: · 39 40: · 41–45: G · 46:
22. ESTIMATED COMPLET. DATES	47–51: 1CONm · 52–56: 2 · 57–61: 3 · 62–66: 4 · 67–71: 5

Card "E"

23. PRIORITY (11–14) 24. PROGRAM ELEMENT (15–26)	11–14: 1 · 15–26: 6•11•25•01•1
25. CMR&D CODES	27–29: N/A · 30–32: · 33–35:
26. CDOG REFERENCE a. Paragraph No. (36–44) b. Functional Group (45)	36–39: 1412 · 40: · 41 42: a · 43 44: · 45: 6

Card "F"

27. FUNDING

a. Est. Total Cost (11–15)	11–15: · 16–18: 1 · 19–21: 2 · 22–26:
b. % Spent Intern. (16–18)	
" " Extern. (19–21)	
c. Total Obligation (22–26)	
d. Progrmd. Cur. FY (27–33)	27 28: · 29–33: · 34 35: · 36–40:
e. " " " +1 (34–40)	
f. " " " +2 (41–47)	41 42: · 43–47: · 48 49: · 50–54:
g. " " " +3 (48–54)	
h. " " " +4 (55–61)	
i. " " " +5 (62–68)	55 56: · 57–61: · 62 63: · 64–68:
j. " " " +6 (69–75)	
k. Total Man Years of Effort (76–78)	69 70: · 71–75: · 76–78:

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ANNUAL PROGRESS REPORT

Project No. 3A0125013818 Title: Communicable Diseases and Immunology

Task No. 01 Title: Communicable Diseases

Subtask No. 25 Title: Pathogenesis of Enteric Disease

Description: The pathogenesis of enteric disease is studied to elucidate the mechanisms by which enteric pathogens produce symptoms. By understanding the disease process, improved procedures for prevention and treatment of diarrheal diseases become evident.

Progress:

1. Studies on the pathogenesis of bacillary dysentery continued. It was previously reported (Annual Report, 1962-1963) that an opaque (O) colonial variant of a virulent translucent (T) strain of S. flexneri 2a is avirulent for starved orally challenged guinea pigs. These studies have continued in an attempt to define other characteristics of the T-parent strain which render it pathogenic. Previous work indicated that both the T-parent and the O-variant multiplied equally well in vitro and in vivo in the ligated guinea pig intestine. The lethal effect of the virulent T-parent in guinea pigs could not be ascribed to toxicity for when live or acetone killed and dried AKD cells of either the T-parent or the O-variant were administered to mice by intraperitoneal injection the LD_{50} for each strain was the same. When starved guinea pigs were fed either the virulent T-parent or the avirulent O-variant followed by an intraperitoneal injection of opium and sacrificed at intervals following challenge, the quantitative counts of viable shigella in the small intestine revealed that up to 8 hours both strains multiplied equally well. But, at 24 hours when the effects of opium had worn off, the virulent parent was present in numbers 100-fold greater than the avirulent O-variant. Studies of frozen sections of the ileum by fluorescent antibody (FA) methods confirmed that the T-parent invades the mucosa within 8 hours multiplies there, and by 24 hours large numbers of dysentery bacilli were seen in the mucosa and lumen. Ulcerative lesions containing shigella were a constant feature. In contrast, the avirulent O-variant did not penetrate the epithelial barrier; although large numbers of specifically fluorescing shigella were observed in the lumen at 8 hours, very few were seen at 24 hours. This suggested that the T-parent remains in the small intestine because of its ability to invade and multiply within the intestinal mucosa. The avirulent mutant, although capable of multiplying in the intestinal tract, does not invade tissue and so is subject to the normal cleansing action of bowel motility. Further it was demonstrated that the virulent T-parent reached the lamina propria by transmigration through intact intestinal epithelium cells. The evidence for epithelial cell penetration first obtained by fluorescent antibody methods has been confirmed using electron microscopic techniques (Annual Report 1963-1964 Takeuchi et al.). The end result of transmigration and multiplication in the lamina propria

by virulent S. flexneri 2a is the formation of ulcerative lesions, the pathognomic feature of bacillary dysentery in humans. Avirulence of the opaque colonial variant of S. flexneri 2a for experimentally infected guinea pigs is associated with its inability to penetrate the intact intestinal epithelial barrier. Corollary evidence has been accumulated to show that the above phenomenon is not restricted to one S. flexneri type. Avirulent colonial mutants incapable of penetrating intestinal epithelium in experimentally infected guinea pigs have now been isolated from additional S. flexneri types 1b, 4 and 5.

Studies on the pathology of bacillary dysentery in laboratory animals have been extended to monkeys. We have confirmed that a significant number of monkeys fed an oral dose of approximately 5×10^{10} virulent S. flexneri 2a will experience clinical symptoms of bacillary dysentery. The incidence of clinical disease can be increased if the animals are given subcutaneous injections of morphine. Animals with clinical symptoms of bacillary dysentery were sacrificed and tissue specimens of the intestinal tract examined for pathological changes both by conventional procedures and by fluorescent antibody techniques; ulcerative lesions of the cecum and colon were observed in all of the animals with clinical disease. Fluorescent antibody studies indicated that the lesions consisted of inflammatory cells, necrotic debris and specifically fluorescing dysentery organisms. Although, in most of the animals the lesions were so severe that the normal mucosal architecture was masked, transmigration of virulent shigella across the intact epithelial barrier was seen sufficiently often so as to be a consistent feature of the disease in monkeys. Avirulent S. flexneri 2a incapable of penetrating intestinal epithelium in experimentally infected guinea pigs were not observed in the epithelial cells or in the lamina propria when this strain was fed to monkeys.

From the work in guinea pigs and monkeys it seemed reasonable to explore further the possibility that virulent and avirulent S. flexneri serotypes could be differentiated on the basis of their ability to penetrate epithelial cells. We previously reported (Annual Report, 1961-1962) some preliminary studies which indicated that virulent S. flexneri 2a (strain 2457T) could infect and multiply within Henle Intestinal Epithelial cells while the avirulent S. flexneri 2a (strain 2457 0) could not. Because of difficulties encountered with the Henle cell line the system was changed to one using HeLa cells. HeLa cell monolayers were grown on coverslips in 60 mm plastic tissue culture dishes (5 cover slips/plate). Cell sheets in the logarithmic growth phase were inoculated with 3×10^7 cells/ml of the strain of the S. flexneri serotype to be examined. The infected cultures were incubated in a carbon dioxide incubator for 3 hours. After the initial infection period, the extracellular fluid was removed, the monolayers washed, and fresh medium replaced. This was repeated every two hours thereafter to minimize extracellular multiplication. Coverslips were removed from the dishes at 5 and 7 hours after infection, stained with Giemsa stain and examined for the presence of organisms within the HeLa cells. Cells were considered infected if more than 5 individual bacteria could be found within a single HeLa cell. We have confirmed that the virulent parent S. flexneri 2a (strain 2457T) is able to invade and multiply within HeLa cells, while the avirulent colonial mutant is incapable of invading the cultured cells. A large number of strains from

representative S. flexneri serotypes and several strains of S. sonnei have tested in this system. In every case, strains which were virulent for the starved guinea pig were also capable of infecting and multiplying within HeLa cells. All strains which were avirulent for the starved guinea pig were also unable to infect HeLa cells. Thus, every strain tested could be differentiated as virulent or avirulent on the basis of its ability to infect monolayers of HeLa cells cultured in vitro. Studies on the mechanism of the invasive principle are in progress.

Other workers have shown that virulent S. flexneri serotypes placed in the conjunctival sac of laboratory animals produced a keratoconjunctivitis. We have reproduced this work employing the guinea pig eye as a model. The conjunctival sac of normal guinea pigs were inoculated with 5×10^7 virulent parent S. flexneri 2a (strain 2457T). The animals were observed daily for symptomatic changes indicative of a keratoconjunctivitis, and after 3 days the eyes were removed and examined. Ulcerative lesions were regularly produced by the virulent S. flexneri 2a strain. Further the other consistent feature of the disease was the presence of dysentery bacilli within intact corneal epithelial cells. In contrast, when the conjunctival sac was infected with as many as 5×10^9 avirulent S. flexneri 2a (strain 24570) no ulcerative lesions were formed and invasion of the corneal epithelium did not occur. Also, it was difficult to isolate the avirulent mutant from the eye after 24 hours. These studies were extended to include all the strains studied with the HeLa cell culture system. At the same time starved guinea pigs were fed the same strains as a control for virulence or avirulence. In every instance strains which failed to produce a keratoconjunctivitis also did not invade HeLa cells and were avirulent for the starved guinea pig. Strains which did produce a keratoconjunctivitis also invaded HeLa cells and produced death and pathological lesions in the guinea pig intestine. Thus it appears that epithelial cell penetration is one of the basic pathogenetic mechanisms involved in the disease of bacillary dysentery. The use of this criterion as a control for safety of oral vaccines for protection against bacillary dysentery is reported below.

2. Studies employing orally administered avirulent Shigella cultures as vaccines against bacillary dysentery have continued. On the basis of both old and recent work, tentative criteria for the vaccine strains have been made: 1. The vaccine strain should be sensitive to antibiotic or chemotherapeutic agents commonly used for the treatment of bacillary dysentery. 2. The vaccine strain should cause neither death nor pathology in the experimental guinea pig model. 3. The vaccine strain should not invade HeLa cell cultures. 4. The vaccine strain should not cause keratoconjunctivitis in guinea pigs. 5. The vaccine strain should cause neither symptoms nor pathology when fed to monkeys. 6. Wherever possible the vaccine strain should be tested for its ability to protect monkeys against experimental challenge with a virulent culture of the same serotype. We have five strains of dysentery bacilli which fulfill the first four of the above requirements. Shigella flexneri 1b strain 2381), S. flexneri 2a strain 2457 0, S. flexneri 4, strain 4-12 and S. flexneri 5, strain M90TX are strains which have spontaneously mutated to avirulent forms. S. sonnei (phase I) strain 53GX7 is an avirulent culture obtained by hybridization of S. sonnei with Escherichia coli. Only the strain of S. flexneri 2a has been tested in monkeys and doses of 4×10^{10} cells

fed in various dosage schedules have failed to produce diarrheal symptoms. Monkeys fed the vaccine have been challenged with a virulent culture of the same serotype. In order to observe a significant number of cases of clinical diarrhea in the control group, both immunized and control animals were made more susceptible by injecting morphine after the challenge dose was fed. The results of these studies summarized in the following tables indicate that homologous protection may be achieved with 5 doses of vaccine fed at intervals of 3 days. Three doses of vaccine does not appear to confer protection.

Exp. M-7

Treatment	Diarrhea	Remarks
28 daily Doses Vaccine	0/2	
14 daily Doses Vaccine	0/2	
Control	4/5	1 died

Animals challenged 4 days after last dose

Exp. M-19

Treatment	Diarrhea	Remarks
14 daily Doses Vaccine	2/5	Mild
Control	5/5	Symptoms severe; 2 died

Animals challenged 21 days after last dose

Exp. M-21

Treatment	Diarrhea	Remarks
5 Doses Vaccine	1/5	Mild diarrhea
3 Doses Vaccine	4/6	3 deaths
Control	2/6	2 deaths

Animals challenged 10 days after the last Vaccine

Vaccine fed at 3 day intervals

Exp. M-22

Treatment	Diarrhea	Remarks
5 Doses Vaccine	2/9	1 animal-single diarrheal stool mild diarrhea
Control	6/9	severe symptoms in 2 animals

Animals challenged 10 days after the last Vaccine

Vaccine fed at 3 day intervals

Exp. M-25

Treatment	Diarrhea	Remarks
5 Doses Vaccine	0/5	
Control	2/6	1 died

Animals challenged 10 days after last Vaccine

Vaccine fed at 3 day intervals

3. Guinea pigs given a sublethal dose of CCl_4 become extremely susceptible to the lethal effect of bacterial endotoxin. A study was done to investigate the relationship between endotoxin susceptibility and the hepatic lesion produced by CCl_4 in guinea pigs. Susceptibility was found to be closely correlated with the degree of hepatic necrosis present, being maximal 48 hours after CCl_4 (time of maximal necrosis) and returning toward normal as regeneration takes place. Cell-free homogenates of normal liver can detoxify considerable amounts of endotoxin in vitro (approximately 1 ug endotoxin/mg. liver, as determined by chick embryo assay), but CCl_4-damaged liver is only 1/250 as active as normal liver in this respect. Liver removed 120 hours after CCl_4, when regeneration is virtually complete, is almost as active as normal liver. These temporal relationships suggest that CCl_4 may induce endotoxin susceptibility by damaging the liver, possibly by rendering it incapable of detoxifying endotoxin. Inactivation of endotoxin by liver appears to be an enzymatic process, and CCl_4 is known to disrupt a number of enzymatic functions of the liver. Endotoxin susceptibility of CCl_4-treated animals does not appear to be related to reticuloendothelial blockade or adrenal insufficiency.

4. In another study the effect of endotoxin administration on blood glucose levels was determined in normal animals and in animals given CCl_4 48 hours previously. Normal animals given one LD_{90-100} of E. coli endotoxin (15 mg./kg) intravenously showed initial hyperglycemia, followed by gradual decline of blood glucose to 50 mg/100 ml. at 8 hour,

when 30% of the animals had died. In contrast, animals with liver damage, which had normal fasting blood glucose levels, showed an immediate and progressive fall to a mean level of 4 mg/100 ml. 4 hours after endotoxin. The same response was observed in CCl_4-treated animals when the dose of endotoxin was reduced to 0.063 mg/kg (235-fold). Hypoglycemia in CCl_4-treated animals given endotoxin was due to a decrease in hepatic glucose production. Maintenance of blood glucose levels in these animals by continuous intravenous infusion appeared to prolong survival but did not prevent death.

5. Guinea pig liver preparations inactivate S. marcescens endotoxin as assayed in the chick embryo. The activity is optimal at pH 6.5-7.0 and 8.5-9.0. Mitochondria and the supernatant fraction containing microsomes possess activity. Mitochondria are only active at the acid pH optimum. The activity of acetone powder extracts of mitochondria is enhanced by ATP and NAD while the mitochondria themselves are also activated by malate. It was concluded that the enzymes which inactivated endotoxin involve fatty acid activation and oxidation. Such a finding suggests that the lipid moiety of endotoxin is required for toxicity.

6. The relationship of the liver to response to bacterial endotoxin has been further explored using various types of experimental liver damage. Ethionine administration in female Hartley strain guinea pigs produces a severe degree of fatty change in the liver. Animals with this type of damage are not markedly susceptible to endotoxin, and the liver tissue shows normal ability to detoxify endotoxin in vitro. Allyl alcohol administration causes extensive hepatic necrosis in the peripheral portions of the lobule, sparing the central zone. Unlike CCl_4, allyl alcohol does not greatly diminish resistance of the animals to endotoxin, and the liver tissue maintains its ability to detoxify endotoxin in vitro. If endotoxin degradation by the liver involves fatty acid oxidation, it might be expected that damaged livers which could not detoxify endotoxin would show diminished ability to oxidize fatty acid substrates. Studies of this relationship are in progress.

7. Eleven day chick embryos are susceptible to intravenous inocula of the order of 0.001 to 0.01 ug of purified endotoxins from V. cholerae and other Gram negative bacteria. On the other hand, 15 day embryos tolerate intravenous inoculation with 100 ug of the same endotoxin preparations. Catecholamines could not be implicated in the lethal action of endotoxin in the chick embryo. Histamine, serotonin and acetylcholine were not highly toxic for the endotoxin-susceptible embryos. Heparin did not protect against endotoxin which caused capillary stasis and perivascular hemorrhage in the susceptible mebryos. Natural antibodies to V. cholerae endotoxin could not be detected in the blood during the embryonic state. A marked hypoglycemia resulted following the administration of endotoxin or insulin in the younger embryos after a slight delay in the former case. Older embryos, which were markedly tolerant to insulin, still developed transient hypoglycemia after insulin administration, but endotoxin caused only slight changes in the level of blood sugar in the older embryos. These observations have been summarized in a recent publication (Finkelstein, Proc.Soc.Exp.Biol.Med. 115:702, (1964).

Additional studies performed in collaboration with the group at the Rocky Mountain Laboratories have indicated that the chick embryo assay is indeed a valid method for measuring endotoxin. Results of a study comparing several preparations for chick embryo toxicity and pyrogenicity (Fever Index) in the rabbit indicate good agreement in the two tests. In view of the simplicity of executing chick embryo toxicity tests, this procedure should gain wide acceptance.

8. Previous studies by Dutta and his colleagues demonstrated that experimental cholera could be produced in infant rabbits by intraintestinal inoculation with viable cholera vibrios or by oral administration of multiple doses of sterile filtrates of lysates of heavy suspensions of cholera vibrios following gastric lavage. In this laboratory working on the premise that the choleragenic product(s) may be released by the vibrios cultivated in liquid medium, it was found that sterile filtrates of cultures in aerated brain heart infusion broth were highly potent choleragens; experimental cholera was produced following oral administration of a single dose of 1.0 ml. of filtrate per 100 grams body weight. In an effort to more rigidly control the conditions and the purity of the choleragenic product(s), equally potent choleragen was produced in a simple chemically defined medium supplemented with casamino acids (Syncase medium) but not in the unsupplemented basal medium. Choleraic diarrhea generally commences about 4 hours after administration of the Syncase choleragen to the infant rabbits and then progresses until the animals succumb 12 to 18 hours later. Survival of the animals can be prolonged by administration of Ringer's lactate solution in amounts equivalent to their fluid losses. In some cases over 1/3 of the body weight was administered in 3 days. In the choleraic diarrheal state in infant rabbits, there is virtually complete inhibition of gastric emptying as demonstrated by experiments in which $BaSO_4$ was administered by stomach tube and followed by x-ray photographs at intervals. When a soluble dye, bromsulphalein, was administered, dye appeared at the anus in approximately $1\frac{1}{2}$ hours in choleraic animals, whereas in controls it had not appeared at 8 hours, indicating an acceleration of intestinal transit time. In addition, a significant inhibition of sodium absorption (Table 1) was demonstrated by introducing Na^{24} into an isolated loop of choleraic and control animals, and sacrificing at 5 minutes for counts of radioactivity remaining in the loop and that found in the carcass. The diarrhea of cholera has been attributed recently, by two groups of investigators, to inhibition of the "sodium pump" largely as a result of their in vitro studies on frog skin sodium transport in the presence of products of the cholera vibrio and stools from cholera patients and from the observation that large amounts of sodium are lost in the stool of cholera patients undergoing rehydration. Histological examination of the intestines of the choleraic infant rabbits revealed, as in the human, that the loss of fluid occurs through an anatomically intact intestinal mucosa. Syncase choleragen also causes positive "loops" in the rabbit loop technique advocated by De and others. Depending on the cultural conditions used in its production, the choleragen may be separated by dialysis into two components; a non-dialyzable heat-labile product, designated Procholeragen A, and a dialyzable heat-stable moiety, designated Procholeragen B. Neither of these alone is capable of causing experimental

cholera, but choleragenic activity is restored by combining the two fractions. Under some circumstances, e.g., production of the choleragen in brain heart infusion broth, separation is not obtained in this manner.

Purified cholera endotoxin does not produce experimental cholera in infant rabbits, nor does cholera endotoxin substitute for the Procholeragen A fraction when added to Procholeragen B. Freter and Gangarosa (J.Immunol. 91:724, 1963) recently fed large amounts of cholera endotoxin (in the form of heat-killed cells) to human volunteers with no untoward effects noted.

Additional studies in this laboratory have been directed toward evaluating the role of antibody in resistance to experimental cholera, induced either by infection or by the cell-free choleragenic products. Antibacterial rabbit sera of high vibriocidal and agglutinating antibody activity, inoculated with increasing amounts of live vibrios, exhibited some protective activity against intra-intestinal challenge with living vibrios when the sera were administered passively to the infant rabbits (Table 2). These sera had no neutralizing activity for the choleragen, however (Table 3). On the other hand, anti-choleragen sera, which also contain anti-bacterial antibodies, offer some degree of protection against the intra-intestinal infection and completely neutralize the choleragens (Table 2 and 3). These sera have little or no effect against the choleragen when they are administered passively (Table 3). These results, coupled with our previous observation that the choleragen is without effect when administered parenterally, suggest that there are two loci at which one may interfere, immunologically, with experimental cholera. The first would be by preventing or limiting infection and the second by neutralizing the choleragen. The choleragen appears to act superficially at the surface of the intestinal mucosa and apparently in unavailable to circulating antibody but susceptible to antibody on the same side of the mucosa. Sera from vaccinated humans had no neutralizing activity against the choleragens (Table 3) nor did sera from two human cholera convalescents (Table 3) (the latter observation in accord with the proposed superficial locus of action of the choleragen). Some evidence has been obtained to indicate that some immunity against either challenge (Tables 2 and 3) may be transmitted congenitally to the offspring of immunized mother rabbits. Additional work is in progress to evaluate the effect of purified anti-choleragen antibody and to provide an immunological means of identifying the choleragen.

Table 1

Effect of Experimental Cholera in Infant Rabbits

On Intestinal Absorption of Na^{24}

Na^{24} content (gut loop)*		Na^{24} content (carcass)*	
Cholera	Control	Cholera	Control
92.2**	29.2	8.9	70.0
52.3	15.2	49.9	82.9
72.6	43.5	30.4	58.3
58.0	30.5	42.5	67.3
91.3	31.7	11.0	66.8
69.7	64.8	35.1	37.9
$\bar{Y}$ 72.7	35.8	29.8	63.9
$t_{(10)}$ 3.83***		3.72***	

* Five minutes after administration

** Per Cent of inoculated Na^{24}

*** P = 0.01

Table 2

Effect of Antibody on Intra-Intestinal Infection

with *V. cholerae*

Type of Antibody	Type of Test	Result*		
		Typical	Delayed or Atypical	No Disease
None	Controls	27	2	0
Antibacterial	Passive protection	1	5	8
Anticholeragen	Passive protection	8	18	15
Anticholeragen	Congenital passive protection	0	3	4

*No. of animals in indicated category of response.

Table 3

Effect of Antibody on Choleragenic Activity of

Cell-Free Choleragens Administered *per os*

Type of Antibody	Type of Test	Results
None	Controls	40/42*
Anti-choleragen (Sonicate, BHIB, Syncase)	Neutralization	0/27
Anti-heated choleragen	Neutralization	6/6
Anti-bacterial		
Rabbit	Neutralization	9/10
Vaccinated human (4)	Neutralization	12/12
Cholera convalescent (2)	Neutralization	6/6
Anti-choleragen	Passive protection	15/17
Anti-choleragen	Congenital passive protection	6/11

*No. of animals responding with fatal choleraic diarrhea/total.

9. Virulence of El Tor vibrios for chick embryos. Studies in this laboratory have amply confirmed the earlier findings of Lankford and his colleagues with regard to the virulence of V. cholerae strains for chick embryos inoculated allantoically at 13 days of age. An LD_{50} value characteristic for a given strain is obtained when deaths of the embryos are recorded at 24 hours. The LD_{50} values generally range from 10^2-10^7 organisms. If incubation is extended, generally an inoculum of a single smooth cholera vibrio is sufficient to cause death in 48 to 72 hours; however, deaths are rare prior to 20 hours even in embryos which receive heavy inocula. In contrast, El Tor vibrios uniformly cause death in chick embryos, inoculated with the order or 100 viable cells or less, by 16-18 hours after inoculation. A total of 68 El Tor vibrio strains, with a single exception, was found to exhibit this exalted virulence pattern. The strains tested included isolates at the Quarantine Station of El Tor in the Sinai Peninsula; from Hong Kong, 1961; from the Philippine outbreaks of 1961 and 1962; non-hemolytic El Tor strains from New Guinea; paired hemolytic and non-hemolytic strains from the Celebes; cultures from the 1960 outbreak at Ubol, Thailand; and current isolates from Malaya, Thailand, Korea, Burma and Viet Nam as well as isolates from water in the Calcutta area. The single exception, which was avirulent, was a 1934 isolate from El Tor. The exalted virulence was not associated with any increased ability to multiply in the chick embryo nor was it associated with the hemolytic or hemagglutinative activity. Perhaps the reason for the exalted virulence resides in the production of an additional lethal toxin by the El Tor vibrios, but additional study is needed to define the lethal principle. It is unlikely that it is significant in the ability of these strains to cause cholera in humans since the disease manifestations are identical with those elicited by true V. cholerae.

10. Use of polymyxin as an adjunct in the rapid isolation and identification of El Tor vibrios. Finkelstein and Gomez (1963) recently advocated the use of the oblique light technique recommended by Lankford and demonstrated that ordinary nutrient agar was quite satisfactory as a primary plating medium for isolation of cholera vibrios when coupled with the oblique light technique. However, the simultaneous growth of other enteric organisms could present a problem to inexperienced personnel, and overgrowth by commensal organisms could obscure the results if plates were not examined early or if there was a tremendously high proportion of commensal organisms relative to the cholera vibrios. The observation of Han and Khie (Amer.J.Hyg. 77:184, 1963) that El Tor vibrios were resistant to polymyxin whereas classical cholera vibrios were sensitive suggested to us that it might prove advantageous to incorporate polymyxin in primary isolation media for El Tor vibrios. Preliminary tests confirmed the observations of Han and Khie. Growth of El Tor strains was undiminished on nutrient agar containing 10 u of PM per ml., while V. cholerae strains were markedly or completely inhibited by that level of antibiotic. El Tor vibrios added to normal stool suspensions could readily be recovered on polymyxin agar which markedly inhibited the growth of the normal fecal microflora. The technique was evaluated at the Pasteur Institute in Saigon, Viet Nam during TDY of one

of the investigators (RAF) during the 1964 cholera epidemic. PM agar was tested in parallel with ordinary meat extract agar and with the alkaline lauryl sulfate tellurite agar of Felsenfeld and Watanabe. Parallel determinations on the same specimens were made by the staff of the Pasteur Institute. Over 200 specimens were tested with 123 isolates. The number of isolates was practically identical on each of the three media. However, addition of PM had some beneficial effect in reducing the background commensal microflora. Frequently, the alkaline lauryl sulfate tellurite medium did not yield as many colonies of the El Tor vibrio as did the other media, but it was the most highly selective. The staff of the Pasteur Institute employing Monsur's gelatin tellurite agar isolated 100 strains from the same specimens.

11. Fifty-two fresh isolated strains of E. coli from pediatric patients at Children's Hospital, Washington, D.C. were tested for virulence for 13-day chick embryos by allantoic inoculation of serial dilutions of viable cell suspensions. From the lethal patterns which emerged, no clearcut relationship between dose and response, or death of the embryos, could be demonstrated. Thus, it was not possible to derive clearcut LD_{50} values for each of the strains which could be used as a basis for comparisons of virulence. Rather it appeared that regardless of the size of the inoculum, many strains were capable of killing only a proportion of the inoculated eggs. This proportion, with only slight variation in repeated tests, appeared to be characteristic for groups of strains. From these results, the strains were classified as "Virulent" if 70-100 % of the inoculated eggs succumbed, as "partially virulent" if 20-70 % succumbed, and as "avirulent" if less than 20 % succumbed within a 3-day interval. No association between enteropathogenicity and embryo virulence could be demonstrated (Table 4). However, there was an association between virulence for chick embryos and the ability to cause direct hemagglutination of chicken erythrocytes in a slide test (Table 5).

Additional studies have suggested that virulence may be associated with the production and availability of toxic factors. The age of the embryo and the route of inoculation exert marked effects on the outcome challenge with E. coli strains. Of the ages tested, 8-day embryos were found to be most susceptible and 16-day embryos were most resistant. The 13-day embryo was most useful in differentiating among strains of E. coli while a return to greater susceptibility was manifest in the 19-day embryo. Even "avirulent" strains were lethal when inoculated intravenously in 13-day embryos or when inoculated on the CAM in which case death was somewhat slower than when the intravenous route was employed.

Table 4

Virulence of E. coli strains for chick embryos

Serological classification	Virulence for chick embryos			
	Virulent	Partially Virulent	Avirulent	Total
Enteropathogenic	1	3	7	11
Non-enteropathogenic	1	6	2	9
Non-typable	0	9	15	24
Rough	1	3	4	8
	3	21	27	52

Table 5

Comparison between virulence of E. coli strains for chick embryos and hemagglutinin activity

Virulence category	Hemagglutinin activity No. Positive/No. Tested	% positive
Virulent	3/3	100.0
Partially virulent	6/19	31.6
Avirulent	2/26	7/6

12. This study was undertaken to determine (1) whether it was possible to obtain a satisfactory primary antibody response by injection of reduced amounts of cholera vaccine (present cholera vaccines contain 8 billion organisms per ml) and (2) to determine whether there were any significant differences in responsiveness associated with belonging to different blood groups. There have been some suggestions that members of different blood groups respond differently to bacterial antigens. In a preliminary phase of this study (Annual Report, 30 June 1963), the responses of the few individuals who received 0.1 ml of cholera vaccine produced at WRAIR were similar to those who received conventional doses. Accordingly, to differentiate further between responses of individuals, approximately equal numbers of subjects representing each of the A, B, O, and AB blood groups were inoculated with a single dose, equivalent to 0.05 ml of cholera vaccine. Their sera were titrated for agglutinating and vibriocidal antibodies against both the Inaba and Ogawa serotypes of cholera vibrios before and 2 weeks following inoculation. Titrations for agglutinating antibody were performed by adding equal volumes of viable cell suspensions (standardized at 90% T) of V. cholerae Inaba and Ogawa to 0.5 ml of serial twofold dilutions of serum in clear plastic trays. The Aminco Serological Diluter was employed in making the dilutions of the multiple serum samples. The mixtures were incubated at 37° C for 2 hours followed by storage overnight in the cold. The endpoint was the highest dilution of serum with macroscopically visible evidence of agglutination. Vibriocidal antibody titrations were performed as described by Finkelstein (J.Immunol. 89:264, 1962). Essentially all of the individuals were devoid of agglutinating antibody prior to inoculation. Following inoculation, almost all (97%) responded with agglutinating antibody against one or both serotypes. These results are summarized in Table 6. Differences in responses among the blood groups were not statistically significant.

In contrast with the absence of agglutinating antibody in preinoculation sera, the normal sera had evidence of vibriocidal antibody activity as previously reported (Finkelstein, J.Immunol., 89:264, 1962 and Basaca-Sevilla, Pesigan and Finkelstein, Amer.J.Trop.Med. 13:100, 1964). The distribution of natural vibriocidal antibody activity and the distribution of vibriocidal antibody titers following inoculation are depicted in Table 7. There were no significant differences in vibriocidal activity among the blood groups either prior to or after inoculation (Table 8). However, it is apparent that there were significant rises (approximately 2.5 logs) following inoculation in all groups. The antibody rises (both agglutinating and vibriocidal) observed in this study in response to 1/20th ml of cholera vaccine were nearly comparable to those obtained in a previous study employing conventional doses of cholera vaccines (Basaca-Sevilla, Pesigan and Finkelstein, Am.J.Trop.Med. 13:100, 1964). The results suggest that the dosage of cholera vaccine may be substantially reduced without affecting the primary antibody response insofar as the antibodies measured are concerned. What, if any, bearing serum antibody has on immunity to cholera is still undetermined.

Table 6.

Agglutinating Antibody Response to Inoculation

with 0.05 ml of Cholera Vaccine

Blood Group	A		B		O		AB	
Agglutinating Antigen	Inaba	Ogawa	Inaba	Ogawa	Inaba	Ogawa	Inaba	Ogawa
Geom. Mean Titer	209.8	87.5	131.8	94.5	160.0	103.8	234.3	125.5
95 % Confidence Limits	162.2-264.0	48.0-159.3	84.2-206.2	52.4-169.5	98.2-260.7	57.8-186.1	134.5-406.5	75.5-181.8
Number of subjects	23	23	25	25	24	24	20	20
Per Cent with Monotypic Titer	95.7	82.6	92.0	84.0	95.8	83.3	95.0	95.0
Per Cent without antibody rise	4.3		8.0		0.0		0.0	

Table 7

Distribution of Vibriocidal Antibody Titers

Prior to and After Inoculation

			Titer							
			10^0	10^1	10^2	10^3	10^4	10^5	10^6	10^7
Inaba	Pre-Inoc.	No.	21	29	26	15	2	0	0	0
		%	22.6	31.2	27.9	16.1	2.2	0	0	0
Inaba	Post Inoc.	No.	0	2	8	21	34	23	3	0
		%	0	2.2	8.8	23.1	37.4	25.3	3.3	0
Ogawa	Pre-Inoc.	No.	8	16	35	22	5	0	0	0
		%	9.3	18.6	40.7	25.6	5.8	0	0	0
Ogawa	Post Inoc.	No.	0	1	1	13	37	25	5	0
		%	0	1.2	1.2	15.9	45.1	30.5	6.1	0

Table 8

Relationship of Vibriocidal Antibody Titers to Blood Group

	Blood Group							
	A		B		O		AB	
	In	Og	In	Og	In	Og	In	Og
Pre-Inoc. Geom. Mean Titer	34.8	82.5	36.3	155.1	12.1	56.2	39.8	171.9
95 % C.L.	13.2-92.7	29.4-231.4	15.9-82.9	75.9-316.6	4.5-32.6	15.5-204.6	10.6-149.8	64.2-460.1
Post-Inoc. Geom. Mean Titer*	12.1	13.3	4.8	21.5	4.5	12.3	10.0	21.5
95 % C.L.	5.4-27.2	5.1-35.0	1.7-13.7	7.4-62.7	1.5-13.5	5.1-29.9	2.5-39.9	8.6-54.1

* Post-Inoculation titers X 10^3

13. Five lots of cholera vaccine produced at the Vaccine Laboratory of the Pasteur Institute of Viet Nam at Dalat were obtained through the courtesy of its Director, Dr. Jean Louis. The vaccines were prepared in ampules containing 30 ml. They were clean appearing products with turbidity characteristic of bacterial vaccines. The most recent lots were stated to contain 4 milliards of bacteria (4 billion) per ml while the older lots were stated to contain 6 billion per ml. The vaccine strains, also provided by Dr. Louis, were examined and found to be typical smooth strains of Vibrio cholerae of the Inaba and Ogawa serotypes, respectively. The vaccines were assayed in mice in the variable vaccine dose - constant challenge assay in parallel with a reference standard which has been shown to be equivalent to the NIH provisional reference standards of Inaba and Ogawa serotypes. The relative potency values obtained are given in Table 9.

Table 9.

Relative Potency of Viet-Nam Cholera Vaccines

Lot No.	Exp. Date	Stated Conc.	Inaba	Ogawa
38-1	30-3-64	6 billion	3.44	80.0
186-3	30-8-64	6 billion	0.65	6.7
8-4	15-1-65	4 billion	11.0	-
86-2	25-1-65	4 billion	45.0	0.42
24-4	27-1-65	4 billion	1.0	-

From these results and our previous observations, the potencies of these vaccines appear to be entirely adequate. The ability of cholera vaccines to elicit a protective response against cholera in man has yet to be defined. Additional studies on antibody levels in Vietnamese cholera patients, some of whom received these vaccines, are in progress in the US Army Medical Research Unit in Saigon.

14. Kinetics studies of the Hfr S. flexneri 2a, strain 69 have demonstrated gross homology between the chromosomes of Shigella and E. coli. The order of gene transmission by Hfr S. flexneri is the same as E. coli. Interrupted matings demonstrate that distances between gene loci in Shigella and E. coli are the same. However, the kinetics of marker transfer by Hfr S. flexneri differ from that seen in E. coli, and indication of modification of the function of the sex factor when it is incorporated into the Shigella chromosome. Studies of other Hfr strains of S. flexneri 2a and S. flexneri 5 confirm the observations of modifications of sex factor function and gross genetic homology between

E. coli and Shigella. Difficulties have been encountered in performing interrupted matings experiments between recipient strains of Shigella and donor E. coli or Shigella. These difficulties may be simply due to mating conditions such as temperature or pH, or to inherent differences between recipient Shigella and E. coli strains.

It has been observed that the various Hfr Shigella strains which lose their group antigens as a consequence of the initial recombination event with Hfr E. coli, also lose their type specific antigen soon after primary isolation. These Hfr Shigella are now agglutinated only by polyvalent S. flexneri antiserum Reciprocal agglutinin adsorption studies using wild type recipient Shigella and Hfr Shigella antisera show that these adsorbed antisera retain significant homologous titers. This may indicate that as a result or recombination with E. coli, a new antigen has been added or that an antigen already present has been unmasked by the loss of the group and type specific antigens.

15. Thus far all studies on the genetics of Shigella indicate that this organism is allelic with Escherichia coli K-12. We have previously shown that the substitution of certain critical chromosomal regions of the Shigella genome with E. coli genetic material results in the loss of virulence of Shigella for the guinea pig and other experimental models. With the demonstration that Shigella can incorporate segments of the Escherichia genome it is hardly surprising that hybrids may be isolated which show loss of virulence. However, our screening procedures for virulence alterations were based on the premise that all virulence alleles of Escherichia would be of the avirulent phenotype. Any allele which was of the virulent phenotype in both species would not have been detected. Since the fully virulent phenotype probably is a polygenic phenomenon it is not reasonable to expect that two phenotypically avirulent organisms could occassionally give rise to a fully virulent hybrid by genetic complementation

Most virulent Shigella strains consistently segregate avirulent clones which are characterized by a change in colonial morphology. During the course of our investigations of Shigella genetics, three different avirulent colonial variants of Shigella flexneri 5 were isolated and used as genetic recipients with various donor strains of E. coli K-12. The various hybrid classes isolated from these matings were subsequently examined for virulence in several experimental models. All hybrids derived from two of the variants were avirulent. However, complete virulence restoration was consistently found to segregate among the hybrids isolated from the third variant, M90TXF. Table 1 shows the virulence of the parental strains and the hybrid classes of M90TXF. Clearly, hybrids which have substituted Escherichia genetic material in the chromosomal regions containing the genetic determinants for maltose (mal^+) and fucose (fuc^+) have regained full virulence. Both parental strains have invariably proven avirulent in numerous virulence assays. It is also of interest that hybrids carrying a full complement of Shigella genes and the mal E. coli genes as a persistent exogenote (partial diploids) also exhibited full virulence restoration. Such partial diploids return to complete avirulence by elimination of the exogenotic fragment.

Restoration of Virulence to an Avirulent Strain of *S. flexneri* 5 by Genetic Recombination with *E. coli* K-12

Hybrid Classes derived from cross *E. coli* K-12 x *S. flexneri* 5, strain M90TX	No. virulent clones / No. clones tested
lac$^+$ *ara*$^+$	0/12
ara$^+$	0/5
xyl$^+$	0/5
mal$^+$	5/6
fuc$^+$	1/8
nic$^+$	0/3
mal$^+$ *xyl*$^+$	3/3
lac$^+$ *ara*$^+$ *mal*$^+$	3/3
lac$^+$ *nic*$^+$	0/2
lac$^+$ *nic*$^+$ *mal*$^+$	1/1
fuc$^+$ *nic*$^+$	2/4
ara$^+$ *mal*$^+$	6/7

Parents:

E. coli K-12	Avirulent
S. flexneri 5, strain M90TX	Avirulent

16. Three injections of duck embryo rabies vaccine (DEV) were given either subcutaneously (1.0 ml.) or intradermally (0.2 ml.) within a one-month period to previously unimmunized normal male volunteers, and the neutralizing antibody response determined. Nineteen of 20 subjects in the subcutaneous group (95%) and 14 of 20 in the intradermal group (70%) showed antibody 30 days after the third dose. This difference is statistically significant ($p = 0.04158$). The geometric mean titer of the subcutaneous group was also significantly higher ($p < 0.05$). Of 23 additional subjects who had received 3 or more injections of rabies vaccine (various types) prior to the study, 22 had antibody 30 days after a single subcutaneous injection (1.0 ml.) of DEV. It is concluded that DEV is a suitable agent for the pre-exposure immunization of individuals at high risk of exposure to rabies. Three subcutaneous injections (1.0 ml. each) given within a one-month period will result in the appearance of antibody in a high proportion of recipients. Most individuals who have received 3 or more injections of rabies vaccine within the preceding 4 years will show antibody following a single subcutaneous injection (1.0 ml.) of DEV.

Summary and Conclusions:

1. Pathogenic strains of dysentery bacilli can be distinguished from non-pathogenic strains by virtue of the former's ability to penetrate epithelial cells.

2. An attenuated strain of Shigella flexneri 2a administered orally renders monkeys resistant to challenge with a virulent culture of the same serotype.

3. Guinea pigs with hepatic damage due to CCl_4 are abnormally susceptible to bacterial endotoxin. Normal liver is capable of detoxifying endotoxin *in vitro*, whereas CCl_4-damaged liver shows very little activity. The liver may play a major role in the response of animals to bacterial endotoxin.

4. Animals with liver damage respond to endotoxin with progressive, profound hypoglycemia, which is due to defective glucose production by the liver.

5. Studies of the activity of the enzymes involved in detoxification of endotoxin by liver tissue indicate that endotoxin is inactivated by activation and oxidation of the fatty acid portion of the molecule. Thus the lipid moiety of endotoxin appears to be essential for toxicity.

6. Studies of the effects of various other hepatotoxic drugs, such as allyl alcohol and ethionine, on response to endotoxin, hepatic endotoxin-detoxifying capacity, and ability of liver cell preparations to oxidize fatty acid substrates are in progress.

7. The chick embryo has been demonstrated to be a useful tool for the assay of endotoxin and for investigation of its mode of action.

8. An antigenic cell-free product which causes experimental cholera in infant rabbits is released in young aerated cultures of Vibrio cholerae in a simple chemically defined medium supplemented with casamino acids. The physiological, pathological and immunological attributes of this "Syncase Choleragen" are under investigation.

9. El Tor Vibrios can be distinguished from V. cholerae strains by the exalted virulence of the former for 13-day chick embryos inoculated allantoically.

10. Polymyxin has been found to be a useful adjunct when added to primary isolation medium for detection of El Tor vibrios.

11. Escherichia coli strains vary in their ability to cause death of chick embryos. The age of the embryos and the route of inoculation are important aspects. The results suggest that elaboration and availability of toxic factors may have a bearing on the outcome. No association between virulence for embryos and enteropathogenicity could be demonstrated.

12. A cholera vaccine prepared at WRAIR produced an immune response in human beings when administered in one-twentieth the dose commonly employed for such products.

13. Five lots of cholera vaccine, prepared at the Vaccine Laboratory of the Pasteur Institute in Dalat, Viet Nam, and supplied by its Director, Dr. Jean Louis, were tested for potency in mice in comparison with our reference standard. These vaccines were in use during the recent cholera (El Tor) outbreak in Viet Nam. In preliminary mouse potency tests, the lots of vaccine were found to be nearly equivalent to or considerably more potent than the reference standard.

14. Kinetic studies of an Hfr S. flexneri 2a strain have demonstrated gross homology between the chromosomes of Shigella and E. coli.

15. An avirulent mutant of S. flexneri 5 when mated with an avirulent Hfr strain of E. coli regains its capacity to cause disease in experimental animal models if coligenetic material from the maltose-fucose region is incorporated into the Shigella genone.

16. Pre-exposure immunization against rabies using duck embryo vaccine was found to be more reliable when the subcutaneous route was used than when vaccine was given intradermally.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER 36185

PROJECT, TASK, OR SUBTASK NO. 3A012501A8180126

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
A Walter Reed Army Inst of Rsch
Walter Reed Army Medical Center
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5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER
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WRAIR, WRAMC, Washington, D. C., 20012
576-2677 or Interdepartmental Code 198, Ext 2677 See Continuation Sheet 49

6. TITLE OF: PROJECT ☐ TASK ☐ SUBTASK ☒ Histopathologic manifestations (U)

7. DATE OF REPORT DAY 30 MONTH June YEAR 1964

8. RESUME' (U) The anatomy of the intestinal tract is studied under various conditions and by various methods to further knowledge of general gastrointestinal physiology and the pathogenesis of diarrheal disease. Electron microscopic and histochemical studies of intestinal structure and enzyme distribution are made in normal and diseased states. Neuroanatomical studies are being carried out to determine the effects of bacterial endotoxin and enteric infections on the autonomic nerves of the intestines. Various aspects of the effect of the germfree state on the intestinal tract are being investigated. Collaborative studies with the Department of Applied Immunology on the pathogenesis of cholera and bacillary dysentery are in progress. Other investigations are concerned with the pathogenesis and immune host responses of relapsing fever. Collaborative studies on certain aspects of schistosomiasis are being carried out with the Department of Medical Zoology. A major effort has been expanded in research on malaria.

9. KEY WORDS
Intestine, gut, enteric, cholera, dysentery, bacteria, germfree, schistosome, relapsing fever, malaria.

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36185	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	3A012501A818 / 01 / 26 / 27 28 / 29
14. DATE OF REPORT (30-33) 15. SECURITY OF WORK (34)	0664 / 4
16. TYPE OF REPORT	3 / 36 47 48 49 50 51 / 52 55: 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	010607 / 62 64
18. OSD CLASSIFICATION (65-66) 19. R&D CATEGORY (67)	AR / 67: 1

Card "D"

20. CONTRACT NUMBER	DA / 13 14 / 15 17 / 18 21 / 22 26 / 27
21. GRANT NUMBER	DA / 30 33 / 34 35 / 36 38 / 39 40 / G 41 45 / 46
22. ESTIMATED COMPLET. DATES	1CONT / 2 52 56 / 3 57 61 / 4 62 66 / 5 67 71

Card "E"

23. PRIORITY (11-14) 24. PROGRAM ELEMENT (15-26)	1 / 6.11.25.01.1
25. CMR&D CODES	N/A / 30 32 / 33 35
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	1412 / 40 / a 41 42 / 43 44 / 6 45

Card "F"

27. FUNDING

a. Est. Total Cost (11-15)	11 15
b. % Spent Intern. (16-18)	16 18: 1
" " Extern. (19-21)	19 21: 2
c. Total Obligation (22-26)	22 26
d. Progrmd. Cur. FY (27-33)	27 28 / 29 33
e. " " " +1 (34-40)	34 35 / 36 40
f. " " " +2 (41-47)	41 42 / 43 47
g. " " " +3 (48-54)	48 49 / 50 54
h. " " " +4 (55-61)	55 56 / 57 61
i. " " " +5 (62-68)	62 63 / 64 68
j. " " " +6 (69-75)	69 70 / 71 75
k. Total Man Years of Effort (76-78)	76 78

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ANNUAL PROGRESS REPORT

Project No. 3A012501A818 Title: Communicable Diseases and Immunology

Task No. 01 Title: Communicable Diseases

Subtask No. 26 Title: Histopathologic manifestations

Description:

The intent of the project was to continue to advance our knowledge in the fields listed in the Resumé.

Progress:

1. Malaria.

a. A preliminary study was undertaken on mice infected with P. berghei to investigate the possible applications of enzyme histochemistry to the study of malaria.

The demonstration of enzymes in the parasites themselves is complicated by the presence of pigment that tends to mask any dye precipitated by enzymatic action.

A survey of the enzymes in several organs of the infected host has shown that the liver enzymes appear altered even before morphologic changes can be demonstrated by routine methods.

Since it has been reported that young rats develop a lethal infection from P. berghei while the older rats survive this infection it was decided to compare the enzymatic changes in the liver of young and old rats infected with P. berghei in an effort to recognize possible conditions responsible for survival. To this effect pilot experiments are being conducted to establish mortality rates for different size rats infected with different strains of P. berghei, and to test different methods of handling tissues, counting parasites and demonstrating enzymes.

b. The feasibility of acridine orange fluorescent staining of malarial parasites in peripheral blood smears was investigated for use in machine counting of parasites.

A method for staining malarial parasites in peripheral blood smears with acridine orange has been developed. With presently available instruments for automatic counting, this staining technique has no foreseeable value. It does, however, show some promise of being superior to Romanovsky stains for rapidity of detection of parasites and for

detection of parasites at extremely low levels of parasitemia. It is hoped that trials in detection of human malaria can be arranged to assess its practical value.

c. It was initially hoped that a model of passive immunity could be established against P. berghei infection in mice and by in vivo use of fluorescein isothiocyanate-tagged immune serum, determine on what stage of the asexual erythrocyte cycle antibodies acted and, if possible, gain some insight into how such antibodies could act to reduce parasitemia.

It was found that the mouse model of infection was relatively poor for the degree of immunity needed for such studies. However, a satisfactory model has now been established in rats by infecting them for three successive times at monthly intervals. Immune serum, so obtained, is capable of producing a 50- to 100-fold drop in parasitemia in 48 hours in infected young rats while normal rat serum has no demonstrable effect. Studies using fluorescein isothiocyanate-tagged immune and normal serum are now in progress.

d. Intraperitoneal administration of chloroquine to mice infected with Plasmodium berghei has been shown here to induce a series of morphologic changes in the parasites beginning with objectively demonstrable clumping of pigment granules in the cytoplasm of trophozoites within 35 minutes. We are now attempting to extend these studies by means of electron microscopy hoping to gain some insight into the mechanism of action of chloroquine.

Our findings to date indicate that malaria pigment normally appears to consist of individual crystals, each surrounded by single membranes. One hour after chloroquine administration a condensation of these structures has occurred resulting in a smaller number of larger membrane enclosed spaces containing many individual crystals of pigment. Studies of later stages of chloroquine effect are underway.

2. Gastrointestinal System.

a. Recently a baseline study of tissue artefacts in the neurovegetative periphery was completed in order to fill a gap in current knowledge on this point.

b. A study of Auerbach's plexus of the conventional and germfree cecum has shown that the intramural network has been stretched in response to the distention of the cecum. The greater stretching occurs on the anti-mesenteric side, while there is some compression of the mesenteric side, much as one would expect to see if a mesh work was drawn on a balloon, which was then inflated. There has been no actual growth of the nervous system.

c. The study of enzymatic changes in the intestinal mucosa during the course of Shigella and cholera infections in the monkey and the guinea pig was continued in collaboration with the Department of Applied Immunology.

In the monkey histochemical studies showed that vaccination prevents the changes in the enzymatic activity of the intestinal mucosa observed in the non-vaccinated monkeys, when challenged with Shigella flexneri.

In the guinea pig histochemical studies showed that animals challenged with non-virulent strains of Shigella showed enzymatic activity in the small intestine similar to that in the starved controls, while the animals challenged with virulent strains showed a decrease in the enzymes activity proportional to the severity of the morphologic alterations.

Also in the guinea pig histochemical studies showed that the activity of some enzymes in the mucosa of the small intestine of the guinea pig showed slight changes already 8 hours after challenge with the cholera vibrio, followed by a rapid decline of the activity of most of the enzymes studied 18 and 24 hours after challenge.

Since both the dysentery and cholera studies use, as experimental models, guinea pigs starved for 5 days, a concurrent study is being conducted to determine the effect of the starvation on the guinea pig, and to elucidate which changes take place to make it susceptible to these enteric diseases to which it is naturally resistant.

d. Few lesions have been described in the abdominal ganglia in lead poisoning, although many clinical manifestations and symptoms seem to indicate their involvement. A systematic study of the abdominal autonomic nervous system in experimental lead intoxication has been undertaken in young guinea pigs.

e. The anti-nerve growth factor of Levi-Montalcini has been studied in neonatal rats. It has been found to be effective in preventing proper growth of the sympathetic chain and abdominal sympathetic ganglia. However, the period under study must be increased beyond three months for maximum effectiveness. A study of the intramural innervation of the gut is in progress, utilizing this model. A number of physiological parameters (gut motility, BP, pulse, respiration, etc.) were unaffected by the loss of sympathetic neurons. A study of the antibody response of this model has been projected for the coming year.

f. Investigation of the pathogenesis and morphologic pathology of cholera is being continued using the suckling rabbit as the main experimental model as well as the modified adult guinea pig. Investigations have centered on fractionation of the vibrio into various components and testing of these for choleragenic activity as well as examining the antigenicity of the various components.

g. A pilot study was undertaken to demonstrate the intramural innervation of the intestine by means of the enzymatic reactions of neurons and ganglia. It was found that: (1) In the guinea pig Auerbach plexus, alkaline phosphatase, acetylcholinestatase and DPNH diaphorase activity could be demonstrated both in the fibers and in the neurons, acid phosphatase only in the neurons; and, (2) In the rat and in the mouse Auerbach plexus, the same enzymes could be demonstrated as in the guinea pig except the alkaline phosphatase, which gave no reactions.

h. A pilot study was made to establish the best method to demonstrate different types of mucus in the goblet cells of the intestine.

Several staining methods were used and it was found that: A combination of the PAS stain with Hale's colloidal iron reaction, with and without methylation, could be used to distinguish neutral from acid mucopolysaccharides.

The same stain combination or the Gomori aldehyde fuchsin stain, following a methylation demethylation sequence, could determine the presence of COOH groups.

The methods were applied to a series of tissues taken from guinea pigs infected with S. flexneri and their controls. In the normal guinea pig the goblet cells in the crypts are filled with either neutral or acid mucopolysaccharides or more often with a combination of both. On the villi the proportion of acid mucopolysaccharides usually increases. In the experimental group, with the increased severity of the infection, the goblet cells appeared progressively and irregularly depleted. The differential stains allowed visualization of even traces of mucus in cells, which with routine stains appeared to have discharged their mucus completely.

i. The results on the survey on "The mucosal enzymes in the cecum of conventional and germfree mice," were presented at the Symposium on Recent Advances in Germfree Animal Experimentation, and are being published in the April 1964 issue of The Anatomical Record.

j. Work was completed on the study of the histopathology of LIVIM virus infection in infant mice. LIVIM lesions were found to be limited to the intestinal tract and to be remarkable and of unique character not seen in other enteritides of mice. There was a difference in the type of response depending upon the age at the time of challenge with the virus. The response in the youngest animals was principally "cytopathologic" with alteration of the epithelial cells and the formation of bizarre syncytial giant cells associated with ineffectual cell replacement and ulceration. In the older animals the response to the virus was a "proliferative" one with intense mitotic activity in crypt glands and a reversal of the crypt-villous ratio.

The results of this work were presented to the American Federation for Clinical Research, D.C. Chapter, in October 1963, and at the Federation of American Societies for Experimental Biology in Chicago, Illinois, in April 1964. A paper has been accepted for publication by the American Journal of Pathology.

It is hoped that in collaboration with Dr. Lisbeth Kraft of the New York Public Health Institute of Research, we can continue our study of the morphologic effects of this viral agent, especially as concerns the morphogenesis of the peculiar syncytial giant cell and other interesting features of the LIVIM complex.

k. Electron microscopic studies on the guinea pig small intestine at different age groups resulted in a thesis accepted by the George Washington University for the degree of Master of Science in the Department of Anatomy, obtained by Mr. Thomas G. Merrill of the Department of Experimental Pathology, WRAIR. A preliminary abstract of this study was presented at the Annual American Association of Anatomists Meeting in Denver, Colorado, 31 March-3 April 1964. Results of this study have shown that ultrastructural changes occur in the organelles of absorptive cells as maturation proceeds from prenatal, to postnatal to adult levels. Dimensions and numbers of microvilli, mitochondrial size, shape and number, and cellular glycogen deposits, show definite alteration from the crypt area to the villus tips, as well as from one age group to another. Studies of ultrastructural changes of guinea pig intestine incident to experimental cholera and Shigella infections are underway.

Summary and Conclusions:

A multifacet collaborative research effort was expanded in the fields of experimental gastroenterology and medical parasitology.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36171

PROJECT, TASK, OR SUBTASK NO.: 3A01250 1A8180127

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
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3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
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6. TITLE OF: PROJECT ☐
TASK ☐
SUBTASK ☒ Zoonotic Diseases (U)

7. DATE OF REPORT | DAY 30 | MONTH Jun | YEAR 64

8. RESUME' (U)Leptospirosis: Methods were developed for the purification of a leptospiral toxin; cross-immunity between hemolysin from diverse types was shown; the usefulness of a new selective media for leptospira was demonstrated; an indirect fluorescent antibody technic was developed for serodiagnosis of human cases; the presence of various serotypes including new types was disclosed in Brazil and Panama; the potential leptospiral infection hazard in the use of dog kidney tissue cultures for viral vaccines was demonstrated experimentally; antibiotics removed this hazard. Listeriosis: Similarities between listeria hemolysin and other O-labile hemolysins were demonstrated. Virus and PPLO: A new pox virus from raccoons was identified as a member of variola-vaccinia group; other viral isolates from wildlife are being studied. Six types of enterovirus from swine were identified, two of which are new types; freeze-dried WEE vaccines gradually lose their potency after prolonged storage; an attenuated WEE vaccine elicited excellent antibodies in horses and was not pathogenic; the duck embryo rabies vaccine is more antigenic for man when given SC rather than ID; a fastidious PPLO capable of causing pneumonia was isolated from dogs; etiology of outbreaks of respiratory diseases in recruited dogs was studied.

9. KEY WORDS
Zoonoses, leptospiral, pox virus, enterovirus, veterinary, PPLO, toxin, Listeria, encephalitis, rabies, pneumonia

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
[x] NO [ ] YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
[x] NO [ ] YES See Continuation Sheet

DA FORM 1 June 63 1309R PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____

ACCESSION NO. 36171	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	3A012501A818 / 01 / 27 /
14. DATE OF REPORT (30-33)	0664
15. SECURITY OF WORK (34)	4
16. TYPE OF REPORT	3 / 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	010609 /
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19. R&D CATEGORY (67)	1

Card "D"

20. CONTRACT NUMBER	DA
21. GRANT NUMBER	DA / G
22. ESTIMATED COMPLET. DATES	1CONT / 2 / 3 / 4 / 5

Card "E"

23. PRIORITY (11-14)	1
24. PROGRAM ELEMENT (15-26)	6.11.25.01.1
25. CMR&D CODES	N/A
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27. FUNDING

a. Est. Total Cost (11-15)	
b. % Spent Intern. (16-18)	1
" " Extern. (19-21)	2
c. Total Obligation (22-26)	
d. Progrmd. Cur. FY (27-33)	
e. " " " +1 (34-40)	
f. " " " +2 (41-47)	
g. " " " +3 (48-54)	
h. " " " +4 (55-61)	
i. " " " +5 (62-68)	
j. " " " +6 (69-75)	
k. Total Man Years of Effort (76-78)	

DA FORM 1 June 63 1309R — Previous Editions are Obsolete — Page 2 of _____

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ANNUAL PROGRESS REPORT

Project No. 3A012501A818	Title: Communicable Diseases and Immunology
Task No. 01	Title: Communicable Diseases
Subtask No. 27	Title: Zoonotic Diseases

Description:

Major objectives are to evaluate the real or potential military significance of selected zoonoses, to study basic biological characteristics of zoonotic agents, their mechanisms and modes of infection, distribution in nature and to establish methods of diagnosis, treatment and control. During the period of this report, studies were conducted on: immunological characteristics of a leptospiral toxin; a new selective medium and a new serological tool for leptospirosis; antigenic characteristics of leptospiral isolates from Latin American sources; potential leptospiral infection hazards in the use of dog kidney cultures for production of viral vaccines; characteristics of listeria toxin; selected zoonoses in wildlife; a new PPLO agent found in dog kidneys; respiratory infection in dogs recruited for experimental use; swine enteroviruses; immunological responses to WEE and rabies vaccine.

Progress:

1. Leptospirosis:

a. Characteristics of a Leptospiral Hemotoxin: The disclosure of a hemolytic toxin in cultures of specific leptospiral serotypes was reported previously from this laboratory. Earlier studies provided evidence that the toxin was a protein with enzymatic and antigenic properties. To obtain more information on the nature, mode of action and properties of the hemolytic toxin, studies were initiated to purify and chemically characterize this substance.

The starting material used for purification of the toxin is allantoic fluid from embryonated chick eggs infected with serotype pomona. The procedure used for obtaining optimum yields of hemolytic toxin was reported previously.

Uric acid, the end product of protein metabolism in the chick embryo, is present in allantoic fluid in high concentration at the time toxin is extracted from infected eggs, during the 13th and 14th days of incubation. Since uric acid has an absorption peak near that of proteins in the visible region of the spectrum and is only slightly soluble in aqueous solutions, it is desirable to ascertain that all uric acid residues

are removed from the allantoic fluid during the final dialysis step employed in the isolation procedure. A recently described enzymatic method employing uricase (Liddle, Lab. Clin. Med. 54: 903, 1959), that presumably is sensitive for detecting trace amounts of uric acid in body fluids, was investigated to determine if it could be adapted for use as a simple routine technique in the toxin isolation procedure. The data obtained in preliminary studies with this method provided indications that consistent, accurate results could not be obtained. Further study revealed that the enzyme, in the commercially available form, was very unstable under ideal conditions over a period of one to two weeks. This observation has since been noted by other investigators, therefore, no further work was done with this method. Further efforts are being made to find a sensitive method for uric acid determination.

The purification of the leptospiral toxin by a gel filtration technique was investigated. The gel employed in this study was Sephadex (cross-linked dextran molecules). In preliminary studies, attempts were made to determine if the toxin could be eluted from columns containing Sephadex of the following porosity grades: G-25, G-50, G-75, and G-100. The columns were prepared essentially by the method described by Flodin (Chromatography, 5: 103, 1961). Generally, a 2 x 22 cm or a 1.25 x 30 cm glass column, containing a specific amount of Sephadex (the amount used varies with the length of the column and the water retaining capacity characteristic of the individual lots of Sephadex) was equilibrated with .15 M NaCl. One and five tenths to 2.5 ml portions of dialyzed allantoic fluid from infected embryonated chick eggs, previously concentrated with ammonium sulfate or carbowax was passed through the column using .15 M NaCl as the eluting agent. Fractions of 10 to 30 ml each were collected and analyzed for protein concentration (U.V. absorption at 280 mm) and hemolytic activity. From the results obtained, it was possible to conclude: (1) that leptospiral toxin can be eluted from Sephadex columns and (2) the hemolytic proteins are eluted from the columns just prior to and simultaneously with the major portion of other proteins present in the crude sample of toxin. The best separations were obtained with the G-50 and G-75 columns.

Further studies were conducted using only G-50 Sephadex columns to determine the eluting agent that would result in maximal separation of crude toxin into fractions with high hemolytic activity. The following eluting agents were used: .15 M NaCl; Michaelis buffer, pH 7.6; Veronal buffer, pH 8.6 and acetate buffer, pH 5.6. The crude toxin preparation was precipitated with crystalline ammonium sulfate at 50% saturation levels and resuspended to one-tenth of its original volume with .15 M NaCl. The ammonium sulfate was removed by dialyzing the preparation against successive changes of tap water, distilled water and .15M NaCl in the cold. In each experiment, the concentrated samples were re-dialyzed in the cold for 24-36 hours in one of the four buffers to be used as the eluting agent. One and eight tenths to 2.0 ml of the concentrated samples were applied to the column and fractions of 4 to 6 ml each

were collected and analyzed for protein content and hemolytic activity.

The best separation of the allantoic fluid preparation into individual fractions was obtained using Michaelis buffer, pH 7.6. The protein content of the sample was distributed between fractions 2 through 6; the remaining fractions, 7 through 20 contained either exceedingly small concentration of protein or none at all. Upon testing these fractions for hemolytic activity, it was observed that fraction 3 contained the maximum activity. The hemolytic titer of the crude sample applied to the column was 1:32; the hemolytic titer of fraction 3 was 1:112, representing a 3.5 fold increase in hemolytic activity. It is possible that better resolution of the protein into fractions can be accomplished by using a longer column for separation and higher grades of Sephadex.

This data indicates that gel filtration techniques can be successfully applied to separate the hemolytic toxin from other extraneous materials present in the allantoic fluid. Further studies are planned in which the gel filtration technique, along with other well-established techniques, will be used in the purification and characterization of the leptospiral hematoxin.

In vivo studies in sheep on cross-protection between leptospiral hemotoxins derived from antigenically diverse strains have been reported previously (Annual Progress Report 1962-1963). It was shown that animals infected with a hemolysin-producing strain (pomona or canicola) were refractory to hemolytic effects produced by the parenteral administration of partially purified leptospiral hemolysin from an homologous or antigenically different strain. Further, it was shown that infection with a non-hemolytic strain (serotype hardjo) does not afford protection against the effects of hemolysin; however, it produced toxic effects in animals previously exposed to infection with a serologically heterologous type. These effects were rarely seen in animals previously infected with the homologous strain, or in controls inoculated with culture medium. Additional studies were conducted to determine if these cross-neutralization effects could be demonstrated in vitro.

For the conduct of neutralization tests, a standard dose of hemolysin was added to two-fold serial dilutions of test sera, and incubated at 37° C for 40 minutes; sheep red blood cells were then added and the mixtures were further kept for 4 hours at 37° C, and then at 4° C for 16-18 hours before readings were made. The neutralization titer was expressed as the reciprocal of the highest final dilution resulting in 75% inhibition of hemolytic activity. The results of these studies are shown in Table I. The normal neutralization titer of sheep sera obtained prior to infection ranged from 1:20 to 1:80; following infection the increase in hemolysin neutralizing antibodies was not remarkable, from a GM titer of 1:38 to 1:56 mean titer of animals infected with hemolysin producing strains increased. The titer for the animals infected with hardjo, a non-hemolytic strain, did not increase. The post infection levels of anti-hemolysin

Table I

Results of In Vitro Hemolysin Neutralization Studies

Exposure of Sheep	Challenge (26 to 28 days post-infection)	Index Neutralization Titer Increase* Day of Challenge, 26-28 days post-infection	7 days post challenge	14 days post challenge
Hemolytic strains (*pomona* and *canicola*)	Homologous or heterologous hemolysin	1.73	7.65	6.86
	Non-hemolytic preparation (*hardjo*)	1.97	2.10	2.10
	Heat-inactivated *pomona* hemolysin	2.90	11.8	8.43
	Concentrated media	1.0	1.0	1.0
Non-hemolytic strain (*hardjo*)	Hemolysin (*pomona* or *canicola*)	1.58	1.87	1.0
	Non-hemolytic preparation (*hardjo*)	1.0	1.0	1.0
Non-infected controls	Hemolysin (*pomona* or *canicola*)	1.0	1.0	1.0
	Non-hemolytic preparation (*hardjo*)	1.0	1.0	1.0
	Heat-inactivated *pomona* hemolysin	1.05	4.21	2.1

* Index = $\frac{\text{Test geometric mean}}{\text{Normal geometric mean}}$, geometric mean neutralization titer of normal sheep sera was 1:38. The range was 1:20 to 1:80.

1.0 = index was 1 or less

titers amongst pomona and canicola infected sheep that elicited hemolytic signs were essentially the same as those animals with no hemolytic manifestation. Animals exposed to hemolytic strains of leptospira and challenged with the homologous or heterologous hemolysins showed a six to seven fold increase in neutralizing antibodies 7 to 14 days post challenge. Similar results were obtained when a challenge of heat inactivated hemolysin was employed. In contrast, animals exposed to the non-hemolytic hardjo strain and challenged with pomona or canicola hemolysin did not show a significant increase in neutralizing antibodies 7 to 14 days post challenge. It is apparent then, that exposure of animals to hemolytic strains of leptospires elicits the formation of hemolysin-neutralizing antibodies; however, their presence is manifested after challenge with either pomona or canicola hemolysins. This rapid increase in neutralizing antibody concentration is reminiscent of an anamnestic response, thereby, substantiating the existence of low level neutralizing antibodies prior to challenge.

b. Indirect Immunofluorescence for the Serodiagnosis of Leptospiral Infections.

The potential usefulness of the Fluorescent Antibody (FA) Technique as a serological tool for the diagnosis of leptospirosis was investigated. This study was prompted by the critical need for a genus-specific antigen useable in the serodiagnosis of leptospirosis in man and animals. A nonpathogenic strain of Leptospira biflexa (Patoc) was selected as antigen. Its selection was based on the reported findings of Roumanian workers (Sturdza, et al., Arch. Roum. Path. Exper., 19: 572, 1960) that the Patoc antigen had genus-specific activity when used in a CF procedure. The CF procedure was used effectively to detect antibodies in human sera but lacked sensitivity when employed with animal sera.

A modified immunofluorescence reaction was patterned after the system used by Fife and Muschel (Proc. Soc. Expt'l. Biol. Med., 101: 540, 1959). A 3 to 5 day-old culture of leptospira in Stuart's medium was used as antigen. A film of antigen was prepared on a slide, air dried and fixed by immersion in 97% alcohol for 3 minutes. Test sera were diluted 1:100 in phosphate-buffered saline solution, pH 7.4, and applied. The"conjugate" used was a commercially-available antihuman globulin, labelled with fluorescein, prepared in either goat or mule. This had undergone one absorption process with rabbit-liver powder. When applied to the test system the "conjugate" was used in a dilution of 1:200 in a 2% Tween 80 phosphate-buffered saline solution, pH 8.5.

To date 118 human sera have been tested by the indirect immunofluorescence procedure. Forty-eight of these comprised single, paired or serial samples from 29 cases of leptospirosis. Thirteen of the 48 samples were acute phase sera, the remainder were obtained after the first week of disease and included 4 specimens obtained approximately 6 months to 1 year after infection. Infection with approximately 12 different serotypes were represented in this series. Seventy other sera were obtained from

the serum files of the Division of Veterinary Medicine and were negative for leptospiral antibodies. Antibodies were demonstrated by FA technique in all 29 leptospirosis cases. The results of FA and MA tests were in complete agreement. Thirty-five samples (convalescent and post-convalescent serums from leptospirosis cases) were positive and 83 serums were negative in both tests. The MA titers of positive sera ranged from 1:100 to 1:25,600; three of the positive sera had titers of 1:100, the remainder were positive at levels of 1:400 or greater. Preliminary findings provide indications that the FA test is a sensitive, genus-specific procedure that may be applicable as an epidemiological as well as a diagnostic tool. Additional preliminary trials are planned with sera from domestic animals to determine if the FA procedure can be more advantageously used than the "patoc CF test" in this regard.

c. Selective Medium for Leptospira. The report by Johnson and Rogers (J. Bact., 87: 422, 1964) that the addition of 5-fluorouracil (5-FU) in leptospiral medium affords selective growth of pomona and canicola from a contaminated inoculum, prompted further evaluation studies with a variety of pathogenic leptospiral serotypes. The growth of 13 different serotypes in Stuart's liquid and Fletcher's semi-solid media containing 5-FU in concentrations of 300 µg/ml was studied. The strains were selected to cover the range of poor to excellent growth characteristics noted amongst stock cultures of leptospiras. The inocula for the modified Stuart's and Fletcher's media and control media were 7 to 10 day-old cultures of strains in the respective conventional media. 5-FU media and control media were distributed into test tubes in 5 ml amounts. Both 0.1 and 0.5 ml amounts of inocula were used in tests. All tests were conducted in triplicate. Growth in Stuart's media was measured by nephelometry, in Fletcher's media by the time of appearance of the subsurface circular disc of growth characteristic of leptospiral cultures in semi-solid media.

There was no remarkable difference in the growth of 13 strains in Fletcher's media containing or lacking 5-FU with the two different levels of inoculum. Growth in Stuart's medium containing 5-FU was essentially the same as that in ordinary Stuart's medium when 0.5 ml inocula were used. However, 5-FU affected the growth of 7 of 13 strains in Stuart's media, inoculated with 0.1 ml amounts of culture. These effects were characterized by a longer lag time in growth although optimum growth eventually approximated that seen in ordinary Stuart's medium. Maximum growth in Stuart's media was attained in 7 to 14 days, correspondingly in Stuart's medium with 5-FU in 14 to 21 days.

Employing 5-FU in leptospiral media, pure cultures of 51 leptospiral strains were recovered from 77 contaminated leptospiral cultures submitted from Malaya. Eight additional strains were recovered in pure culture by the use of 5-FU media in conjunction with filtration or animal inoculation techniques.

d. Serological Studies. Cross agglutination and agglutinin adsorption studies were conducted on 14 strains isolated from animals in Brazil. This study was conducted principally by a visiting scientist as part of his training program at the WRAIR. The source and identity of the strains are as follows:

Serotype	Source
grippotyphosa	2 opossum, 1 rat
icterohaemorrhagiae	1 cow, 1 pig, 1 cavy
hyos	3 pigs
canicola	1 pig
pomona	1 pig
mini	1 opossum
new bataviae type	2 opossum

The bataviae isolates were identified as members of a new serotype (as yet undesignated), the other isolates were serologically homologous with type strains. The presence of grippotyphosa, mini and the bataviae types had not been disclosed heretofore in Brazil.

Studies on leptospiral strains isolated during an investigation of an outbreak of leptospirosis in troops stationed at Fort Kobbe, Canal Zone (see Annual Progress Report, 1962-63) are now completed. A summary of the classification studies is shown in Table II.

Attempts were also made to separate strains in a mixed culture of leptospiras originally recovered from the urine of a Spiney rat in Panama. The presence of a mixed culture was suspected because of the variable agglutination reactions of different subcultures against members of the hyos and hebdomadis serogroups. Portions of the culture in Stuart's media were treated respectively with an equal volume of undiluted anti-hyos rabbit serum and anti-kabura rabbit serum. The antiserum culture mixtures were kept at room temperature for one hour and then centrifuged at 1800 g for 5 minutes. Material near the surface of the centrifuged tubes were then subcultured into Fletcher's medium employing 0.1 ml inocula per approximately 5 ml of medium. Isolated strains were subsequently typed.

A member of the hebdomadis group and a member of the hyos group were recovered respectively from cultures treated with anti-hyos and anti-kabura sera. These particular techniques had not been applied previously for the separation of strains in mixed cultures of leptospiras.

e. Viability of Leptospiras in Dog Kidney Tissue Cultures. The use of dog kidney tissue cultures for the preparation of attenuated viral vaccines posed questions on the potential leptospiral infection hazards of such preparation. Previous studies (see Annual Progress Report, 1962-63) were conducted on the viability of leptospiras in monolayer tissue cultures prepared from kidneys of hamsters and dogs experimentally infected with serotype canicola. The antibiotics used in the maintenance fluids

Table II

Summary of Results of Classification

Studies on Panamanian Leptospiras

Strain Studied	Serotypes Used in Reciprocal Agglutinin Adsorption Tests	Identification and Designation
Bravo	hyos (Mitis Johnson), hyos (bakeri) hyos (guida), atlantae	New type - hyos group Serotype - bravo
CZ-320	bataviae, paidjan, djatzi	New type - bataviae group Serotype - kobbe
CZ-390	icterohaemorrhagiae (RGA), icterohaemorrhagiae (M20), mankarso, sarmin, ndambari, birkini, smithii, javanica, alexi, zanoni	New type - icterohaemorrhagiae group Serotype - weaveri
CZ-188	cynopteri, butembo, diasiman, grippotyphosa	New type - cynopteri group Serotype - canalzoni
CZ-285	hebdomadis (hebd.), hebdomadis (mona), wolffi, kremastos, worsfoldi, jules, borincana, kabura, mini (mini), medanensis, saxkoebing (saxk.), sejroe (sejroe), hardjo, ricardi, hemolyticus	New type - hebdomadis group Serotype - maru
CZ-299	pomona, cornelli	New subserotype of pomona - tropica
CZ-214	None	New type Serotype - panama

were a combination of penicillin, dihydrostreptomycin and amphotericin B. Viable leptospiras were found in tissue culture at the time of preparation, surviving trypsinization, but not after 24 hours of incubation of cultures. Loss of viability of leptospiras was attributed to antibiotic effects. By the time this study was completed, the use of penicillin in tissue culture vaccines was prohibited by new federal regulations. Additional studies were conducted employing other antibiotics. The source of kidneys for culture was a 6-week old dog that was infected with serotype canicola.

The procedures employed for infecting a dog and for the preparation of monolayer kidney cultures were essentially the same as those described previously except that the initial maintenance fluid used for planting the kidney cells contained 5% bovine serum; after 3 days incubation, the substituted maintenance fluids contained 2% bovine serum. The kidneys from an infected dog were processed under careful aseptic conditions without the use of antibiotics in the trypsinization and washing of kidney cells. From a common tissue cell suspension, four different final cell suspensions (dilution 1:500) were prepared as follows:

Suspension I contained no antibiotics
Suspension II contained 100 µg/ml of neomycin
Suspension III contained 100 µg/ml of chloramphenicol
Suspension IV contained 100 U/ml of penicillin and
100 µg/ml of streptomycin

The preparations were planted in multiple test tubes. Tubes were examined for leptospiras over a 25-day observation period employing microscopic, cultural and hamster inoculation technics. Each examination was made on triplicate tubes. Maintenance fluids were not changed after the third day of incubation. The pH of tissue cultures was maintained by the addition of $NaHCO_3$ when necessary.

The kidneys employed for the preparation of tissue cultures contained profuse numbers of leptospiras. A drop (ca 0.05 ml) of the final 1 in 500 cell suspension, placed on a slide and distributed under a 22 x 22 mm cover slip, had approximately 4 to 5 leptospiras per field, when examined microscopically (450X magnification). Viable leptospiras were detected in antibiotic-free tissue cultures up to the 25th day of incubation. There was no evidence of leptospiral multiplication in the antibiotic-free tissue cultures. After one day of incubation, leptospiras were still detectable microscopically; thereafter, they could only be demonstrated by culture or animal inoculation procedures. Tissue cultures containing penicillin and streptomycin were negative for leptospiras after 24 hours of incubation. Leptospiras were recovered from the neomycin-treated cultures after one day of incubation but not thereafter. In the tubes containing chloramphenicol, leptospiras were recovered after 2 days of incubation.

It was apparent from findings that leptospiral infection hazards may occur in the use of tissue culture vaccines containing no antibiotics. Such hazards can be eliminated by employing neomycin or chloramphenicol at concentrated levels of 100 µg/ml.

2. Listeriosis.

Some of the described characteristics of a hemolysin found in cultures of Listeria monocytogenes were reminiscent of those described for oxygen-labile hemolysins derived from other microorganisms. Studies were conducted to determine if other attributes of O-labile hemolysins were present in listeria hemolysin. Specific attention was directed to action of divalent cations, chelating agents, and cholesterol on the activity of Listeria hemolysin, also on the spectrum of the activity of this hemolysin on diverse animal erythrocytes.

The listeria strain employed was 5349, serotype I. Excellent hemolytic activity was found in filtrates of 96 hour old culture in a broth containing Bacto-Tryptose, yeast extract and glucose. The hemolysin was precipitated from culture filtrates by treatment with ammonium sulfate and concentrated according to the method of Girard, et.al. (J. Bact. 85: 349, 1963). The concentrated hemolysin solution in 0.85% NaCl was stored at -65° C. A standard 1% suspension of sheep red blood cells in Michaelis' buffer, pH 7.25, was used to assay hemolytic activity. Gelatin in 0.1% concentration was employed in all diluents of erythrocytes except for the divalent cation studies. The degree of hemolysin was measured spectrophotometrically. A reduced environment was maintained in all hemolytic tests by utilizing neutralized cysteine hydrochloride in a final concentration of M/20 in Michaelis' buffer.

To determine the effect of varying concentrations of disodium ethylenediaminetetraacetate(EDTA), and divalent cations (Co^{++}, Mg^{++}, Mu^{++}, Ca^{++} and Fe^{++}) on the action of hemolysin, various concentrations of the test reagents in a standard volume (0.5 ml) of reduced buffer were added in equal volume to a standard solution of hemolysin in cuvettes. Ten minutes were allowed for exposure of lysin to test substance, then sheep cells were added (1.0 cc) and mixtures were incubated at 37° C for one hour after which time cuvettes were centrifuged and the freed hemolysin was measured spectrophotometrically. As shown in Table III, EDTA had no inhibitory effect on the hemolytic capabilities of concentrated hemolysin. The ineffects of excess divalent cations - Mg^{++}, Ca^{++}, Fe^{++}, Mu^{++} and Co^{++} - on the activity of listeria hemolysin is shown in Table IV. High concentration of Co^{++} and Fe^{++} give results similar to those of the other cations, except that these ions impart a color to the medium which partially interferes with the spectrophotometric quantitation of hemolysis. The possibility of cysteine interference was ruled out by employing the same procedures with a leptospiral hemolysin whose activity is contingent on the presence of Mg^{++}. The failure of divalent cations to effect the activity of listeria hemolysins is characteristic also of other enzymes and hemolysins which are cysteine activated.

Table III

Hemolytic Activity of L. monocytogenes Hemolysin

on Sheep RBC in the Presence of EDTA

EDTA Concentration Micro-moles per ml x 10^{-2}	Per Cent Hemolysis with Dilution of Hemolysin* 1:8	1:16	1:32	1:64	1:128
0.00	100	95	70	10	-+
1.25	99	95	55	10	-
2.50	96	92	66	10	-
5.00	95	90	55	10	-
7.50	93	93	52	12	-
37.50	95	90	52	10	-
125.00	93	89	63	12	-

* Final concentration of cysteine in all dilutions was M/20.

+ Less than 10% hemolysis.

Table IV. Effect of Magnesium, Calcium, Manganese, Cobalt and Ferrous Ions on the Action of L. monocytogenes Hemolysin on Sheep Erythrocytes

EDTAuM per ml x 10^{-2}	Test Ion uM per ml x 10^{-2}	Per Cent Hemolysis with Dilution of Hemolysin 1:8	1:16	1:32	1:64	1:128	1:256	1:512
1.25	0.0	98	95	92	87	45	+-	-
1.25	Mg^{++} 5.0	100	95	94	90	51	11	-
1.25	Mg^{++} 12.5	100	99	94	92	53	10	-
1.25	Mg^{++} 125.0	100	98	94	85	57	10	-
1.25	Ca^{++} 5.0	100	97	94	87	54	-	-
1.25	Ca^{++} 12.5	100	96	95	94	57	12	-
1.25	Ca^{++} 125.0	100	100	97	92	63	16	-
1.25	Mn^{++} 5.0	99	96	92	87	50	11	-
1.25	Mn^{++} 12.5	99	97	94	90	55	10	-
1.25	Mn^{++} 125.0	96	92	90	83	51	13	-
1.25	Co^{++} 5.0*	98	94	93	83	38	-	-
1.25	Co^{++} 12.5	94	93	91	85	44	-	-
1.25	Fe^{++} 5.0*	97	94	94	89	43	-	-
1.25	Fe^{++} 12.5	100	98	95	87	40	-	-
1.25	0.0*	99	96	94	91	58	10	-

+ Less than 10 per cent hemolysis.

* Performed 24-hr after preceding titrations.

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The O-labile are known to be adsorbed from aqueous solutions by petroleum ether solutions of cholesterol. This characteristic was tested for listeria hemolysin according to techniques of Howard, et al., (Brit. J. Exp. Pathol., 34: 174, 1953). The results of tests are shown in Table V. The activity of solutions of hemolysins was greatly reduced after contact with freshly crystallized cholesterol.

Table V. Ability to Lyse Sheep RBC after Absorption of L. monocytogenes Hemolysin with Cholesterol

Non Aqueous Phase	Aqueous Phase Per Cent Hemolysis with Dilution of Hemolysin 1:8	1:16	1:32	1:64	1:128	1:256	1:512
0.3 Cholesterol in Petroleum Ether	23	-*	-	-	-	-	-
Petroleum Ether	100	100	96	70	18	-	-

* Less than 10 per cent hemolysis.

A further characteristic which serves to differentiate O-labile hemolysins from other bacterial hemolysins is their ability to lyse readily a great variety of erythrocytes. Mouse and chicken erythrocytes have been found to be more refractory to the action of O-labile hemolysins. Table VI illustrates that sheep, goat, horse, rabbit and bovine erythrocytes are similar in their susceptibility to the hemolysin.

Table VI. Hemolysis of Erythrocytes from Various Species

Erythrocyte Species	Per Cent Hemolysis with Dilution of Hemolysin 1/3	1/16	1/32	1/64	1/128	1/256	1/512
Rabbit	99	97	91	76	43	12	-*
Chicken	99	95	83	57	10	-	-
Sheep	100	100	96	95	69	19	-
Goat	100	95	94	80	44	15	-
Mouse	94	85	71	59	49	37	23
Bovine	100	98	95	68	34	-	-
Horse	99	96	92	85	68	27	-

* Less than 10 per cent hemolysis.

Chicken cells were somewhat more resistant, but an aberrant pattern can be seen with mouse erythrocytes. In comparison with red blood cells of the other species, the mouse cells were more resistant to lysis in low dilutions of hemolysin and more susceptible at higher dilutions.

3. Virological Studies.

a. Screening of Wildlife for Viruses. Virological aspects of a study of the role of wildlife in the epizootiology of selected zoonoses were continued (see Annual Progress Report, 1962-63). These studies were conducted on 97 raccoons, 104 striped skunks, 64 opossums, 10 woodchucks, 1 muskrat and 5 feral cats, all trapped in an undeveloped forest area near Aberdeen, Maryland. Further attempts have been made to isolate viruses from brain, respiratory and intestinal tissues. Pools of tissues from three to five animals have been screened extensively by various tissue culture, egg and animal inoculation technics. Approximately 90% of the specimens have been processed completely. To date, pox viruses have been isolated from the respiratory tissues of 2 raccoons, 2 isolates have been obtained from pooled liver and spleen tissues of two skunks.

The pox virus was initially isolated from a pool of respiratory tissues from 5 raccoons. Examination of the individual tissues comprising the pool resulted in isolation of pox viruses from two animals. Both pox agents appear to be identical. Some of the growth and serological characteristics establishing the tentative identity of the pox virus were reported previously. The pox agent would be grown on tissue cultures of bovine, rabbit, hamster, mouse, human (embryonic), monkey, dog and cat kidney cells. It could infect suckling mice by intracerebral or intraperitoneal routes of inoculation, but did not infect adult mice, hamsters and guinea pigs. Vesicular lesions in rabbits were produced in some trials after successive passages. In monkey kidney tissue, growth of the agent was inhibited by 5-iodo-2-deoxyuridine; this inhibition could be reversed by the addition of excess thymidine. Inoculation of the agent on chorioallantoic membrane (CAM) of 12-day old embryonated hen's eggs produce discretely embedded plaques. Fixed sections of infected chorioallantoic membrane stained with hematoxylin and eosin had large cytoplasm inclusion bodies. Acridine orange stained preparations of infected CAM showed fluorescence of cytoplasmic inclusions and cell nuclei when examined under a fluorescent microscope. Treatment of infected cells with deoxyribonuclease eliminated the green cytoplasmic fluorescence. The inhibitory effects of azide and isatin B-thiosemicarbazone were noted in the growth of the virus on CAM. The agent was inactivated by sodium desoxycholate (SDC) and chloroform. Treatment of a suspension of pox virus with diethyl ether (final concentration 20%) for 18 hours at 4° C caused no appreciable loss in titer. On the basis of its serological properties (previously studied), its cultural, physical and chemical characteristics , the raccoon pox isolate was classified as a new member of the variola-vaccinia group of pox viruses.

The two isolates from skunk liver and spleen suspensions were recovered from dog kidney tissue culture in which they produced cytopathogenic effects (CPE). The CPE was inhibited by anti-canine infectious hepatitis serum. The origin of the agents from dog kidney cell cultures was ruled out by re-isolation of the agents from original samples. Two other possible isolates, one from skunk brain, the other from respiratory tissues, have been recovered from embryonated hens' eggs inoculated in the yolk sac. The agents from skunks are being studied further.

b. Enteroviruses of Swine. The isolation of 45 viral agents from feces of pigs before and after animals were exposed to lethal or sublethal doses of irradiation was reported previously. These cultures could be grouped serologically into six types employing plaque-neutralization techniques. The characteristics of selected prototype strains for each group, designated by consecutive numbers WR-1 to WR-6, have been studied further. All prototype strains measured 20 to 35 mμ by gradacol filtration, were resistant to ether, chloroform and sodium desoxycholate, and were stable at a pH of 3.0 when held for 4 hours at room temperature. All strains were relatively heat stable but were inactivated when held at 50° C for 30 minutes. The presence of 1 M $MgCl_2$ stabilized these strains to heat inactivation. Two distinct types of cytopathogenic effects were produced by the prototype strains. Strains WR1, WR3 and WR4 comprise one group in which the onset of cytopathogenic effect (CPE) occurred 12 to 18 hours. The cells became rounded, shrunken, and CPE spread to the whole culture within 24 to 36 hours. Strains WR2, WR5 and WR6 form the second group in which the onset of CPE is delayed, usually beginning in 48 to 60 hours. The infected cells had an irregular margin and the CPE spreads slowly (7 to 10 days), over the cell culture. The CPE type was a stable and characteristic feature of each strain studied and corresponded to two distinct plaque types, when cultured according to the Dulbecco technique. The plaque type of the first group (WR1, WR3 and WR4) appeared 36 to 48 hours after inoculation, and reached a maximum size in 6 to 7 days. These plaques had distinct borders and were relatively much clearer than those produced by the other types. These appeared in 5 to 6 days after inoculation, attained maximum size by the ninth to tenth day, had irregular borders and were very hazy and indistinct. The prototype strains were compared serologically with previously reported ECPO viruses, employing plaque neutralization techniques. Antisera obtained from Ohio State University, as well as sera obtained from a laboratory in England (Dr. Betts) were included in this study. Two of WRAIR types (WR4 and WR5) were related to the Teschan (Talfin) group of enteroviruses - the polio encephalomyelitis viruses of pigs. Two other representative strains were related to types previously described. The serological characteristics of 2 types (WR2 and WR6) have not been described heretofore.

c. Western Equine Encephalomyelitis (WEE) Vaccines. Randall, et al. in 1947, reported on the production and partial purification of

chick embryo vaccines against the three types of equine encephalomyelitis virus (J. Immunol., 55: 1, 1947). Since 1948, over 40,000 doses of (WEE) vaccine, produced and freeze-dried by the Division of Veterinary Medicine, WRAIR, have been administered to human beings. Persons receiving this vaccine have been those considered to be at high risk to infection, i.e., laboratory and field employees of various state health departments, many universities, Canadian Provincial health departments, employees of commercial biological supply firms, other Army, Navy and Air Force laboratories, and personnel at the WRAIR.

Vaccine was reassayed annually in guinea pigs and always passed this potency test. However, no recent studies had been conducted on the antibody response of the human beings receiving the vaccine and no information was available on the response to vaccine which had been in storage for a number of years. Therefore, the cooperation of a number of recipients of this vaccine was requested. Vaccine produced and freeze-dried in 1950 (designated Lot #4) and stored at 4° C, was employed in this phase of the study. Users were asked to furnish pre and post immunization serum samples from each employee, the post immunization specimen being collected 14 days after the final or booster dose of vaccine. It was recommended that the vaccine be administered subcutaneously (SC) in three doses of 0.5 ml on days 0, 7 and 21, or as a booster dose of 0.1 ml intradermally (ID) to previously immunized individuals.

Sera were examined by the mouse neutralization test using the constant serum-varying virus technic. Equal amounts of serum and diluted virus were incubated at 37° C for one hour and 0.03 ml of this serum-virus mixture were inoculated intracerebrally (IC) into each of 5 mice. Mice were observed for 10 days and titers were calculated by the Reed-Muench formula.

A total of 207 individuals from 11 different places received the full course of the vaccine. Significant neutralizing antibodies were detected in 46 (22%) of the post immunization sera. Neutralization indices ranged from 16 to 16,000; approximately 80% had an index of 300 or less. Table VII shows the origins of the individual groups and the number developing antibody.

In 1963, a new lot of WEE vaccine was produced by the Department of Biologics Research, Division of Communicable Disease and Immunology, WRAIR. This lot of vaccine was produced and partially purified by the same procedures as those previously produced by the Division of Veterinary Medicine. It was also freeze-dried and has been stored at 4° C.

This 1963 vaccine (designated Lot #6) has been furnished to seven organizations for use on their personnel. To date, tests have been completed on only two groups which had not previously been

Table VII

Response to Lot #4 WEE Vaccine Prepared in 1950

Source	Number Vaccinated	Number Positive*
Provincial Health Department, Saskatchewan, Canada	16	11
Fort Dodge Laboratories	47	9
New York State Health Department	9	1
Fort Detrick, Maryland	25	0
University of Miami	12	3
University of Arizona	5	0
Arizona State Health Department	8	0
Bandy Laboratories	12	5
University of Wisconsin	27	11
Southern Research Institute	23	2
WRAIR	23	4
TOTALS	207	46

* Protection of 1.2 logs or greater in the post immunization specimen.

immunized and on one group immunized with Lot #4 vaccine in 1963, and which received the new product in 1964 as a booster. The groups not previously immunized totaled 33 individuals of whom 22 (67%) converted from negative to positive.

The group of 13 which received a single injection of this vaccine as a booster contained five individuals who had developed antibody from the previous immunizations. However, at the time the booster dose of vaccine was administered (approximately 13 months after the original immunization), only two workers still had antibody. Ten people receiving the booster dose converted from negative to positive, with neutralization indices ranging from 40 to 300. The two with pre-booster antibody titers did not develop an increase in titer.

Table VIII shows the origins of the group receiving Lot #6 vaccine and the results of the tests on these sera.

Table VIII

Response to Lot #6 WEE Vaccine Prepared in 1963

Source	Number Vaccinated	Number Positive
Norden Laboratories	20	10
Univ. of Alberta	13	12
TOTALS	33	22

Response to a Booster Injection of Lot #6 Vaccine

Source	Number Vaccinated	Number Positive
Bandy Laboratories	13	12

In the summer of 1963, a large outbreak of WEE infection in human beings occurred in the province of Saskatchewan, Canada. This area, where the disease is endemic, provides an excellent opportunity for a field trial of the vaccine. Plans have been made to vaccinate approximately 1,000 people who are considered to be at high or moderate risk to infection. A sufficient amount of Lot #6 vaccine has been furnished the Provincial Department of Health for these immunizations, and vaccination of the first group has been completed. This group consists

of a number of poultry ranchers and their families who live in a part of the province where the disease recurs each year. Pre and post vaccination serums from 106 individuals have been received and will be examined at a later date.

The need for a more potent vaccine for WEE prompted an evaluation of an attenuated strain of WEE - "clone 15" of strain B628 - developed by Johnson (Am. J. Trop. Med. Hyg., 12: 604, 1963). Initial studies were conducted in burros and ponies. Each of the animals employed in these experiments did not have any measurable neutralizing antibody prior to the study.

In the first experiment, two burros were inoculated intracerebrally (IC) with a dose of clone 15 virus equivalent to approximately 16 x 10^6 LD 50's for suckling mice (SM) inoculated IC. Neither animal developed any fever (< 103.0 F.), signs of central nervous system disease, viremia or neutralizing antibody. Thus, no evidence of viral multiplication could be found following IC inoculation of virus. Two additional burros were inoculated SC with the same dose of clone 15 virus. Neither of these animals developed any fever, signs of central nervous system disease or viremia. However, each animal developed a neutralization index of greater than 500 on the thirteenth day post inoculation. A decline in neutralization index to approximately 10 was observed on the forty-second day.

In the second experiment, four ponies were inoculated SC with approximately 32 million SM IC LD 50 of clone 15 virus. None of these animals developed any fever or signs of central nervous system disease. Viremia studies of the plasma from these animals are in progress. Three of the four ponies developed a neutralization index of greater than 250 on the thirteenth day post inoculation and the remaining animal had a neutralization index of 16. Studies of the persistence of neutralizing antibody in ponies are in progress.

These preliminary studies in burros and ponies indicate that clone 15 virus has little neurotropic potentiality and produces good levels of neutralizing antibody. Further studies are required in equines to determine the persistence of neutralizing antibody, the other types of antibody engendered, i.e., complement-fixation, hemagglutination-inhibition, the dose of virus required to immunize, and most important, the ability to protect against field strains of WEE virus.

d. Antibody Response of Human Beings to Rabies Duck Embryo Vaccine. Veterinarians, animal handlers and laboratory workers constitute a group which is potentially at high risk of exposure to rabies virus. Current U. S. Army recommendations for the vaccine prophylaxis of such individuals call for the administration of three ID injections of 0.2 ml of duck embryo vaccine at intervals of 5 to 7 days. In previous studies, it was found that this regimen often does not result in the

formation of detectible humoral antibody. A study was undertaken to compare the results of vaccine administered via the SC route with that given ID. In addition, information was needed on the effect of a single booster injection of vaccine given to previously immunized individuals.

A single lot of duck embryo rabies vaccine (Eli Lilly Company, Lot Number 5047-801371 PH) was used throughout the study. Volunteers who had never previously received any rabies vaccine were divided into two groups. One group received three doses of 1.0 ml SC on days 0, 7 and 30; the other group received three doses of 0.2 ml (0.1 ml in each of two sites) ID, also, on days 0, 7 and 30. Blood specimens were collected at the time of the first and last injections (days 0 and 30) and 30 days after the last injection (day 60). A third group consisted of subjects who had previously received rabies vaccine. These were given a single booster injection of 1.0 ml SC. Blood specimens were obtained from this group on days 0 and 30.

Antibody response was measured by the WHO "standard" mouse neutralization test. The "CVS" rabies virus, obtained from the National Institutes of Health, was used as the test strain. Sera were screened by adding a volume of virus dilution containing 10-100 LD 50 to an equal volume of undiluted serum. This mixture was incubated at 37° C for one and one half hours and 0.03 ml were injected IC into each of 6 mice. Survival without the development of signs of rabies of at least 4 of 6 mice for 14 days was considered evidence of antibody. Positive sera were retested at actual serum dilutions of 1:2.5, 1:12.5 and 1:62.5 against the same amount of virus, with the negative pre immunization serum repeated simultaneously as a control. Titers were calculated by the method of Miller and Tainter as the highest dilution protecting 67% of the mice.

Each of the groups not previously immunized was comprised of 20 individuals. As shown in Table IX, 19 of 20 (95%) subjects vaccinated subcutaneously and 14 of 20 (70%) subjects vaccinated intradermally developed detectible antibody titers after 3 injections of the vaccine. This difference is statistically significant ($P = 0.04158$) when the data are analyzed by Fisher's exact method for probability. Of the 7 subjects who did not respond, 6 received a single injection of 1.0 ml SC, approximately six months after the third injection of the primary series; antibody titers were found in all six when tested 30 days later. Several subjects received only two of the three scheduled injections. Among these individuals, 2 of 4 in the SC group and 3 of 3 in the ID group responded.

The distribution of titers and the geometric mean titer of the SC group is significantly higher than that of the ID group ($P < 0.05$) when tested by means of the t test (Table X).

Table IX

Response of Normal Human Subjects to 3 Injections of Duck Embryo Rabies Vaccine*

Route	Amount	Day blood sample obtained 0	30	60	% Responding
Subcutaneous	1.0 ml.	0/20+	16/20	19/20‡	95
Intradermal	0.2 ml.	0/20	12/19	14/20‡	70

* Injections given on days 0, 7 and 30.

+ No. sera protecting at least 4 of 6 mice/total no. sera tested.

‡ p = 0.04158 (Fisher's exact method (7)).

Table X

Distribution of Antibody Titers after 3 Injections of Duck Embryo Rabies Vaccine

Route	<1:1	1:1-1:2.4	1:2.5-1:12.4	1:12.5-1:62.4	1:62.5+	Geometric Mean Titer
Subcutaneous	1	9	7	2	1	1:2.9*
Intradermal	6	9	4	1	0	1:1.3*

* $p < 0.05$ (t test)

The booster group was comprised of 25 subjects who had received from 1 to 14 injections of rabies vaccine at various times, from 3 months to 21 years prior to the present study. They were given a single SC injection of 1.0 ml and the antibody titer determined 30 days later. Fourteen were serologically negative at the time of inoculation. A booster response was elicited in 11 seropositive subjects. Conversion to positive reactions was attained in 11 of the 14 serologically negative persons. Two of 3 subjects who did not respond to the vaccine received fewer than three injections previously.

4. A New Filterable Infectious Agent for Dogs.

A transmissible, filterable agent, infectious for dogs, was recovered from a routine preparation of primary dog kidney tissue culture (DKTC) in which cytopathogenic effects (CPE) appeared spontaneously. In view of current interest in the use of DKTC for preparation of attenuated vaccines for human use, characteristics of this agent were studied.

The agent, designated S4-63, was isolated first from a monolayer DKTC. CPE, characterized by focal occurrence, rounding of cells and swelling of nuclei, were seen on the eighth day following the preparation of culture. It could be transferred and grown readily in DKTC but not in a large variety of primary cell cultures or cell lines, viz., kidney cell cultures of pig, cow, rabbit, horse, hamster, cat, monkey, rat, mouse, embryonic kidney cell cultures of rat, mouse, chicken and man, human enbryonic diploid brain, hela L929 and AV3. The infectivity titers in DKTC were usually in the order of $10^{-3.5}$, occasionally $10^{-4.5}$.

Earliest CPE can be detected in about 18 hours employing large inocula (DKTC diluted up to 10^{-2}).

Eosinophilic, intranuclear inclusions can be seen in infected cells stained with hematoxylin and eosin. In some cells, the inclusions appear to coalesce, filling the nuclei and pushing the chromatin to the periphery of the nucleus. No cytoplasmic inclusions can be seen. With Giemsa or Dienes stain, single organisms can be seen in vacuoles in the cytoplasm and mycelial elements can be seen forming bridge-like arrangements between infected cells. Small, 1 to 1.5 mm plaques with irregular borders developed in approximately 3 days on agar overlayed cultures of DKTC.

The S4-63 agent measured 150 to 200 mμ by gradacol membrane filtration. It was inactivated by ether, chloroform and sodium desoxycholate and failed to grow in the presence of 5-iodo-2-deoxyuridine but was resistant to the effects of 5-fluorouracil. No hemagglutinins to dog, guinea pig, human O and adult chick red blood cells could be demonstrated when tested from pH 5.0 to 8.0 and no evidence of hemolysis was noted. Spontaneous agglutination of dog red blood cells occurred

below pH 6.0. This agent was inactivated when kept at 50° C for one half hour, the infectivity titer of tissue suspension held at 37° C and at 25° C rapidly declined from approximately $10^{-4.5}$ to negative in 5 to 7 days. At 4° C or at -70° C., the titer decreased about 90% per week. However, if the agent was stored in 40% fetal calf serum, the titer was somewhat stabilized. Freeze-drying of suspensions of S4-63 resulted in an immediate loss of about 99% of the infectivity titer, but the titer remained constant thereafter up to nine months. The infective titer of S4-63 is decreased sharply below pH 5.5; below pH 4.0, it is inactivated.

S4-63 did not produce infection in suckling and weanling mice inoculated by oro-nasal, intraperitoneal, subcutaneous and intracranial routes, nor in chick embryos challenged via allantoic, amniotic and chorioallantoic membrane routes. The agent could be grown in the yolk sac of 5 to 6 day-old embryonated eggs but elicited no detectible lesions.

Inclusion of the following concentrations of antibiotics in tissue culture media had no effect on the growth of S4-63: chloramphenicol - 1,000 µg/ml; tetracycline - 20 µg/ml; tylocine - 200 µg/ml, kanomycin - 200 µg/ml. Only chloramphenicol, tetracycline in concentrations of 5,000 µg/ml and 50 µg/ml respectively, were capable of inhibiting growth of this agent in tissue culture.

During the course of this study, agents which produced a similar type CPE were repeatedly recovered from tissue cultures prepared from kidneys of puppies obtained at the WRAIR animal dog colony. Isolates were also obtained from lungs, liver and kidney of 3 puppies which died 8 to 20 days after birth, with signs of septicemia, and from the lungs of 2 four-week-old pups with a fatal pneumonic infection.

Employing a plaque neutralization technic, paired sera from 50 newly purchased dogs were examined for antibodies to S4-63. Seven developed neutralizing antibodies. These 7 dogs were all from a group of 20 dogs which had been debarked. Five of the 7 dogs became ill in 7 to 14 days and died 18 to 35 days after arrival, following an upper respiratory infection. Additional tests were conducted on 9 dogs that had been housed at the WRAIR animal holding facility for more than a year. All had high neutralizing antibody titers. Low level antibody titers were found also in three different lots of commercial canine globulin.

Infectivity studies were conducted on 8 eight-week-old mongrel pups. A dose of approximately $10^{5.2}$ tissue culture LD 50 was administered to each dog via oral nasal spraying. Three of the dogs died on eleventh, sixteenth and twenty-second day post exposure. The sick dogs had signs of weakness, slight fever and emaciation prior to death.

Petechiae of the pleura and congestive focal pneumonia were the only lesions noted on necropsy examinations. The agent was recovered from lung tissues of 2 of the 3 dogs. S4-63 produced a fatal hemorrhagic septicemia in puppies less than 5 days old that were inoculated by the oro-nasal route. Principal lesions noted on necropsy examinations were: congestion and petechiation of lungs, liver, spleen and kidneys, inflammed joints and meninges. The agent was recoverable from all inflammed tissues.

The cultural characteristics of the agent, its pathogenicity in puppies were reminiscent of those of a PPLO agent isolated from respiratory tissues of puppies with signs of pneumonia. Attempts were made to determine the possible identity of S4-63 as a PPLO. Initial attempts to grow S4-63 on various PPLO media were unsuccessful.

The agent was successfully grown in a medium containing beef heart infusion supplemented with yeast hydrolysate (rather than yeast extract) and 10% horse serum. Microcolonies developed in 48 to 72 hours attaining maximum diameter of 0.8 to 1.0 mm in 7 to 8 days. The colonies at first appeared smooth with no central core; after 4 to 5 days, the central core with radiating margins became evident. Further studies are required to definitively determine the identity of the PPLO agent and the plaque-forming tissue culture done of S4-63 and the relationship of S4-63 to the Cornell PPLO isolate.

5. Respiratory Infections in Newly Procured Dogs at the WRAIR Animal Colony.

Studies were continued on causes and methods of control of respiratory infections in newly procured dogs. These infections are characterized by high morbidity and mortality and appear during the period of quarantine -- 1 to 3 weeks after admission to the WRAIR animal facility.

Preliminary studies on selected numbers of dogs provided no evidence that outbreaks of respiratory disease could be arrested by the use of broad spectrum antibiotics, by the elimination of debarking surgery, or by the application of vaccine prophylaxis for canine distemper and infectious canine hepatitis (ICH). From diseased lungs, cultural studies consistently yielded PPLO. This group of organisms could also be isolated from throat swabs of normal dogs. These findings were reminiscent of PPLO etiology (Eaton agent) of atypical pneumonia in man. It may be significant that streptococcal organisms were isolated from approximately half of the lung specimens cultured. From sick dogs, adenoviruses (related to ICH virus) were commonly isolated from feces and throat swabs. There was also evidence based on histopathological examination of lung tissues that some of the fatal cases were simultaneously infected with canine distemper virus. In addition to streptococci, Bordetella bronchiseptica and Pseudomonas aeruginosa

have been found in relatively few animals and were probably secondary invaders.

Further attempts were made to evaluate the etiological significance of PPLO and adenovirus agents commonly found in dogs. At the same time, the protective effects of types 3, 4 and 7 adenovirus vaccine and an ICH vaccine were studied. The choice of adenovirus vaccine was based on published reports by Sinha (J.A.V.M.A., 136: 481, 1960) and Carmichael (Proc. Soc. Exptl. Biol. & Med., 109: 75, 1962) that the human adenovirus can produce subclinical infections in dogs that can be rapidly spread from dog to dog; furthermore, that such infections can result in a modified ICH infection. The study was conducted on 75 dogs which were procured and screened for absence of overt signs of disease. Animals were divided into four groups of 18 to 20 each. On the day of admission to the colony, one group was vaccinated with a multivalent (types 3,4, and 7) adenovirus vaccine, one with an inactivated ICH virus vaccine, a third with both vaccines, the fourth control group with placebos for the respective vaccines. Vaccines were administered intramuscularly, adenovirus in a 1.0 ml dose, ICH in a 2.0 ml dose. Serum samples, throat swabs and fecal samples for serological or cultural studies were obtained on day animals were procured. Samples were also obtained on the first day of illness in the event of respiratory infections. Additional serum samples were taken 21 days after the study was begun and/or 14 to 21 days following the onset of respiratory infections. Post-mortem examinations were performed on dogs which died and specimens of various tissues were submitted to pathology and for virus isolation. To date, approximately 600 serum and virus isolation samples have been processed, catalogued and stored.

The occurrence of disease and deaths in the 4 groups of dogs is shown in Table No. XI.

Table XI

Results of Vaccine Trial in WRAIR Dog Colony

No. of Dogs	Vaccine Received	Tot. No. of dogs becoming ill during			Deaths
		1st week	2nd week	3rd week	
20	Adeno + ICH	9/20 (45%)	6/11 (55%)	1/5 (20%)	2
18	ICH only	5/18 (28%)	4/13 (31%)	2/9 (22%)	3
19	Adeno only	4/19 (21%)	7/15 (47%)	2/8+1*(22%)	2
18	None	3/18 (17%)	8/15 (53%)	3/7 (43%)	3
TOTALS		21/75 (28%)	25/54 (46%)	8/29+1 (27%)	10

* Dog became ill a second time.

The overall incidence of respiratory disease or mortality in the study group given was not significantly different from that of control dogs. It has not as yet been determined whether any of the vaccine groups varied significantly from one another with respect to number of sick dog days or length of illness.

Sixty-eight specimens obtained from either post-mortem tissue samples or from throat and fecal swabs obtained at the onset of illness have been cultured for viruses. To date agents have been found in 12 of these samples. These and other agents isolated will be identified.

In addition to the isolation and characterization work remaining considerable serology must yet be accomplished. It is hoped that such studies will yield information as to:

1) incidence of disease produced by each agent;

2) the antibody status of newly arrived dogs;

3) the antibody patterns observed in dogs which have not had respiratory diseases.

Although this study is still in an early stage, a sufficiently broad number of different specimens has been collected so as to provide possible clues or answers to the etiology of this disease complex.

Summary and Conclusions:

1. Attempts have been made to purify a leptospiral hemolysin. Employing as starting material dialysed allantoic fluid from infected eggs, it was possible to separate fractions with 3.5 fold greater activity through the use of agar-gel filtration technique. Maximum separation of the hemotoxin from other proteins in a Sephadex (G-50) column was effected by the use of a Michaelis buffer, pH 7.6 as eluting agent.

In vitro aspects of cross-immunological relationships between hemolysins derived from different serotypes were studied. Sheep infected with hemolytic strains and challenged 26 days later with a hemolysin from a serologically homologous or heterologous strain elicited a marked increase in hemolysin-neutralizing antibodies. In contrast, animals infected with a non-hemolytic strain and challenged with hemolysin did not develop detectible antibody levels. It was apparent that infection of animals with hemolytic strains led to the formation of antihemolysins. However, their presence was manifested after challenge with hemolysin, eliciting in effect a booster response. These findings were consistent with those observed in vivo, viz., that infection of sheep with hemolytic strains conferred immunity against the hemolytic affects of hemotoxins from the same or antigenically diverse leptospiras.

The potential usefulness of an indirect fluorescent antibody technic as a genus-specific serological test for leptospirosis was investigated. The antigen was a culture of a non-pathogenic strain of (Patoc)

Leptospira biflexa. The test was applied to 48 sera from 29 proved human cases of leptospirosis encompassing infections with 12 different serotypes and, also, to 70 human sera known to be negative for leptospirosis. Findings correlated completely with those obtained in conventional agglutination tests. These preliminary observations provide indications that the fluorescent antibody procedure can be used as a sensitive, genus-specific serological diagnostic tool for human cases. Its applicability to animal sera requires further study.

Growth of 13 different leptospiral strains in conventional Stuart's liquid and Fletcher's semi-solid media and in the same media containing 5-fluorouracil -- a potent growth inhibitor for most bacteria -- was studied. The concentration of 5-fluorouracil was 300/µg/ml. Growth in the selective Fletcher's media was not remarkably different from that in conventional media, when either small or large inocula were used. In the two types of Stuart's media, no differences in growth were seen when large inocula, e.g., 0.5 per 5 ml media were used. When small inocula (0.1 ml) were used, 7 of the 13 test strains had a longer lag phase of growth, the other 7 strains grew equally well in the selective and conventional Stuart's media. The practical importance of the new selective media was demonstrated by the recovery of 51 pure cultures of leptospiras from 77 contaminated cultures that were received as part of a large shipment of strains from Malaya.

Serological studies were conducted on 14 leptospiral strains isolated from domestic animals in Brazil. Seven different serotypes were disclosed, 6 of which were related to the following types on the basis of cross-agglutination and agglutinin-adsorption tests: grippotyphosa, icterohaemorrhagiae, hyos, canicola, pomona, mini. The seventh type was found to be a new serotype in the bataviae group. These findings extend knowledge on the presence of various leptospiras in Latin-America.

Antigenic studies have been completed on 7 different leptospiral types isolated during the course of a study of an outbreak of leptospirosis at Fort Kobbe, Canal Zone. The serological characteristics of types found had not been described previously. Six of the types had antigenic affinities respectively, with members of the hebdomadis, hyos, bataviae, icterohaemorrhagiae, cynopteri, and pomona serogroups. The seventh strain could not be related to members of any known serological type. The occurrence of mixed infections in animals was bacteriologically verified by the separation of two different serotypes from a culture derived from a spiney rat. This separation was effected by the utilization of a newly developed in vitro techni

Further studies were conducted to evaluate the potential leptospiral infection hazards in the use of dog kidney tissue cultures for the preparation of attenuated viral vaccines Four different types of

monolayer dog kidney tissue cultures were prepared from a trypsinized suspension of dog kidney containing high concentration of pathogenic leptospiras. The types of tissue cultures differed with regard to the presence and kinds of antibiotics added. In cultures containing no antibiotics, leptospiras could be isolated up to the 25th day of incubation. Tissue cultures containing a combination of penicillin, 100 U/ml, and streptomycin, 100 µg/ml were negative after one day of incubation. Leptospiras were recovered from cultures containing neomycin, 100 µg/ml and chloramphenicol, 100 µg/ml, respectively, after 1 and 2 days of incubation, but not thereafter. It was concluded that leptospiral infection hazards may be present in the use of tissue culture vaccines containing no antibiotics. Such hazards can be eliminated by the use of neomycin, chloramphenicol and a combination of penicillin and streptomycin at levels of 100 U or 100 µg/ml.

2. The effect of chelating agent, divalent cations and cholesterol on the activity of a Listeria monocytogenes hemolysin on sheep erythrocytes and the spectrum of activity of the hemolysin against diverse animal erythrocytes was studied. Chelation of divalent cations by ethylenediaminetetraacetate in solutions of hemolysin did not affect the activity of such preparation, nor was its activity affected by the addition of excess magnesium, manganese, calcium, cobalt or iron ions. The addition of freshly crystallized cholesterol suspended in petroleum ether greatly reduced the hemolytic activity of listeria hemolysin. Listeria hemolysin was similarly active against erythrocytes from horse, sheep, goat, cow, and rabbit. Erythrocytes from chickens were relatively less sensitive. The titer of activity of hemolysin for mouse erythrocytes was comparable to that observed with other mammalian red blood cells; however, the degree of lysis was notably less in low test dilutions of toxin, and greater in high dilutions when compared to other cells. These findings were similar to those described for oxygen labile hemolysin derived from other microorganisms.

3. Screening of 97 raccoons, 104 skunks, 64 opossums, 10 woodchucks, 1 muskrat and 5 feral cats for viral zoonotic agents has now been completed on approximately 90% of the animals. These animals were trapped on an undeveloped forest area located near Aberdeen, Md. To date, pox viruses have been isolated from upper respiratory tissues of 2 raccoons and isolates have been obtained from 4 different skunks, 2 from pooled liver and spleen tissues, 1 from brain tissue and the last from respiratory tissues. The isolates from skunks are being studied further. The pox virus of raccoons can infect suckling mice but not adult mice, hamsters and guinea pigs. Vesicular lesions in rabbits were produced in some trials after successive cutaneous passages. It produced discretely embedded plaques on the chorioallantoic membrane of embryonated hen's eggs. Other physical, chemical and cultural characteristics were the same as that of other variola-vaccinia viruses. On the basis of findings, the raccoon pox-virus was classified as a new member of the variola-vaccinia group of viruses.

Additional studies were conducted on prototype strains of 6 serologically distinct types of enteroviruses isolated from swine. The agents measured 20 to 35 mμ by gradacol filtration; they were resistant to ether, chloroform and sodium desoxycholate and were inactivated when kept at 50° C for 30 minutes. The prototype strains could be separated into two groups on the basis of the type of cytopathogenic effects produced in tissue culture. Strains were compared serologically with type strains reported elsewhere. Four of the representative isolates were related to previously reported types. Two types have not been described heretofore. The relationship of the swine enteroviruses to human enterovirus is unknown.

The potency of a lot of freeze-dried Western Equine Encephalomyelitis (WEE) vaccine prepared in 1950 was reexamined in serological studies on 211 persons who were given the recommended course of immunization. Significant neutralizing antibodies were developed in 22 per cent, although the vaccine passed potency tests in guinea pigs. In contrast a new lot of vaccine given to 33 subjects elicited detectible neutralizing antibodies in 22. The new vaccine given to 13 individuals who had been vaccinated one year earlier with the old lot of vaccine provoked a booster response in 12 of 13 persons, 10 of whom converted from negative to positive serological reactors. Indication from findings is that the vaccine slowly loses potency after prolonged storage.

The potency of an attenuated strain of WEE(clone 15, strain B628) was evaluated in burros and ponies. Animals inoculated intracerebrally (IC) or subcutaneously (SC) with a large dose of virus developed no fever, viremia, nor signs of CNS disease. High levels of neutralizing antibodies were provoked in animals inoculated SC but not in those inoculated IC. From preliminary findings it appears that the attenuated virus has little neurotropic but good antigenic properties. Further studies are required in equines to determine the persistence of neutralizing antibodies, the other types of antibody engendered, the dose of virus necessary for immunization and its prophylactic efficacy against field strains of WEE virus.

The recommended regimen for vaccination of human beings with a duck embryo rabies vaccine was reexamined. Trials were conducted in two groups, each comprising 20 human volunteers. In one group the vaccine was administered intradermally (ID), the recommended route; individuals in the second group were inoculated subcutaneously (SC). Nineteen of 20 subjects vaccinated SC and 14 of 20 vaccinated ID developed neutralizing antibody. In addition, the geometric mean titer in the SC group was significantly higher than that of the ID group. It was also found that SC administration of the vaccine to a group of 25 individuals previously vaccinated by conventional method provoked a booster response in 22 of 25 subjects. Eleven of 14 persons that were serologically negative at the time of inoculation became serologically positive. On the basis of these findings, the SC administration of duck rabies vaccine in man has been recommended.

4. A transmissible filterable agent designated S4-63, infectious for dogs, was recovered from a routine preparation of primary dog kidney tissue culture (DKTC). The agent could be maintained in DKTC, producing cytopathogenic effects but could not be grown in a large variety of tissue culture cells. It produced plaques in monolayer tissue cultures covered with agar. The agent was inactivated by ether, chloroform, and sodium desoxycholate and failed to grow in the presence of 5-iodo-2-deoxyuridine, but was resistant to effects of 5-fluorouracil. It did not produce infection in suckling and weanling mice inoculated by various routes nor in embryonated hen's eggs inoculated via allantoic, amnionic and chorioallantoic membrane routes. In tissue culture, its growth was unaffected by presence of relatively high levels of either chloramphenicol, tetracycline, tylocine, or kanomycin. Growth of the agent in DKTC was apparently inhibited by chloramphenicol in concentrations of 5000 µg/ml and also by tetracycline at levels of 50 µg/ml. Plaque neutralizing antibodies were found in sera of 7 of 50 dogs in WRAIR animal colony. When S4-63 was administered to 2-month-old puppies by the oral nasal route, a fatal pneumonic disease was produced in 3 of 8 dogs. In puppies less than 5 days old, it produced a fatal hemorrhagic septicemia. The characteristics of the agent in tissue culture and its pathogenicity in dogs were similar to those described for a PPLO agent isolated at Cornell University from respiratory tissues of puppies with pneumonia. After considerable cultural efforts, it was possible to subculture the agent in modified PPLO medium. The definitive identification of the PPLO agent with the plaque-forming agent requires additional studies. The disclosure of a PPLO agent associated with pneumonic disease in dogs is reminiscent of PPLO etiology of primary atypical pneumonia in man. The findings also serve to draw attention to the possible presence of fastidious PPLO in tissue cultures used in preparation of attenuated viruses; moreover, they pose questions on the possible PPLO identity of viral isolates in tissue culture.

5. Studies on causes and method of control of outbreak of respiratory infections in newly procured dogs were continued. Specific attention was directed to the possible prophylactic efficacy of inactivated infection, canine hepatitis virus vaccine, and an adenovirus vaccine (types 3,4 and 7) used either singly or in combination. Trials conducted in a total of 75 dogs provided no evidence that the administration of the vaccines affected the morbidity or mortality rates. During the study, respiratory disease occurred in 55 of the 75 animals; 10 of the sick animals died. Specimens have been collected at appropriate intervals for serological studies and viral isolation attempts. To date agents have been recovered from 12 of 68 specimens processed.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36186

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8180203

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
A Walter Reed Army Inst of Rsch
Walter Reed Army Medical Center
Washington, D. C., 20012
723-1000, Ext 3552 49

5. PRINCIPAL & ASSOC. INVESTIGATORS/PROJECT OR ACTION OFFICER
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576-5208 or Interdepartmental Code 198, Ext 5208 See Continuation Sheet 49

6. TITLE OF: PROJECT ☐
TASK ☐
SUBTASK ☒ Development of biological products. (U)

7. DATE OF REPORT DAY 30 MONTH June YEAR 1964

8. RESUME' (U) Freeze-dried typhoid seed cultures, prepared for vaccine production, have maintained their viability after storage at 4°C or 22°C for 3 years. A new lot of acetone-killed and dried typhoid vaccine, similar to that produced for the 1960 WHO field trials, was prepared for use as a national reference vaccine and for additional human field studies. Phase 1 and phase 2 Q fever vaccines were prepared for comparative laboratory and human field studies. An experimental lot of phase 2 Q fever vaccine, employing extraction with genetron-113 in place of ether, is currently being evaluated. Studies on the efficacy of Eastern Equine Encephalomyelitis vaccine, and on methods for measuring the human responses to this vaccine, are continuing. A lot of freeze-dried purified chick embryo Western Equine Encephalomyelitis vaccine was prepared and is being evaluated. Studies on the production of WEE vaccine in tissue culture have been initiated. Additional experimental investigations on the method of preparation of a freeze-dried cholera vaccine were carried out. Surveillance of the viability of freeze-dried cholera seed cultures prepared for vaccine production, has indicated that, for long term storage, a temperature of -20°C may be required.

9. KEY WORDS
Biological products, maintenance of biological activity, evaluation of vaccines, freeze-drying.

10. SUPPORTING PROJECTS
Not applicable.

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

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ACCESSION NO. 36186	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	11–22: 3A012501A818 · 23 24: 02 · 25 26: 03 · 27 28: · 29:
14. DATE OF REPORT (30-33)	30–33: 0664
15. SECURITY OF WORK (34)	34: 4
16. TYPE OF REPORT	35: 3 · 36–47 48 49 50 51: · 52–55: 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56-61) b. Functional Class (62-64)	56–61: 010608 · 62–64:
18. OSD CLASSIFICATION (65-66)	65 66: AR
19. R&D CATEGORY (67)	67: 1

Card "D"

20. CONTRACT NUMBER	11 12: DA · 13 14: · 15–17: · 18–21: · 22–26: · 27:
21. GRANT NUMBER	28 29: DA · 30–33: · 34 35: · 36–38: · 39 40: · 41–45: G · 46:
22. ESTIMATED COMPLET. DATES	47–51: 1CONT · 52–56: 2 · 57–61: 3 · 62–66: 4 · 67–71: 5

Card "E"

23. PRIORITY (11-14)	11–14: 1
24. PROGRAM ELEMENT (15-26)	15–26: 6•11•25•01•1
25. CMR&D CODES	27–29: N/A · 30–32: · 33–35:
26. CDOG REFERENCE a. Paragraph No. (36-44) b. Functional Group (45)	36–39: 1412 · 40: · 41 42: a · 43 44: · 45: 6

Card "F"

27. FUNDING

a. Est. Total Cost (11-15)	11–15: · 16–18: 1 · 19–21: 2 · 22–26:
b. % Spent Intern. (16-18)	
" " Extern. (19-21)	
c. Total Obligation (22-26)	
d. Progrmd. Cur. FY (27-33)	27 28: · 29–33: · 34 35: · 36–40:
e. " " " +1 (34-40)	
f. " " " +2 (41-47)	41 42: · 43–47: · 48 49: · 50–54:
g. " " " +3 (48-54)	
h. " " " +4 (55-61)	55 56: · 57–61: · 62 63: · 64–68:
i. " " " +5 (62-68)	
j. " " " +6 (69-75)	69 70: · 71–75: · 76 78:
k. Total Man Years of Effort (76-78)	

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PRINCIPAL & ASSOC. INVESTIGATORS - Item 5, Continued

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Vivona, S., Lowenthal, J. P., Berman, S., Benenson, A. S. and Smadel, J. E.: Report of a Field Study with Q Fever Vaccine. Amer. Jour. Hyg., 79:143-153, 1964.

ANNUAL PROGRESS REPORT

Project No. 3A012501A818 Title: Communicable Diseases & Immunology

Task No. 02 Title: Immunology

Subtask No. 03 Title: Development of biological products

Description: This task is concerned with the development of manufacturing methods for the production of new effective vaccines and for the modification of existing biological products to afford greater stability under adverse storage conditions, to minimize logistic requirements and to increase the purity, thereby increasing effectiveness and reducing the reactivity.

Progress:

1. Typhoid Vaccine

a. Surveillance of the viability of the freeze-dried Salmonella typhosa, Ty2 strain, seed culture prepared by the Department of Biologics Research, WRAIR, in 1960, has continued during this period. This seed material was prepared at the request of the World Health Organization for use in the production of typhoid vaccine for current and future human field studies. A summary of the results of titrations of the number of viable organisms, as determined by plate counts, on samples stored at various temperatures over a period of 3 years is as follows:

	Temperature of Storage			
Time	4°C	22°C	37°C	45°C
12 mos.	1.89×10^9	1.12×10^8	$>3.00 \times 10^5$	$<10^1$
25 mos.	1.15×10^9	4.17×10^7	2.30×10^4	$<10^1$
37 mos.	7.17×10^8	8.20×10^6	$<10^1$	-

From these results it is apparent that the viability of the seed culture is satisfactory after storage in the dried state at 4°C and 22°C for 3 years.

b. Another large lot of acetone-killed and dried typhoid vaccine was produced, employing the original S. typhosa, Ty2 strain, seed culture and using the production techniques previously described (annual report, 1960). Ten thousand bottles, each containing the equivalent of 10 ml of final vaccine, were prepared for the Division of Biologics Standards, National Institutes of Health, to serve as a national reference standard vaccine. Four hundred bottles of this acetone-killed and dried vaccine, together with an equal amount of freshly prepared

freeze-dried heat-killed phenol-preserved typhoid vaccine, were sent to the WHO International Laboratory for Biological Standards, Copenhagen. In addition, approximately 100,000 ml of the acetone-killed and dried vaccine were set aside for human field studies. Mouse potency tests, performed by the Bioassay Section, Department of Applied Immunology, WRAIR, as well as in vitro chemical assays, indicate that the new lot of acetone-killed and dried vaccine is comparable to vaccine K, the lot produced for the 1960 WHO human field studies.

2. Q Fever Vaccine

a. The human field studies with a freeze-dried purified Q fever vaccine (Henzerling strain, phase 2) initiated in 1962, have continued (annual report, 1963). To date, of 444 recipients of 3 doses (0.1 ml, 1.0 ml and 1.0 ml) of the dried vaccine, 222, or 50%, converted or had a post-immunization CF titer of 1:4 or greater. These results are similar to those reported by Benenson (Med. Science Publication No. 6, Walter Reed Army Inst. Res., 1959, 47-60), in which 30 out of 59 individuals, or 51%, given three 1.0 ml doses of fluid Q fever vaccine, developed CF antibody titers of 1:5 or greater.

b. In cooperation with the Commission on Epidemiological Surveys and Commission on Rickettsial Diseases of the Armed Forces Epidemiological Board, two new lots of freeze-dried purified Q fever vaccine were produced for use in comparative laboratory and human field studies. One lot was prepared from the Henzerling strain in phase 2 and is therefore comparable to the lots of vaccine previously prepared and extensively tested in humans. The other lot was prepared by the same procedures, but from seed material which consisted of the Henzerling strain in phase 1. The phase conversion of the seed was accomplished by Dr. R. Ormsbee, Rocky Mountain Laboratory, NIH, by guinea pig passage of the phase 2 seed material. A comparison of the two vaccines is given below:

	Phase 1 (Lot 1)	Phase 2 (Lot DP-5)
Nitrogen (mg/ml)	0.0080	0.0127
Total Solids (mg/bottle)	70.3	71.05
Moisture (%)	1.22	0.86
Antigenicity (CF):		
vs phase 1 antiserum	1:1	1:1
vs phase 2 antiserum	-	1:1

Immunogenicity (in guinea pigs) - in progress.

c. Current methods for the preparation of Q fever vaccine involve the extraction of the crude yolk sac membrane suspensions with diethyl ether. Because of the hazard involved in handling large vol-

umes of ether and in the processing of ether-containing material, experimental studies were initiated to find other reagents which would effectively remove the fats and extraneous proteins from the crude suspensions without destroying the antigenicity of the rickettsiae. In preliminary studies with several reagents (chloroform, carbon tetrachloride, and genetron-113), the most satisfactory results were obtained with genetron-113 (trifluorotrichloroethane). Additional investigations were therefore carried out to determine the optimum conditions for purification of the rickettsial suspensions with genetron. As a result of these studies the following procedure was followed for the production of an experimental lot of vaccine. A 10% yolk sac membrane suspension (pH adjusted to 5.7) was homogenized in a Waring Blendor with 1½ volumes of genetron-113 at room temperature. Separation of the aqueous and genetron layers occurred on standing overnight at 5°C. The aqueous phase was collected, adjusted to pH 7.0, and passed through the Sharples centrifuge (48,000 rpm) at a flow rate of 20 ml per minute. The sediment from the Sharples bowl was resuspended to 1/5 the original volume with buffered physiological saline (pH 7.2) containing 0.05% formalin. The resulting suspension was diluted with an equal volume of formalinized saline containing 4% human serum albumin. After neutralization of the formalin with sodium bisulfite, the product was filled in 2 ml quantities into 10 ml bottles and freeze-dried. The dried material, when rehydrated to 10 ml with physiological saline, consisted of a purified rickettsial suspension, equivalent to that in a 5% yolk sac membrane suspension, in saline containing 0.4% human serum albumin. Immunogenicity tests in guinea pigs are currently underway to compare this lot of genetron-extracted vaccine with the standard ether-extracted Q fever vaccines.

3. Russian Spring Summer Encephalitis Vaccine

Experimental studies on the preparation of RSSE vaccine by the growth of the virus in chick embryo fibroblast tissue culture were suspended during this period because of other commitments. In the meantime, distribution of an experimental freeze-dried RSSE vaccine of mouse brain origin to laboratories for immunization of personnel at risk, has continued.

4. Eastern Equine Encephalomyelitis Vaccine

a. The evaluation of the response of human recipients to the dried purified Eastern Equine Encephalomyelitis (EEE) vaccine of whole chick embryo origin was continued. The results to date of serum neutralizing antibody levels, as measured by the standard mouse neutralization test, are summarized in the following table:

Serum Neutralizing Antibody Responses to EEE Chick Embryo Vaccine
(Individuals with Pre-Immunization Index Less Than One Log)

No. of Immunization Doses	Neutralization Index (Log_{10}) Increase* Negative	Equivocal	Positive
3 Doses (original series)	163 (63%)	24 (9%)	70 (28%)
1 Dose (booster)	67 (53%)	13 (10%)	47 (37%)

*Negative-Neutralization index increase (post-pre) less than 1 log.
Equivocal- " " " " " between 1 and 1.3 logs.
Positive- " " " " " greater than 1.3 logs.

These results show that about one-third of the recipients who received either three 0.5 1 doses as an initial series, or one 0.1 ml dose as a booster, converted from negative to positive.

b. A new lot of freeze-dried purified EEE vaccine of chick-embryo origin was prepared by the same methods used for earlier lots of this product. This lot of vaccine is currently being evaluated.

c. Attempts to use the plaque-reduction technique in a tissue culture system for quantitatively measuring the neutralizing antibody levels in sera of immunized individuals have continued to give erratic results. An investigation of the factors which have been reported by others to affect the efficiency of plaque formation by viruses, and a critical evaluation of the optimal conditions for plaque production by EEE virus in chick embryo fibroblast monolayers, is currently underway. It has been found, as was expected, that the efficiency of plaquing is dependent upon the volume of fluid in which the virus particles are suspended during the adsorption phase. The results of a titration, on monolayers in 2-ounce prescription bottles, in which a constant number of virus particles is suspended in volumes of inoculum ranging from 0.1 to 0.5 ml, are shown in the following table:

Effect of Volume of Inoculum on Efficiency of Plaquing

Total Volume of Inoculum* (ml.)	Number of Plaques	Average Number of Plaques
0.1	41, 48, 47	45
0.2	34, 25, 26	28
0.3	24, 18, 18	20
0.4	12, 12, 20	15
0.5	11, 17, 13	14

*0.1 ml of EEE virus suspension + appropriate amount of medium (MEM, Eagle's) to make up the total volume.

Although the greatest number of plaques is consistently found when the total volume of the inoculum is 0.1 ml, some difficulty has been encountered in distributing this small inoculum over the entire monolayer. Certain areas of the cell sheet are crowded with plaques, while other areas have none. Since, in a plaque-reduction test, relatively large numbers of plaques, uniformly distributed, are required to quantitatively evaluate the effect of specific antisera, more reproducible results can be obtained by using an inoculum of 0.2 ml, the minimum volume necessary to distribute the virus particles uniformly over the entire monolayer in 2-ounce bottles. The temperature of incubation following infection of the cells and overlaying with agar, found to be critical by some investigators for plaquing efficiency with Group A arboviruses, did not seem to have a significant effect in our system. Results of a typical test are given in the following table:

Effect of Incubation Temperature on Plaquing Efficiency

Virus Dilution	Average Number of Plaques	
	31°C	37°C
$10^{-9.3}$	47	44
$10^{-9.6}$	24	23
$10^{-9.9}$	15	15

The effect of the addition of DEAE (diamino-ethyl aminoethane) -dextran to the overlay agar was investigated because of reports in the literature of the ability of this substance to offset the plaque-inhibitory action of the sulfated polysaccharides found in agar. In our system, using three different concentrations of DEAE-dextran (50 µg/ml, 100 µg/ml and 200 µg/ml), no significant effect on the number of plaques was observed. However, the addition of DEAE-dextran increased the plaque size, thus making the plaques more readily discernible. The following table lists the results obtained with 100 µg/ml of DEAE-dextran in the overlay agar:

Effect of DEAE-dextran on Efficiency of Plaquing

Virus Dilution	With DEAE-dextran		Without DEAE-dextran	
	Aver. No. Plaques	Range of Plaque Size	Aver. No. Plaques	Range of Plaque Size
$10^{-8.0}$	51	2.5 - 5.0 mm	57	1.0 - 2.5mm
$10^{-8.3}$	26	"	24	"
$10^{-8.6}$	15	"	12	"

Investigations of other potential causes of inconsistencies in the plaquing technique are planned, in order to determine whether the plaque-reduction method may be used for quantitatively evaluating the human

responses to the EEE vaccine.

5. Western Equine Encephalomyelitis Vaccine

a. During this period, approximately 700 bottles of freeze-dried purified WEE vaccine (chick embryo origin) for human use were prepared by the method of Randall, Mills and Engel (J. Immunol. 1947, 55: 41). An evaluation of the human responses to this vaccine is being carried out by the Division of Veterinary Medicine, WRAIR. The results to date indicate that the responses are excellent.

b. Studies have been initiated on the production of a WEE vaccine in chick embryo fibroblast tissue culture. The results of preliminary investigations have been encouraging. Current studies include determination of the optimum conditions for the growth, harvesting and inactivation of the virus.

6. Dried Cholera Vaccine

a. Because of other commitments, the studies on cholera vaccine in this laboratory were limited to improvement of production techniques and to periodic assays of the viability of the freeze-dried Inaba and Ogawa seed cultures which were prepared for production of cholera vaccines for WHO field studies.

b. During the preparation of the freeze-dried cholera vaccine in 1963 for the WHO pilot field studies (annual report 1963), it was observed that the harvests of cholera vibrios, inactivated with 0.1% formalin, became extremely viscous upon standing. This phenomenon, apparently due to lysis of the organisms, resulted in technical difficulties in the processing of the harvests. Experimental studies showed that lysis of the vibrios can be prevented by increasing the formalin concentration to 0.25%. This modification resulted in a more fluid product, which could be processed readily. No significant loss in potency was observed as a result of the increased formalin concentration, as is shown in the following table:

Effect of Formalin Concentration of Fluid Divalent Cholera Vaccine on Mouse Potency

Challenge Strain	Inactivation	ED_{50}*	Relative Potency
Inaba	0.1% formalin	0.0000774 (56-178%)	3.23
	0.25% formalin	0.000110 (70-144%)	2.27
	0.5% phenol (Standard vaccine)	0.000250 (50-201%)	-
Ogawa	0.1% formalin	0.0000552 (76-138%)	2.14
	0.25% formalin	0.0000786 (71-141%)	1.50
	0.5% phenol (Standard vaccine)	0.000118 (59-170%)	-

*ED_{50} (and range of one standard deviation expressed in per cent) calculated by method of Wilson and Worcester (Proc. Nat'l Acad. Sci. 29: 207, 1943).

c. The stability of the freeze-dried Inaba (NIH 35A-3) and Ogawa (NIH 41) seed cultures, prepared in September 1962 (annual report, 1963) was studied by periodic titrations of the number of viable organisms in samples stored at various temperatures. The results, as determined by plate counts, are as follows:

Surveillance of Freeze-Dried Cholera Seed Cultures

	Temperature of Storage					
	-20° C		+4°C		+22° C	
Time	Inaba	Ogawa	Inaba	Ogawa	Inaba	Ogawa
0 mos.	7.6×10^8	2.6×10^9				
2 mos.	2.2×10^8	2.1×10^9	2.6×10^8	1.4×10^9	2.7×10^4	1.9×10^6
4 mos.	1.9×10^8	1.5×10^9	9.7×10^6	1.1×10^7	1.0×10^4	3.1×10^4
7 mos.	1.4×10^8	2.9×10^8	8.9×10^7	3.0×10^8	2.0×10^2	3.1×10^3
12 mos.	1.5×10^8	8.9×10^8	7.0×10^6	3.7×10^7	1.7×10^2	8.2×10^2

From these results, it is apparent that the cholera seed cultures showed

no appreciable loss in viability over a 12-month period when stored at -20°C. Storage at 4°C for the same time interval resulted in a drop of about 2 logs in the number of viable organisms. However, these cultures are still satisfactory for use as seed material for vaccine production. It would appear, though, that for long-term storage, -20°C is the temperature of choice.

Summary and Conclusions:

1. Surveillance of the stability of the dried typhoid seed culture, prepared in 1960 for the World Health Organization, has demonstrated that these cultures have maintained their viability after storage at 4°C or 22°C for 3 years. Another large lot of acetone-killed and dried typhoid vaccine, similar to vaccine K produced in 1960 for WHO field trials, was prepared. Part of this vaccine was distributed to the Division of Biologics Standards, NIH, for use as a new national reference typhoid vaccine, part was sent to the WHO International Laboratory for Biological Standards, Copenhagen, and approximately 100,000 ml were set aside for additional human field studies.

2. The human field studies of dried Q Fever vaccine (Henzerling strain, phase 2) have continued. To date 50% of the recipients with no pre-immunization CF titer converted to positive. Two new lots of freeze-dried purified Q Fever vaccine were produced for comparative laboratory and human field studies, one from the Henzerling strain in phase 2, the other from the Henzerling strain in phase 1. An experimental lot of vaccine, employing extraction with genetron-113 in place of ether, was prepared and is currently being evaluated.

3. Experimental studies on Russian Spring Summer Encephalitis vaccine were temporarily suspended due to other commitments.

4. Evaluation of the human response to dried purified chick embryo Eastern Equine Encephalomyelitis vaccine continued. To date, approximately one-third of the recipients who received either three 0.5 ml doses as an initial series, or one 0.1 ml dose as a booster, converted from negative to positive. Studies have continued on the plaque-reduction method for quantitatively measuring the neutralizing antibody content of immune sera. An evaluation of the optimal conditions for plaque production by EEE virus has shown that plaquing efficiency is affected by volume of inoculum but not by temperature of incubation. It has also been demonstrated that an inhibitor present in agar affects the size of plaques, but not the number of plaques.

5. A lot of dried purified chick embryo Western Equine Encephalomyelitis vaccine was prepared. Evaluation of the human responses is currently being carried out by the Division of Veterinary Medicine, WRAIR.

Studies on the production of WEE vaccine in chick embryo fibroblast tissue culture have been initiated.

6. The inactivation of cholera harvests with 0.25% formalin, instead of 0.1% as previously used, has reduced the lysis of the vibrios and has facilitated the processing of the suspensions for the production of a freeze-dried cholera vaccine. Studies on the stability of freeze-dried Ogawa and Inaba seed cultures indicated that, although samples stored at $4^{\circ}C$ for one year still contain sufficient viable organisms, $-20^{\circ}C$ is the temperature of choice for long term storage.

ARMY RESEARCH TASK REPORT

REPORTS CONTROL SYMBOL CSCRD-6(R2)

ACCESSION NUMBER: 36187

PROJECT, TASK, OR SUBTASK NO.: 3A012501A8180204

1. REQUESTING AGENCY
The Army Medical Service
Office of The Surgeon General
Washington, D. C., 20315

2. FUNDING AGENCY
Army Medical R&D Command
Office of The Surgeon General
Washington, D. C., 20315

3. CONTRACTING AGENCY
NA

4. CONTRACTOR AND/OR GOV'T LABORATORY
A Walter Reed Army Inst of Rsch
Walter Reed Army Medical Center
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576-3544 or Interdepartmental Code 198, Ext 3544 See Continuation Sheet 49

6. TITLE OF: PROJECT ☐
TASK ☐
SUBTASK ☒ Sero-recognition of microbial infections (U)

7. DATE OF REPORT | DAY 30 | MONTH June | YEAR 1964

8. RESUME' (U) The work performed in this task is concerned with the mechanisms and patterns of the immune response. In vivo and in vitro methods are being used to study host response to various antigenic stimuli. The antigens employed include those from the infectious organism as well as those from normal and altered mammalian tissue.

Studies of the in vitro detection of antibodies involve isolation, purification, and identification of antigens and the continuing development and/or evaluation of immunodiagnostic methods for the laboratory diagnosis of infectious diseases and autoimmune disorders.

Investigations pertaining to the immune response and its potential harmful or beneficial effect on the host are supplemented by attempts to immunize the host with soluble antigens obtained from infectious agents (e.g., parasites) and by investigations directed toward the development of a laboratory model for studies of autoimmune diseases. Examples of the latter are investigations of runt disease in the albino rat and allergic encephalomyelitis in the guinea pig.

9. KEY WORDS
Serology, immunology, antibody, parasite, antigen, bacteria.

10. SUPPORTING PROJECTS
Not Applicable

11. COORDINATION WITH OTHER MILIT. DEPARTMENTS & GOV'T AGENCIES
☒ NO ☐ YES See Continuation Sheet

12. PARTICIPATION BY OTHER MILIT. DEPTS. & GOV'T. AGENCIES
☒ NO ☐ YES See Continuation Sheet

DA FORM 1 June 63 1309R PREVIOUS EDITIONS ARE OBSOLETE PAGE 1 of ____

ACCESSION NO. 36187	ARMY RESEARCH TASK REPORT

Card "C"

13. PROJECT, TASK OR SUBTASK NUMBER	11–22: 3A012501A818 · 23 24: 02 · 25 26: 04 · 27 28: · 29:
14. DATE OF REPORT (30–33)	30–33: 0664
15. SECURITY OF WORK (34)	34: A
16. TYPE OF REPORT	35: 3 · 36–47 48 49 50 51: · 52–55: 1263
17. SCIENTIFIC FIELD a. Topical Classific. (56–61) b. Functional Class (62–64)	56–61: C10602 · 62–64:
18. OSD CLASSIFICATION (65–66)	65 66: AR
19. R&D CATEGORY (67)	67: 1

Card "D"

20. CONTRACT NUMBER	11 12: DA · 13 14: · 15–17: · 18–21: · 22–26: · 27:
21. GRANT NUMBER	28 29: DA · 30–33: · 34 35: · 36–38: · 39 40: · 41–45: G · 46:
22. ESTIMATED COMPLET. DATES	47–51: 1 [illegible] ONT · 52–56: 2 · 57–61: 3 · 62–66: 4 · 67–71: 5

Card "E"

23. PRIORITY (11–14) 24. PROGRAM ELEMENT (15–26)	11–14: 1 · 15–26: 6.11.25.01.1
25. CMR&D CODES	27–29: NA · 30–32: · 33–35:
26. CDOG REFERENCE a. Paragraph No. (36–44) b. Functional Group (45)	36–39: 1.12 · 40: · 41 42: a · 43 44: · 45: 6

Card "F"

27. FUNDING

a. Est. Total Cost (11–15)	11–15: · 16–18: 1 · 19–21: 2 · 22–26:
b. % Spent Intern. (16–18) " " Extern. (19–21)	
c. Total Obligation (22–26)	
d. Progrmd. Cur. FY (27–33)	27 28: · 29–33: · 34 35: · 36–40:
e. " " " +1 (34–40)	
f. " " " +2 (41–47)	41 42: · 43–47: · 48 49: · 50–54:
g. " " " +3 (48–54)	
h. " " " +4 (55–61)	
i. " " " +5 (62–68)	55 56: · 57–61: · 62 63: · 64–68:
j. " " " +6 (69–75)	
k. Total Man Years of Effort (76–78)	69 70: · 71–75: · 76–78:

DA FORM 1 June 63 1309R — Previous Editions are Obsolete — Page 2 of _____

ACCESSION NUMBER 36187	ARMY RESEARCH TASK REPORT Continuation Sheet

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ANNUAL PROGRESS REPORT

Project No. 3A012501A818 Title: Communicable Diseases and Immunology

Task No. 02 Title: Immunology

Subtask No. 04 Title: Sero-recognition of microbial infections

Description: This task is concerned with the mechanisms and patterns of the host's immune response to natural or experimental antigenic stimuli caused by microbial organisms or by altered and normal mammalian tissue. Studies on the in vitro detection of antibodies involve the isolation, purification and identification of antigens and continuing efforts to develop and improve immunodiagnostic procedures. In vivo investigations on the beneficial or harmful effects of immune response include studies on host response to immunization with selected microbial antigens and efforts to develop a laboratory model for autoimmune diseases.

Progress:

1. Studies on the exoantigens of Schistosoma mansoni cercariae. Although serologic tests employing "somatic" antigens of Schistosoma mansoni (i.e. antigens extracted from the whole organism) provide a reliable and sensitive means for the detection of schistosomiasis, the procedures are of little value in determining the status of infection or in appraising the efficacy of therapy; antibodies usually persist for long periods following the disappearance of ova in the stools and relief from clinical symptoms that accompany an apparently adequate course of therapy. On the other hand, it is well known that the so-called "metabolic" antigens (excretions and secretions of living helminths) evoke a considerably higher degree of protective immunity in animals than do somatic antigens. In view of the latter, consideration was given to the possibility that these secretory and excretory products, designated "exoantigens", could serve as antigen in serological procedures and that such tests might overcome the limitations inherent in standard serologic tests employing somatic antigens. Studies therefore were initiated to investigate the chemical, serological and immunological properties of the cercarial exoantigens. The present report describes the methods developed for collection and concentration of the exoantigens and presents results of chemical analyses and serological evaluations conducted to characterize the antigen component(s).

Preliminary studies revealed that exoantigens of S. mansoni cercariae possessed in vivo as well as in vitro antigenic capacity (Ann. Prog. Rept., 1963-64). However, the amount of exoantigen available for these studies was extremely limited and this precluded comprehensive chemical and serological evaluations necessary to characterize the antigen component(s). These problems were overcome by the recent development of technical innovations which improved the productivity of snail colonies and made it possible to obtain 1.0 - 1.8 million freshly shed cercariae at a given time. In addition, the use of siliconized glassware throughout prevented adherence of the cercariae to the vessels and more importantly, significantly increased survival of the organisms during the incubation required for elaboration of the exoantigens.

Cercarial exoantigens were obtained by incubating freshly shed cercariae in charcoal-conditioned tap water for 18 hours at 23°C. Following removal of the cercariae by centrifugation and subsequent filtration through an F-porosity sintered glass filter, the supernate containing the antigen was concentrated by flash evaporation to a volume corresponding to 1 ml/70,000 cercariae. The concentrate then was frozen and thawed four times to free the antigen components from the mucoid-like precipitate that developed during concentration, and the insoluble fraction removed by centrifugation for 1 hour at 10,000 rcf. Since initial experiments revealed that antigens prepared in this manner contained dialyzable components that inhibited the fixation of complement by immune complex, the preparations used in the present studies were dialyzed vs distilled water for 72 hours prior to lyophilization and storage in the dried state. A control consisting of an aliquant of the water into which the snails had shed the cercariae also was processed in a similar manner to provide a basis for determining the products that were of snail rather than cercarial origin.

Chemical analyses of the exoantigen indicated that the product was primarily a polysaccharide, or possibly a glycoprotein. It is noteworthy that although the antigen appeared to contain some protein it is not yet clear whether this is an essential component of the antigen. Nevertheless, protein was present only in low concentration and the chemical behavior of the antigen was essentially that of a polysaccharide; only a trace of gel was obtained in the highly sensitive chloroform-gel fractionation procedure and no visible precipitate was formed by isoelectric precipitation methods in the pH range 7.2 - 4.0, either in the presence or absence of 10% ethanol. In contrast, treatment with acetone did precipitate a component and this suggested the presence of polysaccharide and/or nucleic acid. The latter however could be reasonably excluded since no absorption maximum could be demonstrated spectrophotometrically at wave length 260 lambda. Quantitative analyses revealed a carbohydrate:protein ratio of approximately 3:2 in both the exoantigen and the snail water control. Moreover, the respective carbohydrate and protein values of the two products were essentially the same. Although the near identity of these values was not anticipated and could not be explained on the basis of available data, further analyses revealed certain significant differences between the two products. For example, the hexosamine content of the exoantigen was $3\frac{1}{2}$ times that of the control, even though the total carbohydrate content of the two products was essentially the same. Moreover, the total amino nitrogen of the antigen was $2\frac{1}{2}$ times greater than that of the control but the total protein values of the products were the same. Since the exoantigen is highly reactive in complement fixation tests with homologous antibody whereas the control is nonreactive, it appears that hexosamines constitute a major component of the serologically active antigen complex. Some insight concerning the linkage of the component units of the antigen molecule was obtained from the amino nitrogen assays. It was observed that hydrolysis of the antigen with 0.57 N HCL resulted in a fourfold increase of detectable amino nitrogen, thus indicating that considerable linkage of the molecule was through amino groupings. Finally, in contrast to certain other hexosamine-containing polysaccharides of biological origin, no N-acetyl hexosamine was detected in either the exoantigen or control.

Descending paper chromatography of the hydrolyzed and unhydrolyzed exoantigen using n-butanol:acetic acid:water solvent (4:1:5 v/v/v)revealed the presence of two reducing components, one with an Rf corresponding to that of glucose and the other to glucosamine and/or galactosamine. Other solvent systems will be employed in future studies in an effort to determine which of the two amino sugars is present in the antigen. Repeated efforts to detect and identify amino acids, however, were unsuccessful, presumably because the concentrations were below the levels detectable with the ninhydrin reagent. Development of the chromatograms with Oil Red O stain revealed the presence of lipid in trace amounts. Studies now are under way to determine whether lipid is an essential component of the antigen molecule.

The exoantigen showed excellent specificity and sensitivity in complement fixation tests on a group of 50 sera from healthy persons and from individuals with a variety of parasitic diseases. All tests with normal sera were nonreactive. Of the phylogenetically unrelated parasitic diseases, reactions were observed only with sera from cases of trichinosis. It is well known that Schistosoma antigens frequently cross react with Trichinella antisera and thus the latter reactions were not entirely unexpected. The exoantigen was further evaluated in tests on a group of 87 sera obtained from individuals residing in a S. mansoni endemic area and the results compared with those obtained in tests performed with the standard somatic antigen. Complete agreement was shown in tests with 70 (80%) of the specimens; 54 reacted in both tests and 16 were nonreactive in both tests. Of the sera giving discrepant results, 10 were reactive with the somatic antigen and nonreactive with the exoantigen, whereas 7 were reactive with the exoantigen and nonreactive with the somatic antigen. In view of the near identity of the number of reactors (10 & 7) in the two categories of disagreement, it seems unlikely that the divergent results were due simply to different levels of test sensitivity. If this had been the case, one would have anticipated a significantly greater number of reactions with the more sensitive antigen. The findings, on the other hand, appear to indicate that the exoantigens and somatic antigens may not react with precisely the same antibodies, and that the relative concentrations of the respective antibodies may vary significantly during different stages of infection.

2. Studies on the exoantigens of Schistosoma mansoni adult worms. Following the initial successes in the studies concerning the exoantigens of S. mansoni cercariae (No. 1, this Progress Report), investigations were initiated to determine if similar antigenic material was liberated by the adult worm during in vitro cultivation.

S. mansoni adult worms were recovered aseptically by perfusion of the hepatic portal veins of infected mice. Sterile saline with penicillin and streptomycin was used for the perfusion and for rinsing the worms until they were free of host blood. The harvested worms were transferred aseptically to sterile T-flasks containing TC 109, a protein-free, chemically defined medium. The supernatant culture medium was removed at selected time intervals and replaced with fresh TC 109. The worms survived two to four weeks if the medium was replaced every 1-2 days and the cultures incubated at 22°C. Following incubation the medium contained substances which fixed complement (C') in the presence of homologous antibody but did not fix complement in the presence of normal sera. Preliminary chemical tests show approximately 46 micrograms of protein per ml (Lowery) and 26 micrograms of carbohydrate per ml (Shetlar). However, the reactive material declined rapidly during the first 5-6 days of incubation.

Attempts will be made to determine if the reactive material represents exoantigen(s) produced by the worms during cultivation or if it is a complex of host-worm material produced in the worm in vivo and then released during the first 5-6 days of in vitro cultivation. It is also possible that the reactive material may result from breakdown of worm tissue during cultivation.

If it can be shown that the reactive material is an exoantigen(s) detailed serological, immunological and chemical analyses will be undertaken.

3. Studies on the exoantigens of Trypanosoma cruzi. During recent years considerable effort has been devoted to the development and improvement of serodiagnostic tests for American trypanosomiasis (Chagas' disease). These studies culminated with the isolation of a somatic protein antigen (Fife and Kent, Am. J. Trop. Med. & Hyg. 9:512, 1960) that proved to be highly specific in complement fixation tests conducted at the diagnostic level. Subsequently, Weitz (Nature 185:788, 1960) observed that soluble antigens were present in the serum of rats infected with T. brucei and demonstrated that these "exoantigens" were considerably more specific than the "somatic" antigens obtained from the whole organism. In view of these findings, consideration was given to the possibility that exoantigens of T. cruzi might prove superior to somatic antigens in the serodiagnosis of Chagas' disease. The present report deals with preliminary studies along these lines.

The mass-culture technics developed during investigations on somatic antigens (Fife & Kent, op. cit.) proved to be ideally suited for the collection of in vitro exoantigens of T. cruzi. In this procedure the organisms were cultivated within cellulose sacs, thus excluding solid medium components from the material to be harvested. Following incubation for 35 days at 23°C, the sac contents containing the trypanosomes, peptone medium and exoantigens were pooled and the trypanosomes removed by centrifugation. The supernatant fluid then was dialyzed against buffered salt solution to remove excess salts and dialyzable medium components. The dialysant, designated crude or unfractionated exoantigen, finally was lyophilized and stored in the dried state.

Preliminary studies revealed that the crude exoantigen reacted well in complement fixation tests with homologous antibody and showed essentially no anticomplementary activity. Various physico-chemical fractionation procedures were employed in an effort to characterize the serologically active component(s) of the exoantigen. It was soon evident, however, that both carbohydrate and protein were essential for serologic activity and thus it appeared that the major specifically reactive component of the antigen was a glycoprotein. Quantitative chemical and serological analyses of the products obtained by the various fractionation procedures revealed that maximum serologic activity occurred with fractions exhibiting a carbohydrate:protein ratio of 1:2 to 1:2.5. Fractions with ratios outside this range showed significantly reduced activity. The fractionation method of choice was isoelectric precipitation at pH 4.6 wherein the antigen resided in the soluble fraction.

The specificity and sensitivity of the pH 4.6 soluble exoantigen was evaluated in complement fixation tests with a variety of homologous, heterologous and normal sera. Parallel tests with the unfractionated exoantigen, a somatic protein antigen and a delipidized somatic antigen were included for reference (Table 1). It is noteworthy that the somatic antigens had previously been extensively evaluated by the test originators, thus providing a sound basis for appraising the efficacy of the exoantigens. All of the antigens showed excellent sensitivity; complement fixing antibodies were demonstrated in each homologous serum tested. Marked differences of specificity, however, were observed, particularly in tests with the delipidized somatic antigen. It was obvious that the latter was not suitable for use in diagnostic tests in view of the high frequency of reactions with heterologous sera. On the other hand, the specificity of the exoantigens and the somatic antigen was excellent. With the exception of certain phylogenetically related diseases, reactions with heterologous sera were virtually nonexistent. The single leprosy serum that reacted with all antigens was from a patient residing in a T. cruzi endemic area and the possibility of a concurrent T. cruzi infection must be considered. It was surprising to note that the specificity of the unfractionated exoantigen was essentially the same as that of the purified somatic protein antigen and far surpassed that of the delipidized somatic antigen. In fact, with the single exception of one hookworm infection, the unfractionated exoantigen showed cross reactions only with the closely related Leishmania and T. rhodesiense sera. Moreover, even the latter reactions were eliminated by the fractionation procedure; the pH 4.6 soluble exoantigen exhibited no false reactions whatsoever, but still maintained a level of sensitivity comparable to that of the less specific antigens.

In addition to being excellent complement fixing antigens in in vitro tests, the exoantigens also exhibited in vivo antigenic properties. Relatively small amounts of exoantigen injected into rabbits elicited a rapid antibody response. Adjuvant was not required and antibody titers of 32 were detected in CF tests within 20 days following the first inoculation.

Although additional evaluation is necessary, studies thus far indicate that the exoantigens of T. cruzi may play an important role in the serodiagnosis and immunology of Chagas' disease. These products are readily obtained, are highly specific, and do not require the intricate fractionation procedures necessary for obtaining satisfactory somatic antigen. In addition, the capacity of small amounts of exoantigen to rapidly induce production of relatively high levels of antibody indicates the desirability of studies on the immunogenicity of the antigen and its potential use as a vaccine.

Table 1

The relative sensitivity and specificity
of exo and somatic antigens of Trypanosoma cruzi

Diagnostic status	No.	Results* obtained in CF test with: Exoantigen Unfract. R	wr	-	pH 4.6 sol. R	wr	-	Somatic Antigen Protein** R	wr	-	Delipid.# R	wr	-
T. cruzi	22	21	1		21	1		21	1		22		
T. rhodesiense	15	1	1	13			15			15	4	4	7
L. brasiliensis	18		3	15			18		2	16	10	4	4
L. donovani	9		1	8			9		1	8	6	1	2
L. tropica	2			2			2			2			2
Schistosomiasis	10			10			10			10	1	1	8
Filariasis	11			11			11			11	3	2	6
Trichinosis	9			9			9			9	2	1	6
Hookworm	8		1	7			8			8	2		6
Tuberculosis	5			5			5			5		2	3
Leprosy	8##	1		7	1		7	1		7	4	2	2
Malaria	7			7			7			7	2		5
Enteric	5			5			5			5			5
Syphilis	25		2	23			25			25	17	6	2
Healthy	54		2	52			54			54	13	11	30

* Reaction (R), weak reaction (wr), and no reaction (-).
**Fife and Kent, 1960.
Chaffee, et al., 1956.
##Leprosy sera from T. cruzi endemic area.

4. Serodiagnosis of African trypanosomiasis. Previous studies indicated that antigens derived from a culture strain of T. rhodesiense did not detect antibodies in early or advanced cases of Rhodesian sleeping sickness (Annual Progress Report, 1963). It was suggested that the employed trypanosome strain, having been maintained in vitro for many years, had undergone significant variation and consequently shared only remote antigenic relationships with wild strains transmitted by the tsetse. It was necessary, therefore, to temporarily discontinue the studies until freshly isolated strains could be obtained. This problem was solved by the recent acquisition of newly isolated strains of T. rhodesiense and T. brucei from the trypanosome library of the East African Trypanosomiasis Research Organization, Tororo, Uganda. Experiments now are being designed to determine whether the above postulate is correct, giving special attention to the possible role of "exoantigens".

5. Serological studies on filariasis. Deficiencies in the sensitivity and specificity of tests currently employed for the serodiagnosis of filariasis have severely limited the usefulness of these procedures. Studies therefore were initiated to determine whether serodiagnostic tests could be improved by employing various fractions of Dirofilaria immitis as antigen in complement fixation (CF), hemagglutination (HA) and hemagglutination-inhibition (HI) procedures.

Crude extracts of adult D. immitis were prepared by extraction of desiccated worms with pH 8 borate buffer in a motor driven glass tissue grinder. After removal of cellular debris by centrifugation, the supernate was fractionated by the chloroform-gel procedure described by Fife and Kent (Am. J. Trop. Med. and Hyg., 9:512, 1960). The resulting protein and carbohydrate fractions in conjunction with an unfractionated sample were evaluated in complement fixation tests with 13 sera from human cases of filariasis, 3 sera from dogs infected with D. immitis, and a serum from a rabbit infected with Dirofilaria uniformis. All of the human sera reacted strongly with the unfractionated control antigens. With the protein fraction, nine of the sera were reactive, one was weakly reactive, and three were nonreactive. Only two of the human sera gave reactions with the carbohydrate fraction and one of these was of low order (weakly reactive). Of the three sera from infected dogs, one was reactive, and the other two were weakly reactive with the protein fraction, whereas in parallel tests with the carbohydrate fraction, one specimen gave a weak reaction and the other two were nonreactive. Both antigen fractions were highly reactive in tests with the D. uniformis rabbit serum.

To evaluate the efficacy of another fractionation procedure, the crude D. immitis extract was fractionated according to the method of Sleeman and Muschel (Am. J. Trop. Med. and Hyg., 10:821, 1961). The ethanol soluble (ES) fraction contained the carbohydrates, and the ethanol insoluble (EI) was the protein fraction. The EI fraction reacted in CF tests with D. uniformis rabbit serum, D. immitis dog serum, and sera from humans with filariasis. However, the antigen lacked specificity and reacted with the serum of uninfected dogs, uninfected rabbits and with sera from humans who had a wide variety of diseases. The ES fraction reacted only with D. uniformis rabbit serum.

Additional studies on the antigens of D. immitis were performed with the hemagglutination (HA) test described by Kagan et al. (Am. J. Trop. Med. and Hyg., 9: 248, 1960). Twenty-four sera from human cases of filariasis gave only weak reactions in the HA test. Twelve sera from human cases of onchocerciasis also were tested. Two of these were reactive, one was weakly reactive, one gave a doubtful reaction, and eight were nonreactive. When negative and positive D. immitis dog sera were tested, a serological reversal was obtained; the negative sera agglutinated the cells, whereas, the positive sera showed no agglutination. The Hemagglutination inhibition (HI) test therefore was performed on these specimens. Thus, an immune rabbit serum was incubated for 1 hour at 37°C with an equal volume of serum (dog or human) to be tested. Tannic acid treated sheep erythrocytes which had been sensitized with ES antigen were added to all tubes, the mixture incubated overnight at room temperature, and then read. None of the twenty-five human sera and only one of seven dog sera that were tested inhibited the hemagglutination of the immune rabbit serum.

In view of the results of these preliminary studies, it is apparent that many problems still must be solved before the reliability of serodiagnostic tests for filariasis approaches that attained in other parasitic diseases.

6. Isolation, purification, and fractionation of malarial antigens. Efforts to develop methods for the isolation of malarial antigens were continued. Plasmodium knowlesi was selected for the experimental model since relatively large volumes of this species of parasite can be obtained from experimentally infected Macaca mulatta. Crude extracts of the parasites were fractionated by various methods and the serologically reactive components subjected to chemical, serological and immunological evaluation.

In preliminary studies, various methods for separating the parasite from the monkey red blood cell were evaluated. A pool of erythrocytes from 12 infected monkeys (Parasitized rbc's 25% - 75%) was washed, lysed and extracted by a variety of methods. The results indicated that saponin was superior to water for the lysis of the rbc's and liberation of the parasitic material. Phosphate (pH 7.8) or triethanolamine buffered salt solution (pH 7.2) extracts prepared either by homogenization or sonication of the parasite yielded antigenic preparations which were more reactive in complement fixation tests than were similarly prepared borate buffer (pH 8.5) extracts. Dialysis against water at 5°C for 16 hours or heating at 60°C for 30 minutes destroyed the serologic activity of the extracts. All of these crude extracts showed some anticomplementary (AC) activity in the CF test. Several fractionation methods including ammonium sulfate precipitation, isoelectric precipitation at controlled pH, and ethanol precipitation have not been successful in eliminating the AC activity. The purity of the serologically active component could be increased approximately ten-fold by passing the crude extract through a column of Sephadex G-200. However, this treatment failed to completely eliminate AC activity.

Lysates from the saponin and the water lysed preparations also exhibited antigenic activity in complement fixation tests, presumably due to the release of soluble antigen components from the plasmodia during lysis of the erythrocytes. These products are being investigated concurrently with the studies on extracts of the whole parasite.

Experience has shown that the maximum yield of parasites is obtained from monkeys who show a 40-50% parasitemia with a preponderance of mature segmented forms. In practice, however, this is difficult to achieve because P. knowlesi produces an asynchronous fulminating parasitemia in monkeys during the near terminal stage of infection. Frequently the animal develops a severe toxemia at this time and the investigator may be forced to exsanguinate the animal when ring forms of the parasite predominate rather than risk death of the animal before the parasites mature to the segmented stage. In an effort to overcome these difficulties and increase the yield of parasite material, ancillary studies concerning the in vitro maturation of intracellular plasmodia were initiated. Preliminary experiments were designed in accordance with the methods of McKee et al., (J. Exp. Med., 84: 569, 1946) who obtained in vitro maturation of ring forms to segmented forms by incubating parasitized simian erythrocytes in a synthetic medium for 36 hours at 39°C. Maturation was observed in the initial in vitro cultivation experiments. However, development was more rapid than anticipated and the parasites progressed through the segmented state into merozoites. This development delayed investigations on the efficacy of in vitro matured parasites as a source of antigen but studies along these lines are being continued.

Although P. knowlesi infection in rhesus monkey (Macaca mulatta) usually results in death within 7-10 days, efforts have been made to obtain sera with high antibody titer by inducing chronic infections by treatment with subcurative regimens of sulfadiazine. The titers of sera obtained thus far have been relatively low in CF and indirect fluorescent antibody tests. Nevertheless, it is anticipated that high titered sera required for immunodiffusion and immunoelectrophoresis studies still may be forthcoming.

Although the major efforts thus far necessarily have been directed toward development and improvement of methods for collecting malaria parasites, initial studies indicate that satisfactory CF antigens can be obtained from these materials. In addition, preliminary investigations on soluble antigen fluorescent antibody procedures (Item 10, this Annual Progress Report) suggest that soluble plasmodial antigens also may be employed in this technic.

7. Serological tests with treponemal infections other than syphilis.

a. As part of a comprehensive study of pinta (Treponema carateum) in Bolivia (D. Joseph Demis, M.D., Director of Dermatology, Washington University School of Medicine, St. Louis, Missouri), 337 sera were collected from patients with clinical pinta, persons without anamnestic or clinical evidence of the disease and from individuals residing in presumed nonendemic areas. The sera were tested in the Treponema pallidum immobilization (TPI), fluorescent treponemal antibody (FTA) and slide flocculation (SF) tests. The results have been reported to the coordinator (Dr. Demis), who at present is correlating the findings in the serological tests with the clinical status of

the patient. In addition to evaluating FTA test in pinta, the results should shed some light on whether latent pinta exists in Bolivia. Small volumes of these sera have been stored for future evaluation of other treponemal tests.

b. In a similar study, serum was collected from 69 individuals in Iran. Many of these individuals have endemic syphilis (Bejel). At present the sera are being tested in TPI, FTA and CM tests. The serological and clinical results are to be correlated. This is a rapidly disappearing disease and the unused stored sera will be invaluable for evaluation of future treponemal tests.

8. Participation in World Health Organization inter-laboratory evaluation of the fluorescent treponemal antibody (FTA) test for syphilis. The Department of Serology, WRAIR, has accepted an invitation by the WHO to be the American laboratory participating in the Second WHO Inter-Laboratory Evaluation of the FTA Test. The main objectives of this evaluation are four-fold: (1) To study inter-laboratory reproducibility of the FTA test; (2) To study the reproducibility within a given laboratory over an extended period; (3) To compare lyophilized versus liquid antigen; and (4) To determine the effect of incubation temperature on the sensitivity and specificity of the test. This study has been in progress since 24 March 1964 and will continue through 16 January 1965. At the conclusion of the study a statistical evaluation of the results will be made by the WHO and recommendations based on these findings will be presented. It is hoped that carefully designed comprehensive evaluations such as this will ultimately provide a basis for devising an FTA procedure that will receive universal acceptance.

9. Evaluation of the rapid plasma reagin (RPR) card test for treponemal infections. The RPR card test employs the cardiolipin, lecithin and cholesterol complex for antigen as does the standard cardiolipin microflocculation (CM) test used by the Army as a primary screening procedure for syphilis. Choline chloride and ethylenedinitrilotetroacetic acid (EDTA) also are added to the RPR antigen to stabilize the emulsion and to permit the testing of plasma as well as serum. In addition, activated charcoal is combined with the RPR emulsion in order that tests can be read against a white background without the use of a compound microscope. Disposable cards containing a dried anticoagulant and a red cell agglutinin are obtainable with the test kit. Complete kits are commercially available. The RPR card test has obvious advantages for field work and in areas where venipunctures are difficult to obtain.

Since the RPR card test and the plasma extraction cards still are undergoing development and improvement, this Department has performed only a limited evaluation of the procedure. Nevertheless, a preliminary evaluation was performed under laboratory conditions to determine whether the test yielded sufficient specificity and sensitivity to be used in areas where it was impossible or extremely difficult to perform the standard CM test. Since battery driven rotators were available, the test method utilizing mechanical rather than manual rotation was evaluated on serum specimens; the method employing plasma extraction cards was not evaluated because some difficulty had been encountered when these were used in similar tests employing parasitic disease antigens.

The tests were performed on printed cards, a total of 0.05 ml of unheated serum and 1/60 ml of antigen being placed within the designated circle on the card. The cards were rotated on a rotating machine (100 rev/min) for 8 minutes and the results visually read immediately without magnification. The findings were recorded as reactive or nonreactive and correlated with the results obtained with the same sera in the standard CM test, Treponema pallidum immobilization (TPI) test and/or the cardiolipin complement fixation (CCF) test.

In the first trial, 358 sera submitted for routine diagnosis were tested. Seventeen of these sera reacted in both the RPR and CM tests whereas 337 were negative in both procedures. The remaining 4 reacted in the CM but not in the RPR. Moreover, the latter sera showed no reaction in the CCF.

An additional 316 specimens; all presenting diagnostic problems, also were tested. There was agreement between the RPR and CM with 232 of the sera; 55 reacted solely in the CM and 28 solely in the RPR. Fifteen of the 55 CM reactive RPR negative sera showed a reaction in the TPI test. The remaining 40 sera were TPI negative. Of the 28 sera which were RPR reactive - CM negative, 21 were TPI reactive and the other 7 were TPI nonreactive.

These results indicate that the version of the RPR test using sera and low speed rotation is an acceptable procedure for field use.

Since the RPR procedure is still in the process of being modified by the manufactures and since the U. S. Public Health Service and others are performing large scale evaluations on the procedures, only limited evaluation of the test will be performed in this laboratory. Moreover, the evaluation will involve only those modifications believed to be of primary importance in field use.

10. Soluble antigen immunofluorescence. All of the previously described fluorescent antibody (FA) technics for the serodiagnosis of microbial infections have employed the whole organism as antigen. In these procedure, the organisms usually are fixed (e.g. with 10% formalin) and the surface antigens of the organism and/or those which diffuse through the cell membrane are used to detect the presence or absence of antibody. Thus, the investigator has no control over the antigens that enter into the reaction and must depend entirely on those present at the surface of the organism. It is highly desirable, therefore, to develop methods wherein soluble antigens might be employed in lieu of intact organisms. The availability of such technics would allow an investigator: (1) To use selected purified antigens; (2) To use an amount of antigen sufficient to minimize the fading of fluorescence that usually is encountered in tests employing whole microorganisms, and (3) To permit use of a fluorometer in reading the results, thereby eliminating the highly subjective reading methods normally employed in tests with whole organisms as antigen.

Preliminary studies were based on the methods of Paronetto (Proc. Soc. Biol. & Med., 113: 394, 1963) who used bovine serum albumin (BSA) as antigen and rabbit anti-BSA in soluble antigen studies. When Schistosoma mansoni cercarial antigen was used instead of BSA, nonspecific fluorescence was encountered. To determine which protein fraction contained the factor responsible for this nonspecific fluorescence, human serum was fractionated by Cohn's method 10. The nonspecific factor was found in Fraction III which contains approximately 75% of the serum lipids. Attempts to reduce nonspecific fluorescence by extracting the lipids from the serum were unsuccessful. Furthermore, serum euglobulins fractionated by isoelectric precipitation in dilute HCl (Erickson, et al., Am. J. Syph. Gonorr. & V. D., 31: 374, 1947) also contained the factor causing nonspecific fluorescence with human serum. However, it was observed that nonspecific fluorescence could be prevented by incorporating 1% BSA in the antigen solution. In subsequent studies, excellent results were obtained by treating cellulose acetate paper with S. mansoni cercarial antigen containing 1% BSA and testing against homologous human serum. Moreover, a marked difference of fluorescence with reactive and nonreactive human sera was observed in tests employing the antigen from the adult stage of S. mansoni to which had been added 1% BSA.

Ancillary studies were conducted to determine the relative efficacy of various papers for use in this procedure. Thus, ion exchange paper (anion and cation), Whatman filter paper #1, S & S cellulose acetate electrophoresis paper and E-D filter paper #613 were tested as carriers for soluble antigen in the system. S. mansoni cercarial antigen was fixed to these papers and then tested against reactive and nonreactive rabbit sera. Of the five papers evaluated, the S & S cellulose acetate paper gave the most promising results and was used in subsequent studies.

The technic is still under investigation and additional modifications and improvements are anticipated. However, the methods currently in use have yielded very satisfactory results. Small discs (1/4" dia.) of cellulose acetate paper were immersed in undiluted soluble antigen containing 1% BSA. After allowing the excess antigen to drain from the paper, the wet discs were placed in a Petri dish and dried at room temperature. Antigen was then fixed to the discs with 95% ethanol and the alcohol removed by three washings with Tris-buffered saline (pH 8). The wet papers then were immersed in a small volume (0.1 ml) of serum and allowed to incubate at room temperature for 60 minutes. The papers were washed free of serum with three changes of buffered saline. To determine whether antibody had reacted with the antigen, an optimal concentration of fluorescein-labeled antiglobulin diluted in buffered saline containing 2% Tween-80 was added to each test disc. These were allowed to stand in a moist chamber at room temperature for 60 minutes. Unreacted antiglobulin was removed by washing the discs with three changes of buffered saline. The discs finally were blotted, dried and read macroscopically with short range ultraviolet light (150-180 mu) illumination.

To determine whether soluble antigen FA could be used in antigen-antibody systems other than S. mansoni, Plasmodium knowlesi soluble antigen containing 1% BSA was fixed on cellulose acetate paper and tested with P. vivax human serum and with normal human serum. The tests with serum containing malarial antibody exhibited strong fluorescence whereas those conducted with normal serum showed no fluorescence.

In a series of adjunct experiments, attempts were made to label S. mansoni cercarial antigen with fluorescein isothiocyanate. The conjugation procedures developed by Clark & Shepard (Virology, 20: 642, 1963) were followed and the product tested in direct FA tests. The results, however, indicated that the antigen did not conjugate with the dye. Investigations along these lines are being continued in an effort to devise methods for coupling a fluorochrome to schistosomal and other antigens.

The feasibility of employing a fluorometer for reading the fluorescence on cellulose acetate paper also was investigated. Preliminary studies were performed with S. mansoni cercarial antigen and various dilutions of normal and homologous rabbit antiserum. Results thus far indicate that the fluorometer can readily detect fluorescence in the tests with homologous antiserum, and of even greater importance, can quantitate the magnitude of fluorescence obtained in each individual test.

Although the results obtained thus far must be considered to be preliminary in nature, the findings indicate that technics using soluble antigens in immunofluorescence can be put on a practical basis. Moreover, a further advantage of the system is the ability to read test results mechanically on a fluorometer, thus eliminating the subjective readings necessarily employed in systems utilizing whole organisms as antigen.

11. Effect of antimalarial drugs on immune mechanisms. During studies on the effects of antimalarial drugs on the various parameters of normal and abnormal immune mechanisms, it was demonstrated with R reagents that quinacrine selectively destroyed the second (C'2) and fourth (C'4) components of complement in vitro, but not in vivo. Accompanying electrophoretic changes were observed in quinacrine treated serum. (Annual Progress Report 1963).

Further studies with cell intermediates (sheep cells sensitized with hemolytic antibody and those complement components preceding the component being tested in the reaction sequence) have confirmed the previous work done with R reagents. In addition it has been found that cell bound C'2 is inactivated by quinacrine, while cell bound C'4 is resistant. Studies on recently recognized complement components previously classified collectively as C'3, have shown that C'3a and C'3d are also attacked by quinacrine; however, the remaining C'3 components are not.

In an attempt to study further the relationship of observed electrophoretic and immunoelectrophoretic changes in quinacrine treated serum to loss of C'2, C'4, C'3a and C'3d activity, a new micromethod for preparative electrophoresis in agar gel has been developed. This method which allows preparative separation of 0.011 ml of material, has the advantage over other methods of preparative electrophoresis in that time required for electrophoresis is short (1-1/2 - 3 hours). This results in sharp peaking of the serum proteins into albumin, alpha$_1$, alpha$_2$, beta$_1$, beta$_2$, and gamma regions, with very little tailing. Time required for processing of 50 samples from a single separation is approximately seven minutes. Protein recovery after eight hours' elution is 97%. In addition, the method allows a check for antigenic material present by means of simultaneous gel diffusion against specific hyperimmune antisera, thus providing a simple method for the correlation of functional properties of proteins with their specific precipitin band in immunoelectrophoresis.

To date, the method has been applied to several complement components. Guinea pig C'4 has been localized to a single precipitin band in the beta$_1$ region analagous to that recently described for human serum. Following quinacrine treatment this protein shows an increase in mobility, indicating a loss of positive charge (veronal buffer, pH 8.2, u = .035). It has been possible to localize precisely other complement components (C'2 and C'3d) by means of their functional activity. However, in these instances no corresponding precipitin band has been detected when checked against hyperimmune antisera from 9 rabbits.

12. Bactericidal reactions of the antibody-complement system against gram negative organisms. Research on this project was curtailed in March 1963 when the principal investigator left WRAIR. Manuscripts dealing with the studies completed at this time were prepared and published. Investigations along the lines indicated (Annual Progress Report, 1963)have been delayed pending the hiring of a replacement. The replacement started work 1 April 1964. Consequently, there is no further progress to report at this time.

13. Studies on allergic encephalomyelitis in the guinea pig and runt disease in the rat. As was previously noted (Annual Progress Report 1963-64) experimental allergic encephalomyelitis in the guinea pig and runt disease in the rat are being used as experimental models in efforts to elucidate immunological mechanisms that might be involved in the development of autoimmune diseases in humans. For a considerable portion of the present report period, severe restrictions in animal quarters allotted to this Department dictated that these investigations temporarily be curtailed. Thus, the progress todate already has been published in scientific journals. It is noteworthy, however, that the physical problems recently were alleviated and the studies now are being continued.

14. The influence of x-irradiation upon the mechanism and pattern of the immune response. Autoantibody formation in x-irradiated animals was studied to learn if ionizing radiation would cause rabbits to readily produce antibodies against their own tissue. The purpose of the investigation was to learn if such autoantibodies could be used for the purpose of serologically detecting and evaluating radiation injury of tissue. Antigens extracted from irradiated rabbit testes were found to contain less protein, carbohydrate, phosphorous and deoxyribonucleic acid, but more lipid than non-irradiated controls. Electrophoretic properties of irradiated tissue antigens differed markedly from non-irradiated preparations. Despite these differences no evidence was found to indicate that radiation-altered testes antigens were more active as autoantigens than non-irradiated tissue. Conversely, irradiated tissue was found to be less immunogenic and less serologically reactive than non-irradiated control antigens. Fractionation of tissue antigens and serums by cold ethanol was carried out to characterize the protein constituents involved in the complement fixation reaction. Antigen activity was found predominantly in the beta-lipoprotein fraction III-0 and the cold insoluble fraction I + III-3. Naturally occurring rabbit antibody to rabbit testes was found by ethanol fractionation and ultracentrifugation to be a 19 S, beta-2-globulin. In serum from rabbits immunized with rabbit testes, antibody was also found in the 7 S gamma globulin fraction.

a. Autoantigens from rabbit testes. To learn if X-irradiation could alter tissue so that it became immunologically "foreign" to its host, antigens were made from testes of rabbits which had been exposed to 2000 r with the remainder of the animal protected by shielding. Tissue was obtained 2, 10 or 42 days after irradiation and antigen extracted with cold 0.85 per cent saline. Spermatazoa and debris were removed by centrifugation and the supernatant antigens used to immunize the same rabbits from which the tissue had been obtained. In all cases, antigens obtained 10 or 42 days after 2000 r were less immunogenic and less serologically reactive than antigens prepared at the same time from non-irradiated control tissue. No evidence of new antigenic characteristics was observed in the irradiated tissue. Tissue obtained 42 days after 2000 r was much less anticomplementary than antigen extracted from non-irradiated animals.

Antigens from testes were fractionated by the cold ethanol method of Cohn, et al. (J.A.C.S., 72: 465, 1950) and each fraction tested for ability to fix guinea pig complement in the presence of normal rabbit serum and antiserum to normal rabbit testes. Antigen activity was found to be contained in fraction III-0 (beta-1-lipoprotein and beta-1-lipid-poor euglobulin) as well as fraction 1 + III-3 containing the cold insoluble material. Albumin, alpha-globulins and gamma globulin fractions showed no detectable antigen activity.

Chemical and electrophoretic characteristics of antigens from irradiated rabbit testes were studied to learn how these tissues differed from non-irradiated tissue. Tissue obtained 2, 10 and 42 days after 2000 r were assayed for dry weight, protein, carbohydrate, deoxyribonucleic acid, phosphorous, and lipid content. Electrophoretic properties of protein constituents in agar gel were also compared. Irradiated testes did not show large differences in content of solids in comparison with non-irradiated controls. On the other hand, antigens extracted 10 or 42 days after 2000 r revealed markedly lower amounts of protein, deoxyribonucleic acid, carbohydrate and phosphorous. There was a sizeable increase in lipid content of the irradiated tissue extracts. Electrophoresis in agar gel followed by protein staining showed a sizeable increase of fast moving component (resembling albumin) in the irradiated tissue associated with a concomitant decrease in protein electrophoretically resembling alpha-globulin. There appeared little change in relative amounts of beta-2 or gamma globulins.

b. Rabbit serum components reactive with antigens from rabbit testes. An effort was made to test the hypothesis of Allegretti (Bull. Scien., 5: 77, 1960) which states that ionizing radiation may cause mutation of antibody forming cells so that they lose their ability to recognize their own tissue as "self" and thus produce autoantibodies. Rabbits were given 250 r or 500 r whole body radiation (WBR) followed 42 days later by either a single intravenous injection of normal rabbit testes antigen, or a series of intramuscular injections of this antigen in combination with adjuvant. Subsequent serum samples obtained at periodic intervals showed that the rabbits did not produce enhanced amounts of antibody to rabbit tissue. Thus, no evidence was obtained indicating that auto-antibody production is likely to occur in irradiated rabbits as a result of failure of the antibody forming mechanism to recognize rabbit tissue as "self".

Inhibition of antibody formation in rabbits was observed when a single injection of rat testes, sheep erythrocyte, or Salmonella typhosa "O" antigen was given 1 or 10 days after 500 r WBR. This indicated, that under the conditions of our experiments, WBR had a strong inhibitory effect on antibody production to even immunologically "foreign" antigens if they were injected after irradiation.

Fractionation of normal rabbit serum by cold ethanol revealed that fractions III-1.2 (beta globulins) and III-0 (beta-1-lipoproteins and beta-1-lipid-poor euglobulins) possessed complement fixing activity with antigens extracted from rabbit testes. In serum from rabbits immunized with normal rabbit testes antigen, antibody activity also appeared in the gamma globulin (fraction II) as well as enhanced levels of activity in serum fractions III-1,2 and III-0. Anticomplementary activity appeared in fraction I + III-3. Ultracentrifugal analysis showed the natural antibody to have a higher molecular weight (19 S) than antibody arising as a result of deliberate immunization with rabbit testes antigen (7 S).

Summary and Conclusions:

1. Recent technical innovations have significantly increased the available amounts of S. mansoni cercarial exoantigens, thus making possible more comprehensive studies on the chemical nature and serological properties of these products. Chemical analyses indicate that the antigen is a polysaccharide or possibly a glycoprotein, with considerable linkage through the amino groups. Hexosamine (either glucosamine and/or galactosamine) and glucose appear to be the major carbohydrate components of the antigen. Small amounts of protein and lipid also are present but it has not yet been determined whether either is essential for serologic activity. Preliminary evaluations with homologous and heterologous sera indicate that the exoantigen is highly specific and sensitive in complement fixation tests, but may react with a spectrum of antibodies different from those detected with the standard somatic antigens. Studies to investigate the immunological properties of the exoantigen and to determine whether it can provide a basis for appraising the status of infection and efficacy of therapy are in progress.

2. The fruitful progress made in the study of the exoantigens from S. mansoni cercariae encouraged the initiation of investigations on possible exoantigens of the adult stage of the parasite. Products that fix complement in the presence of homologous antibody have been shown to be present in a chemically defined culture medium following incubation of adult worms. It has not been determined if this is a true exoantigen. The study is being continued.

3. Previous work from this Department has shown that purified protein somatic antigen of Trypanosoma cruzi is highly specific in complement fixation tests. The current study was undertaken to determine if the products liberated by the parasite during cultivation could serve as antigen in serological procedures and , if so, to study the chemical composition of the material. The results obtained show that a substance is liberated which can serve as antigen in the complement fixation test and also cause the production of antibodies when injected into experimental animals. The sensitivity and specificity of the antigen liberated by T. cruzi was determined in complement fixation tests employing homologous sera, sera from patients with other diseases, and sera from normal healthy individuals. The results indicate that the antigen is highly sensitive and specific. Chemical studies revealed that both carbohydrate and protein are essential components of the antigen and thus it appears that the major specifically reactive component is a glycoprotein.

The serological and chemical findings indicate that the antigen obtained during cultivation of the parasite is different from the crude or purified somatic antigens and support the hypothesis that the product is an exoantigen.

4. Previous studies showed that antigens from a culture strain of T. rhodesiense did not detect antibodies in individuals with the disease. Recently, organisms freshly isolated from Africans with the disease have been obtained from the trypanosome library of the East African Trypanosomiasis Research Organization, Tororo, Uganda. The study is to be continued employing the fresh isolates.

5. The problems encountered in the serodiagnosis of filariasis are best summarized by the fact that at present no acceptable tests are available. Studies were performed to determine whether various fractions of Diofilaria immitis would yield an improvement in the results obtained in complement fixation, hemagglutination or hemagglutination inhibition tests. The results so far indicate that many problems still must be solved before reliable tests for the serodiagnosis of filariasis can be achieved.

6. Studies were continued in an effort to purify and characterize the soluble antigens from P. knowlesi. Much of the work completed to date has dealt with various methods for separating the parasite from the monkey rbc's. Several fractions have been employed in efforts to separate antigens from the parasite material. However, anticomplementary (AC) activity of these antigens has been one of the major problems encountered. For example, an antigenic extract, although purified approximately ten-fold by using Sephadex G-200, still retained some AC activity. Ancillary studies to obtain maturation of intracellular parasites are in progress. If successful, this would permit exsanguation of the infected animals earlier in the infection, and thus reduce the risk of losing the animal as a result of the toxemia which can occur very rapidly in later stages of the infection.

7. As part of a more comprehensive study, sera from selected Bolivians have been evaluated for serological evidence of Pinta. The cardiolipin microflocculation, the fluorescent treponemal antibody and the Treponema pallidum immobilization tests were employed. The serological findings are being correlated with clinical and anamnestic evidence of the disease. Sera from persons living in Iran are being tested for endemic syphilis (Bejel) in a similar study employing the same tests. The findings should serve as an evaluation of the fluorescent treponemal antibody test and should indicate whether latent Pinta exists in Bolivia.

8. The World Health Organization is currently evaluating the fluorescent treponemal antibody test (FTA) on a world-wide basis. This Department is participating as the American laboratory representative. It is hoped that the results of this study will permit the development of an internationally standardized procedure for the FTA test.

9. A commercial company has developed and is selling a simplified flocculation test kit for syphilis serology. This kit has practical applications in situations where it is difficult to perform the standard screening test (cardiolipin microflocculation) used by the U. S. Army. A preliminary survey indicates that the commercial test (RPR) has adequate sensitivity and specificity for field use.

10. In order to eliminate inherent difficulties concerned in the whole organism fluorescent antibody tests, investigations were initiated to develop methods wherein soluble antigens can be employed. Preliminary results indicate that soluble antigen fluorescent antibody procedures are practical and warrant continued investigation. Concurrent studies are being performed to determine if the results obtained with the soluble antigen fluorescent antibody procedure can be read in a fluorometer. The results obtained, to date,indicate that the tests can be easily read by fluorometric means. While the studies accomplished so far have dealt principally with soluble schistosome antigens, the procedures should be adaptable to other protein antigens.

11. Additional studies on the in vitro inter-reaction of quinacrine with guinea pig complement have shown that cell bound C'2 remains susceptible to inactivation by quinacrine while cell bound C'4 is resistant. Complement components C'3a and C'3d are also inactivated by quinacrine. A micromethod for preparative electrophoresis has been developed to study further the electrophoretic and immunoelectrophoretic changes in quinacrine treated serum. This method shows promise not only in the study of serum factors other than the complement system, but also in the analysis of complex antigenic materials from varied sources.

12. Due to personnel problems, no new research on the bactericidal reaction was performed. Manuscripts dealing with previously completed studies were published. The personnel problems have been resolved and plans are being formulated to continue the study of the bactericidal reactions of the antibody-complement system against gram-negative organisms.

13. Limitations of available animal space necessitated a temporary curtailment of studies on allergic encephalomyelitis in the guinea pig and runt disease in the rat. Progress to date has been published in scientific journals. However, the physical problems have been alleviated and the studies are now being continued.

14. Autoimmunity in X-irradiated rabbits was studied by irradiation of testes, surgical removal, and extraction of antigen, followed by reinjection of antigen into the same animal in combination with adjuvant. No evidence of enhanced immunogenic activity was observed with radiation-altered testes. On the contrary, antigens extracted from irradiated testes were generally less immunogenic and serologically less reactive than antigen from non-irradiated testes.

Several chemical and physical differences between antigen extracted from irradiated and non-irradiated testes were observed, but there was no indication that tissue had become immunologically "foreign" as a result of X-ray induced changes.

Fractionation of antigen from rabbit testes with cold ethanol revealed antigenic activity to be predominantly associated with the beta-1-lipoprotein fraction.

No evidence was found to indicate that radiation-induced mutation of the antibody forming mechanism of rabbits resulted in production of autoantibody.

Naturally occurring complement fixing antibody in rabbit serum against rabbit testis antigen was found to be associated with 19 S, beta-2-globulins. After immunization with rabbit testis antigen, antibody also appeared in the 7 S gamma globulin of fraction II.