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WALTER REED ARMY INSTITUTE OF RESEARCH

WALTER REED ARMY MEDICAL CENTER

WASHINGTON 12, D.C.

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ANNUAL PROGRESS REPORT

Reports Control Symbol MEDDH-288

1 July 1962 - 30 June 1963

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Volume I

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WALTER REED ARMY INSTITUTE OF RESEARCH

Walter Reed Army Medical Center

Washington 12, D. C.

ANNUAL PROGRESS REPORT

1 July 1962 -- 30 June 1963

Volume I

Reports Control Symbol MEDDH-288

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ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 802, Combat Surgery

Task 01, Combat Trauma (infections and antibiotics)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Experimental Surgery
Division of Clinical Surgery

Department of Germfree Research
Division of Basic Surgical Research

Department of Bacteriology
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Capt Raymond C. Doberneck, MC, Howard E. Noyes, Ph.D.
Maj Alexander Pogrebniak, MC, Jimmy R. Evans, M. S.

Assistants: Capt Daniel B. Nunn, MC, Maj Maria LaConte, ANC, Alexander Kimler, Ph.D., Maj Ronald E. Easterling, MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (infections and antibiotics)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Howard E. Noyes, Ph.D., Jimmy R. Evans, M. S.

Reports Control Symbol: MEDDH-288

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Studies on Wound Infection and Cultures During Operation. A standard operation was performed in 14 dogs in the surgical isolator and in 14 dogs in the usual operating room environment. No difference in the healing of the wounds made in the two environments was observed but cultures taken at the conclusion of the operation from the skin adjacent to the wound were less frequently positive after operation in the isolator ($p = 0.03$).

Clostridial Toxins. A mono-clostridial gas gangrene was produced in goats by intramuscular injection of washed bacteria suspended in adrenalin. Specific lethal and/or hemolytic toxins were identified in the wound exudates of these goats. A new hemolysin detection plate was devised which can be used to identify specific hemolysins or antitoxins to these hemolysins.

Therapeutic Regimens in Contaminated Soft Tissue Wounds of Rabbits. Studies of therapeutic regimens in bacterially contaminated soft tissue wounds of rabbits indicated that aerosol preparations of penicillin or peptide antimicrobials could be of value in delaying infections in mass or battle casualties. This led to further studies evaluating surgical debridement with and without topical antibiotics at varying times post-wounding.

BODY OF REPORT

Project No. 3A 0 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (infections and antibiotics)

Description:

Studies on Wound Infections. Modified forms of a surgical isolator used in studies with germfree animals have been suggested for use during human surgery in an effort to eliminate environmental bacteria as a cause of wound infection. While a germfree atmosphere has been shown to be present in the isolator, no comparison has been made regarding the healing of surgical incisions made in both environments and between cultures obtained during operation in both environments.

Clostridial Toxins. The object of this study was to characterize toxins formed in vivo in experimental animals as a basis for formulation of toxoids and antitoxins to be used for prophylaxis and therapy of clostridial infections.

Therapeutic Regimens in Contaminated Soft Tissue Wounds of Rabbits. The object of this study was the evaluation of such therapeutic procedures as antibotics and surgical debridement in the management of wound infections.

Progress:

Studies on Wound Infections. Two groups of dogs were submitted to a standard operative procedure. One group of dogs had the operation in the isolator and the other under usual operating room conditions. Serial cultures were obtained during the operation and the wounds were observed postoperatively. No difference was apparent in the healing of the wounds of the two groups but cultures taken at the conclusion of the operation from the skin adjacent to the wound were less frequently positive after operation in the isolator ($p = 0.03$).

Clostridial Toxins. Prior studies of clostridial toxins in goats were from animals with a mixed aerobic and anaerobe bacterial flora frequently consisting of two or more species of clostridia. This report is concerned with pure culture infections of goats created by intramuscular injection of pure cultures of washed bacteria suspended in an equal volume of sterile 1/1000 Adrenalin. This procedure invariably resulted in an infection which pathologically resembled gas gangrene.

Wound juices were collected at intervals or immediately following death. These juices and saline extracts of diseased muscles taken at death were sterilized by filtration and assayed in vivo and in vitro for the presence of known clostridial toxins. These assays were carried out by mixing sterile exudates with calibrated antitoxins prior to intravenous injection of mice or addition to specially devised blood agar plates to demonstrate specific

hemolysins. Results indicated that most of the lethality and hemolytic activities of type A clostridium perfringens and clostridium septicum wound exudates were caused by their alpha toxins. The lethality of type A clostridium novyi wound exudate was attributable to its alpha toxin and the hemolysin to its gamma toxin. In this study wound exudate containing clostridial toxins could be characterized as to the infecting organism within six hours of sampling. In addition the hemolysis plate can be used to assay sera for antitoxins to specific clostridia.

Therapeutic Regimens in Contaminated Soft Tissue Wounds of Rabbits. Studies on the use of topical antibiotics on traumatic wounds to increase the period between wounding and necessary surgical debridement have continued. Varying regimens involving the use of antibiotics, debridement, or both are being evaluated in a standardized wound in the rabbit. Preliminary results indicate that the use of an aerosol preparation of penicillin G administered 2 hours after wounding decreased mortality from 70 per cent to 30 per cent and the combination of polymyxin, neomycin and bacitracin resulted in a mortality rate of only 12 per cent. Surgical debridement alone carried out six hours after wounding enabled 92 per cent of the rabbits to survive while the same procedure carried out 24 hours after wounding was without benefit. Combinations of the two therapeutic measures are currently being investigated.

Summary and Conclusions:

Studies on Wound Infections. No difference in healing of surgical wounds made in the isolator and under standard operating room conditions was observed. Skin adjacent to the wound less frequently showed growth at the conclusion of operations in the isolator ($p = 0.03$).

Clostridial Toxins. Clostridial toxins formed in vivo have been identified and measured by animal studies and a new technique for the demonstration of hemolysins in wound exudates. This new technique can also be used to measure minute amounts of circulating antitoxins in sera.

Therapeutic Regimens in Contaminated Soft Tissue Wounds of Rabbits. Topical application of penicillin G and a combination of polymyxin, bacitracin, and neomycin significantly reduced mortality rates of rabbits wounded by a standardized technique. Debridement alone six hours after wounding decreased mortality rates but was of no value when delayed 24 hours after wounding.

List of Publications:

1. Noyes, H. E., Pritchard, W. L., Brinkley, F. B., Mendelson, J. A. Analysis of Wound Exudates for Clostridial Toxins. Bacteriol. Proc. 1963, p. 91.
2. Sherman, R. T., Noyes, H. E. Lethality Studies of the Blood of Irreversibly Shocked Dogs. J. Surg. Res. In press.
3. Pulaski, E. J., Noyes, H. E. Infections and Antibiotics. Chapter II in Physiological Principles of Surgery. Edited by L.M. Zimmerman and R. Levine. 2nd Ed. In press.
4. Noyes, H. E., Evans, J. R., Baker, H. J. Effects of a Nuclear Detonation on Swine. Bacteriologic Studies. Ann. N. Y. Acad. Sci. In press.
5. Kalas, J. P., Noyes, H. E. Interaction of Biological Toxins and Adrenergic Mechanisms in Alimentary Excretions of Guinea Pigs. In preparation.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 802, COMBAT SURGERY

Task 01	Combat Trauma (Acute renal injury and failure)
Reporting Installation:	**Walter Reed Army Institute of Research** **Walter Reed Army Medical Center** **Washington 12, D. C.** **Department of Surgical Physiology** **Division of Basic Surgical Research**
Period Covered by Report:	1 July 1962 - 30 June 1963
Principal Investigator:	**Lt. Col. Paul E. Teschan, MC**
Assistants:	**Capt. Gerald P. Murphy, MC** **John A. Gagnon, M. S.** **Natalie L. Lawson, M. S.** **Nancy B. Cummings, M. D.** **SFC John Talabesky**
Reports Control Symbol:	**MEDDH-288**
Security Classification:	**UNCLASSIFIED**

ABSTRACT

Project No. 3A 0 12501 A 802, COMBAT SURGERY

Task 01	Combat Trauma (Acute renal injury and failure)
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
Period Covered by Report:	1 July 1962 - 30 June 1963
Authors:	Lt Col Paul E. Teschan, MC, Capt. Gerald P. Murphy, MC, John A. Gagnon, M. S., Natalie L. Lawson, M. S., Nancy B. Cummings, M. D., SFC John Talabesky
Reporting Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

Acute renal failure induced in rats by methemoglobin-ferrocyanide injection may be prevented by a timely injection of 1 ml of 6.5% sodium sulfate but not by equal volumes of 6% dextran or human serum albumin. Equiosmolal doses of mannitol of 3, 4 or 6 hours prior to induction failed to prevent the lesion, while the small dose of epinephrine may have intensified it.

Discrepancies between chemically and directly measured renal blood flow were found during hypotension in dogs associated with sustained renal perfusion even at 50 mm Hg pressure and anuria. Infusion of hypertonic mannitol exaggerated this discrepancy and reversed its direction with wash-out of clearance substances; but with pressure unchanged a new steady state was reached, and with good agreement the two measures of flow described a progressive increase in renal perfusion. Study of concurrent extraction ratios and filtration fractions indicated that increased perfusion occurred due to decreased postglomerular resistance and perfusion of non-secretory renal tissue.

Urine reinfusion served to induce an experimental uremic state in operant-conditioned primates. Behavioral deficits were quantitated, and were found to be correlated with the degree of azotemia but not with wide ranges of plasma, sodium and potassium concentrations. Characteristics of the setting promised a meaningful approach to the direct and quantitative study of uremia.

BODY OF REPORT

Project No. 3A 0 12501 A 802, COMBAT SURGERY

Task No. 01 Title: Combat Trauma (Acute renal injury and failure)

Description:

The studies reported herein concern (1) the prevention of acute renal failure (ARF), (2) the role of hemodynamic factors (renal blood flow, ischemia) in the pathogenesis of ARF, and (3) attempts to define the nature of uremic toxemia, with a view toward effective prevention and/or better treatment of the uremic syndrome in human renal failure.

Progress:

(1) Prevention of ARF

Acute renal failure with azotemia was produced in rats by a procedure developed at the USA Surgical Research Unit, Fort Sam Houston, Texas, comprising intravenous injection of 0.5 gm/kg methemoglobin and 14.3 mg/kg Na ferrocyanide into sodium-depleted, dehydrated rats. Renal histology corroborated occurrence of ARF defined by significant azotemia. The sections were prepared by the Department of Experimental Pathology, Walter Reed Army Institute of Research.

In previous experiments, a number of substances (hypertonic NaCl, mannitol, mannose, sucrose, and THAM) given intravenously were shown to prevent ARF if administered within 1 hour of the induction-injection. These experiments were extended using isosmolal doses of sodium sulfate, and equal volumes of 6% clinical dextran and 6% salt-poor human albumin, administered 15 minutes and 2 hours following the induction-injection in separate groups along with concurrent controls. Incidence of ARF was reduced from 83% in controls to zero by sodium sulfate given at 15 minutes, but no protection was afforded if the agent was delayed to 2 hours after induction (92% incidence). Neither dextran nor albumin lowered the incidence of ARF; indeed, dextran may have raised it.

In another experiment, epinephrine in an intravenous dose (equivalent to 1 ml of a 1:2000 solution in a 70 kg man) produced a questionable increase in the severity of the lesion (average BUN in 12 experimental animals, 174 mg%, compared with 12 controls, average 127 mg%) when injected 15 minutes following induction but not at 2 hours (average BUN 122 mg%).

Twenty-five per cent mannitol administered 3, 4 or 6 hours prior to induction-injection did not affect the incidence.

(2) Renal Hemodynamics and ARF

a. Effects of hemorrhagic hypotension (with anuria or oliguria) on renal hemodynamics: Renal blood flow was measured directly by cannulating the left renal vein or electromagnetically by implanting a transducer around the left renal artery. During hemorrhagic hypotension at 50 mm Hg, blood continued to perfuse the kidney at 10-30 ml/min despite the cessation of urine flow. Onset of urinary flow at this pressure in response to osmotic load indicated that glomerular filtration did not cease at this low filtration pressure; but because of the small amount of filtrate formed and the slow rate of flow through the tubules, formed filtrate was presumably completely reabsorbed with resultant anuria.

b. The validity of renal clearances in hypotensive and normotensive states: Simultaneous measurements of renal blood flow by the clearance of PAH corrected for E_{PAH} and hematocrit (RBF) and from the cannulated renal vein (DRBF) have shown in both hypotension and normotension that correlation depends on adequate urinary flow rates for a sufficient time to achieve a steady state. At 50 mm Hg RBF was greatly exaggerated when the urine flow suddenly returned following the institution of an osmotic diuresis (hypertonic mannitol or dextrose). This discrepancy presumably occurred as the static column of tubular fluid was washed out of the kidney in the osmotic diuresis. This discrepancy was also observed in normotensive animals when urine flow rates varied.

c. The effect of hypertonic mannitol on renal hemodynamics: Infusion of hypertonic mannitol at 5 ml/min in the normotensive or moderately hypotensive dog, resulted in a 15 to 20% increase in DRBF, a decrease in the filtration fraction, increase in the plasma volume and a subsequent decrease (20-50%) in the hematocrit. A similar infusion directly into the renal artery produced no increase in DRBF. When hematocrits were depressed by plasma infusion or by plasma-for-red-cell-exchange, extractions of inulin, creatinine and PAH declined and DRBF rose to a limited degree. It appeared, therefore, that the increase in renal perfusion by the intravenous route was primarily due to a decrease in physiologic blood viscosity rather than to an intrinsic renal effect of the osmotically-active material.

(3) The Nature of Uremic Toxemia.

A behavioral performance schedule involving paced-avoidance was utilized. Male and female monkeys (Macaca mulata) were placed in a chair-type restraining apparatus and trained to respond during one-hour test periods by pressing a lever every 21 seconds to avoid an electric shock. A system for intravenous reintroduction of bladder

urine was devised, utilizing Foley catheter, sterile polyethylene tubing, millipore filter, and a relay-activated finger pump. The system could be opened to stop the inflow of urine to allow recovery.

Behavioral performance deficits (e.g. a 10-fold increase in average rate of error) were correlated with elevated BUN concentrations at or above 95 mg%, but not with plasma potassium concentrations between 2.0 and 8.5 mEq/l, or sodium concentrations between 135 and 170 mEq/l, nor with the mechanics of the infusion itself. Recovery of behavioral performance followed cessation of the urine reinfusion.

Summary and Conclusions:

(1) Prevention of ARF: A variety of substances sharing known or potential value as osmotic diuretics was shown to prevent experimentally induced ARF in rats when administered in a timely relationship to the event inducing the disease.

(2) Renal Hemodynamics and ARF: Renal blood flow continued to perfuse the kidney during anuric hypotension in the dog. That this flow is sufficient to maintain renal integrity over a more prolonged period of time is suggested by other experiments. When hypertonic mannitol or dextrose was infused into the animal, chemically determined RBF was greatly exaggerated due to a combined wash-out of PAH and a fall in PAH extraction. After this initial wash-out, the chemically-determined RBF closely approximated the directly-measured renal blood flow (cannulated renal vein). A simultaneous fall in inulin and creatinine extraction indicated a decrease in post-glomerular resistance while the decline in PAH extraction suggested increased perfusion of nonsecretory tissue. Increase in renal perfusion following infusion of hypertonic mannitol appeared to be due primarily to a fall in hematocrit and hence a decline in physiologic blood viscosity.

(3) The Nature of Uremic Toxemia: An experimental uremic state was induced by urinary infusion in primates conditioned to a behavioral schedule requiring modes of performance analogous to those which appear to be deficient in the uremic man. Objectively measurable quantitative behavioral deficits ensued at significant levels of azotemia, but these deficits were not directly related to changes in plasma sodium and potassium concentration in the ranges noted nor to the process of the infusion itself. The uremic state was completely reversible on cessation of infusion, permitting repeated studies in individual animals.

List of Publications:

1. Cerilli, G. J., Geever, E. F., and Gagnon, J. A.: The effects of suprarenal aortic occlusion and hemorrhagic hypotension on the renal function of primates, J. Surg. Res., 2: 233-240, 1962.

2. Teschan, P. E.: Management of posttraumatic renal insufficiency. J. Trauma, 3: 181, 1963.

3. Murphy, G. P. and Schirmer, H. K.: The diagnosis and treatment of hypernephroma. Geriatrics (in press).

4. Murphy, G. P., Gagnon, J. A., and Teschan, P. E.: Measurement of renal function in hemorrhagic hypotension: effect of mannitol (in press).

5. Teschan, P. E.: "Acute Renal Insufficiency" and "Peritoneal and Hemodialysis," chapters in Modern Treatment (in press).

6. Murphy, G. P. and Lawson, Natalie L.: Experimental acute renal failure: functional alterations induced by serotonin. J. Invest. Urol. (in press).

7. Gagnon, J. A., Murphy, G. P., and Teschan, P. E.: Renal hemodynamic effects of hypertonic mannitol in the dog. Fed. Proc., 22 (Part I): 173, 1963. (Abstract)

8. Teschan, P. E., Sharp, J. C., and Murphy, G. P.: A method for the study of uremia in primates. (In press) (Abstract)

9. Teschan, P. E., Gagnon, J. A., and Murphy, G. P.: Renal function in hypotension with observations on the action of hypertonic mannitol. Clin. Res., 11: 241, 1963. (Abstract)

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 802, Combat Surgery

Task 01, Combat Trauma (acute renal injury and failure)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Metabolism
Department of Experimental Surgery
Division of Medicine
Division of Clinical Surgery

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Capt Raymond C. Doberneck, MC

Assistants: Lt Col Kevin G. Barry, MC
Capt F. D. Schwartz, MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (acute renal injury and failure)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Capt Raymond C. Doberneck, MC, Lt Col Kevin G. Barry, MC, Capt F. D. Schwartz, MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

A Comparison of the Effects of Loading with 20% Mannitol and with Water on Renal Dynamics in the Human. Mannitol loading did not increase the clearances of PAH, inulin or creatinine observed after water loading in patients with normal or diseased kidneys.

A Comparison of the Histologic Changes in Kidney after the Infusion of 20% Mannitol and 5% Glucose in Water. Multiple needle biopsies showed that the renal changes induced by intra-arterial injection of 5% dextrose or 20% mannitol were similar to those attributed to needle biopsy alone.

BODY OF REPORT

Project No. 3A O 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (acute renal injury and failure)

Description:

Studies on the Effects of Mannitol. Hypertonic mannitol has been advocated for use in prevention of acute organic renal failure. The purpose of these experiments was to determine whether loading with mannitol increases clearances of PAH, inulin and creatinine in patients with normal or diseased kidneys.

Progress:

A Comparison of the Effects of Loading with 20% Mannitol and with Water on Renal Dynamics. Six patients with normal renal function and 15 patients with renal disease were given water (20 ml/kgm) on the first day and 20% mannitol (100 gm) on the second day of study and the ensuing diuresis sustained. Five patients with renal disease received both mannitol and the water load on the second day of study. Following loading clearance of inulin (C_I), para-aminohippurate (C_{PAH}), and creatinine (C_{CR}) were performed by standard techniques. No significant difference was observed in C_I, C_{CR} and C_{PAH} when values after water loading alone were compared to those after mannitol loading.

A Comparison of the Histologic Changes in Kidney After the Infusion of 20% Mannitol and 5% Glucose in Water. Twelve dogs had laparotomy and dissection of the right renal artery between its origin from the aorta and the renal hilus. Four dogs had injection of 35 cc of 20% mannitol into the right renal artery and four others, injection of 35 cc of 5% dextrose. The dogs having injection of 5% dextrose intra-arterially also received 5% dextrose IV in a dose of 20 ml/kgm prior to dissection of the artery. In an effort to separate changes due to the agents per se and those due to the operative manipulation, a third group of four dogs underwent all the manipulations but did not receive injection of any agent. Needle biopsies of the right kidney were obtained after dissection of the artery but prior to injection of the agents and 15, 30, 60, and 120 minutes after injection.

Histologic sections showed that the renal changes characteristic of osmotic nephrosis are of the same magnitude after mannitol infusion as those occurring after dextrose infusion or merely multiple needle biopsy alone.

Summary and Conclusions:

A Comparison of the Effects of Loading with 20% Mannitol and with Water on Renal Dynamics in the Human. Infusion of hypertonic mannitol does not increase the clearance of PAH, inulin or creatinine in patients with normal or diseased kidneys, irrespective of the state of hydration.

A Comparison of the Histologic Changes in Kidney After the Infusion of 20% Mannitol and 5% Glucose in Water. The histologic changes produced in the kidney by infusion of 20% mannitol are of the same degree as those produced by the infusion of 5% glucose in water or those produced by repeated needle biopsy.

List of Publications:

1. Barry, K. G., Doberneck, R. C., McCormick, G. J. The Effect of Hypertonic Mannitol Infusion on Renal Clearances of PAH (C_{PAH}) and Inulin (C_I) in Man: A Comparison with Water Loading. Clin. Res. 10: 245, 1962.

2. Doberneck, R. C., Schwartz, F. D., and Barry, K. G. On the Nature of the Nonspecific Nephropathy Attributed to Mannitol. Proc. Soc. Exp. Biol. and Med. 110: 795, 1962.

3. Doberneck, R. C., Schwartz, F. D., and Barry, K. G. Comparison of the Prophylactic Value of 20% Mannitol, 4% Urea and 5% Dextrose on the Effects of Renal Ischemia. J. Urol. 89: 300, 1963.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 802, Combat Surgery

Task 01, Combat Trauma (experimental orthopedics)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Experimental Surgery
Division of Clinical Surgery

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Maj Austin D. Potenza, MC

Reports Control Symbol: MEDDH-288

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ABSTRACT

Project No. 3A 0 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (experimental orthopedics)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Author: Maj Austin D. Potenza, MC

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Security Classification: UNCLASSIFIED

Tendon Healing. The problems of tendon healing and reconstructive surgery of the long flexor tendons within the digits continue to be of major military surgical interest. Continuing an intensive and comprehensive experimental approach to these problems the following areas have been critically evaluated using unique, specially designed, atraumatic standard-wound procedures: (1) the effect of sublimis tendon excision on profundus tendon healing and adhesion formation, (2) the mechanism of healing and fate of autogenous flexor tendon grafts, (3) the mechanism of formation of adhesions to autogenous flexor tendon grafts, (4) the mechanism of healing and potential clinical usefulness of freeze-dried flexor tendon homografts.

Synthetic Cartilage Prostheses. Since destruction of joint cartilage in major weight-bearing joints of the lower extremities continues to be a major problem in military orthopedics, in vivo experiments with plastic hip-arthroplasty-cups have begun. This work has been made possible by collaboration with the Army Prosthetics Research Laboratory in the synthesis of a plastic which duplicates some of the physical properties of normal joint cartilage.

Acceleration of Rate of Fracture Healing. A technique for discretely elevating the temperature of long bones within physiologically tolerable limits has been devised. By selectively localizing this effect the rate of fracture healing may be accelerated by effecting local changes in vascularity and metabolic activity of individual fractured bones. Such a technique might prove a major advance in military orthopedic practice if it could offer a significant potential for accelerating healing of fractures of the major long bones; average length of hospitalization and manpower losses for such patients might be greatly reduced. Pilot studies have begun.

BODY OF REPORT

Project No. 3A 0 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (experimental orthopedics)

Description:

Tendon Healing. Tendon healing in the hand and its many attendant problems and complications continue to be major military surgical problems. The intensive and comprehensive investigative studies devoted to these problems, which were begun several years ago, have continued; many previously misunderstood and unsolved problems have been defined and brought to solution. This work is a direct progressive extension of the work covered in the last annual report. In the period covered by this report, the following problem areas have been studied: (1) the effect of sublimis tendon excision upon profundus tendon healing and adhesion formation, (2) the mechanism of healing and fate of autogenous tendon grafts in the digits, (3) the mechanism of formation of adhesions to autogenous tendon grafts in the digits, (4) the fate and potential usefulness of freeze-dried flexor tendon homografts.

Synthetic Cartilage Prostheses. As an area of direct progress made since the experimental work reported in the last annual report, in vivo experiments have begun. Polymeric materials have been used to manufacture canine femoral head arthroplasty cups at the Army Prosthetics Research Laboratory; these devices have been placed in dogs for study.

Acceleration of Rate of Fracture Healing. In progress of work reported in the last annual report, in vivo pilot studies have been performed. A technique for discretely elevating the temperature of bone has proved feasible.

Progress:

Tendon Healing. The present study is a direct continuation of the study of flexor tendon healing processes reported in the last annual report. In clinical practice the flexor digitorum sublimis tendon is often surgically excised before repair of the profundus tendon or prior to free tendon grafting in the finger. In the last annual report, it was related that experimental evaluation of sublimis tendon excision following profundus tendon repair resulted in greater adhesions and poorer functional results as a consequence thereof than if the sublimis tendons were left undisturbed. The extensive studies already completed and reported in previous years had established the fact that flexor tendons do not manifest an intrinsic tenoblastic reaction of their own after injury; it was considered unlikely, therefore, that sublimis division per se could initiate a profound inflammatory (healing) reaction resulting in the significant adhesions observed in the studies just cited. It was thought that perhaps the technique of sublimis excision injured some other proximate tissue or structure that responded by extensive adhesion formation. Since the sublimis vincula are vascular structures, broadly based upon the

underlying osseous floor of the digital canal, and they are continuous with the periosteal floor of the underlying phalanx, it was considered that total sublimis excision, since it effectively excises and destroys the integrity of the periosteal floor of the digital canal, might so disturb the vascular bed of that floor so as to cause an extensive inflammatory reaction resulting in the marked adhesions observed. In a test of this hypothesis, forty canine digits divided into two groups of twenty each were studied after atraumatic division and repair of each profundus tendon by the techniques previously described. All sublimis tendons were then excised by one of two techniques. In one group the sublimis tendons were completely excised, down to and including their slips of insertion. In the second group the sublimis tendons were excised by their transverse division immediately proximal to their broadly based vincula. Thus, in the first group, total sublimis excision effectively disturbed the vascular and periosteal floor of the digital canal, while in the second group the technique of sublimis excision by transverse division proximal to the sublimis vincula preserved the cellular and vascular integrity of the bony floor of the digital canal. The differences in results in these two groups were striking. Sub-total sublimis excision by transverse sublimis division immediately proximal to their vincula resulted in only a few filmy, loose adhesions between the healing profundus tendons and the digital sheaths at wound sites and at suture tracts. In marked and profound contrast, total sublimis excision resulted in dense, restrictive adhesions to the osseous floor of the digital canal. Careful histologic evaluation of all digits revealed that in those digits in which total sublimis excision was performed, there was a profound cellular reaction from the disturbed periosteal floor; this proliferative cellular reaction took part in and contributed to the healing of the profundus tendons, as did the digital sheaths. Since total excision of the sublimis with its vincula involves the vascular bony bed of the digital canal over the distal half of the proximal phalanx and base of the middle phalanx, the granulation tissue arising from this bed produced broadly based adhesions to profundus wounds. Adhesions between the osseous floor of the digital canal and the profundus tendons were so broadly based on the underlying bone in these digits that they effectively tethered the healing tendons, severely limiting motion. As these adhesions matured, the healing tendons were effectively anchored to the underlying phalanxes by the broadly based adhesions. In contrast, in those digits in which sub-total sublimis excision by transverse division immediately proximal to their vincula had been performed, the healing granulation tissue arose only from the repaired digital sheaths, and not the periosteal floor of the digital canal. Since the latter had been preserved, it remained intact and did not give rise to granulation tissue. Thus, no adhesions formed between the healing tendons and the bony floor of the digital canal in this group of digits. It was thus demonstrated in this study that the anatomic integrity of the digital canal is an essential factor in the surgeon's efforts to minimize tendon adhesions. Once the integrity of the bony floor of the digital canal is violated as in total sublimis excision, a common clinical practice, there results a profound inflammatory response; this produces tendon adhesions that are broadly based on the rigid, ungliding bony phalanxes of the digits. Tendon healing is then accompanied by the formation of dense, restrictive adhesions that assure an unacceptable functional result. In contrast, if the sublimis tendons are excised by transverse division immediately proximal to their vincula, the integrity of the periosteal floor of the digital canal is preserved, and the healing profundus tendons remain free of any adhesions to them.

An extensive study was performed to determine the fate of autogenous tendon grafts within the digits. Using a unique, specially designed atraumatic technique, fifty-four free autogenous profundus tendon grafts were used as autotopic tendon grafts. They were studied at varying periods from two to 160 days postoperatively. Evaluation was performed by gross observation and by careful detailed histologic analysis. In particular the investigations conducted were concerned with the sequences in the healing processes observed at the proximal and distal tendon stump-tendon graft wounds, the reaction about the tendon grafts within the flexor digital sheath, and the fate of the grafts, and where present, the mechanism of adhesion formation. In all digits, the annular ligaments, sublimis tendons and digital sheaths were preserved anatomically intact in order to eliminate surgical trauma to these structures as uncontrolled variables. It was found that, at the proximal and distal anastomosis sites, the paratenon and synovial layer of the digital sheath, respectively, alone provided the active healing elements for effecting tendon-tendon graft union. The cells of the grafts and tendon stumps were observed to take no active role in the healing processes. Quite remarkably, and in complete contradistinction to what has been reported extensively in the literature by other investigators, the autotopic tendon grafts remained viable and normal by all histologic criteria upon detailed study of their cells and collagen. There was no evidence that the grafts in any way lost their viability or that they in any way acted as tissue struts for gradual replacement by other tissue cells, processes which have been ascribed to such tendon grafts by other investigators. Furthermore, adhesions were observed to occur to the grafts only at those points where the surface integrity of the grafts was disrupted by sutures or by prolonged suture pressure. Quite remarkably, no adhesions occurred between the grafts and sublimis tendons, annular ligaments, or digital sheaths in "no-man's land" both on gross observation and on en bloc longitudinal and cross section histologic analysis. The results of this study strongly suggest that it might be well to experimentally reappraise our long-standing attitudes about tendon surgery within the flexor digital sheath. This unique anatomic area may not truly be a "no-man's land" for the skilled atraumatic surgeon. Perhaps the poor results seen following surgery in this area are due not to flexor tendon surgery per se, but rather to surgical trauma to associated digital structures (annular ligaments, sublimis tendons, and digital sheaths) during so-called "meticulous dissection". The special techniques designed and employed by the investigator have avoided injury to these essential structures. As a consequence, our gross, histologic and functional results in the dog have been most remarkable.

An extensive study of the fate, mechanisms of healing, and mechanism of adhesion formation to freeze-dried flexor tendon homografts in the dog is well underway. These grafts are being compared experimentally in the dog with fresh homografts. Thus far, sixty-eight individual tendon grafting procedures have been performed. Gross observations have been completed following many of these grafting procedures, varying from 14 to 70 days postoperatively. Quite remarkably, and in marked contradistinction to what has been reported by other investigators, the freeze-dried homografts, on re-exploration of the experimental digits, appeared normal grossly with absolutely no adhesions in "no-man's land." It is too early to draw definite conclusions concerning this work since not all of the operated digits have been re-explored and since histologic evaluation

is actively in progress at the time of this report. However, the gross results observed thus far until 70 days postoperatively are exceedingly encouraging and should they remain consistent through the total time interval for which this experiment is planned, this project may truly represent a major contribution to the problems of reconstructive hand surgery in the military.

Synthetic Cartilage Prostheses. Thus far, eighteen specially fabricated plastic form-fitting canine femoral head cups have been placed in dogs and compared to an appropriate control group. The cups have been observed to hold up well through six weeks. However, beyond that period of time, our present acrylic amide terpolymer plastic prostheses have split and fragmented, resulting in marked chronic inflammatory changes in the synovium of the hip joints and in the marrow of the underlying denuded femoral head cancellous bone. Thus, this work must still be considered a pilot study and definitely preliminary, pending the development of more stress-resistant suitable polymeric materials at the Army Prosthetics Research Laboratory, and the development of a special colony of surgically-produced arthritic hips in dogs. This work is progressing along both lines.

Acceleration of Rate of Fracture Healing. The apparatus for conducting these experiments as described in earlier reports, has been completed and has been used in three pilot studies in dogs. The basic instrumentation has been proven feasible, but further studies have been impossible because of the lack of adequate laboratory facilities for conducting the experiments and the lack of adequate technician support for conducting these experiments on a larger scale. It is hoped that this situation may be remedied in the coming year.

Summary and Conclusions:

Tendon Healing. It has been shown that total sublimis tendon excision within the digits results in profound adhesion formation to healing flexor digitorum profundus tendons, because such a technique of sublimis excision disturbs the intensely vascular and cellular periosteal floor of the digital canal. This results in an intense inflammatory reaction from the bony floor of the digital canal which effectively tethers the healing profundus tendon to this bony floor, resulting in extremely poor functional results. In contrast, where sublimis excision for one reason or another must be performed, if the sublimis is excised immediately proximal to its vincula, thus preserving the integrity of the bony floor of the digital canal, no adhesions form between the bony floor and the healing profundus tendon. In a study of the fate and mechanism of healing of autogenous flexor tendon grafts, using a unique atraumatic technique, it has been found that these grafts survive and show no evidence whatsoever of any degeneration or loss of cellular detail. Moreover, employment of truly atraumatic techniques results in a complete absence of any adhesions between the grafts and the other structures in the area known as "no-man's land." Adhesions to the grafts do occur in the palm and at the distal anastomosis site at each point at which the physical integrity of the grafts is broken by sutures. Thus, adhesions to flexor tendon autografts occur only at those points at which the physical integrity of the surface of the grafts has been disturbed. Atraumatically repaired autogenous flexor tendon grafts retain their viability by

all gross and histologic criteria and do not act as tissue struts for gradual replacement by other tissue cells as has been reported by other investigators. Thus, once again true atraumatic technique has been proven to be of the essence in flexor tendon surgery. By these experiments we have added further to a truer and more valid understanding of what atraumatic technique is. The gross results of an extensive study presently underway evaluating the potential usefulness of freeze-dried flexor tendon homografts is extremely encouraging, since once again at periods up to 70 days postoperatively we can find no adhesions between the freeze-dried homografts and the other normal structures of the digital sheaths. Final evaluation of this experiment will await complete gross and histologic analysis and will be reported in the next annual report.

Synthetic Cartilage Prostheses. Initial experiments with our specially synthesized plastic femoral head cups have been disappointing. However, the experiments have been of extreme value in adding to our ever-increasing wealth of knowledge about the reaction of the body to polymeric materials. Furthermore, we are increasing our knowledge in a definition of specific parameters to be evaluated in studying the use of plastic polymeric materials in biologic systems. Our work in this area will continue with the development of better suited plastic materials and better structural design.

Acceleration of Rate of Fracture Healing. Pilot studies have shown the feasibility of discretely elevating specific areas in bone by electronic means. Further studies await the availability of greater laboratory space and technician assistance.

List of Publications:

1. Potenza, A. D.: Tendon Healing Within the Flexor Digital Sheath in the Dog. An Experimental Study. J. of Bone and Joint Surg., 44-A: 49-64, 1962.

2. Potenza, A. D.: The 1961 Wellcome Prize Essay -- Detailed Evaluation of Healing Processes in Canine Flexor Digital Tendons. Mil. Medicine, 127: 34-47, 1962.

3. Potenza, A. D.: Effect of Associated Trauma on Healing of Divided Tendons. J. of Trauma, 2: 175-184, 1962.

4. Potenza, A. D.: Critical Evaluation of Flexor Tendon Healing and Adhesion Formation Within Artificial Digital Sheaths: An Experimental Study. Accepted for publication in the J. of Bone and Joint Surg.

5. Potenza, A. D.: Mechanisms of Healing of Digital Flexor Tendons. To be published in the Transactions of the First Latin American Congress of Plastic Surgery, Northern Zone.

6. Potenza, A. D.: The Healing of Autogenous Tendon Grafts Within the Flexor Digital Sheath. Submitted for publication.

7. Potenza, A. D.: Prevention of Adhesions to Healing Digital Flexor Tendons. To be presented at the Military Section of the annual meeting of the American Medical Association with subsequent publication.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 802 COMBAT SURGERY

Task 01, Combat Trauma (Resistance to Shock)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Germfree Research
Division of Basic Surgical Research

Period Covered by Report: 1 July 62 - 30 June 63

Principal Investigators: Albert Einheber, Ph. D.

Assistants: Robert E. Wren, B.A.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 A 802 Title: COMBAT SURGERY

Task 01 Title: Combat Trauma (Resistance to Shock)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 62 - 30 June 63

Authors: Albert Einheber, Ph. D.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

No practicable, safe, atraumatic method has yet been devised or attempted for subjecting man or experimental animal to repeated sublethal bouts of hypotension for the purpose of evoking salutary autogenous adjustments that may eventually result in a state of augmented tolerance for severe and prolonged hypotension due to blood loss. Towards this goal, we have devised, constructed and are testing a prototype mechanical device for applying controlled pneumatic suction (negative pressure) to the hind limbs of the anesthetized, supine monkey. With this non-surgical, non-exsanguinatory technique, we hope to produce atraumatic, reversible episodes of controlled systemic hypotension, as necessary, to establish a suitable regimen that will consistently provide resistance for hemorrhagic shock that is otherwise "irreversible" to transfusion. If successful, study of the "shock-adapted" monkey may yield important information for formulating rational prophylactic and/or therapeutic approaches to combat shock in man.

BODY OF REPORT

Project No. 3A 0 12501 A 802 Title: COMBAT SURGERY

Task 01 Title: Combat Trauma (Resistance to Shock)

DESCRIPTION:

There is presently one experimental method for reproducibly inducing tolerance for ordinarily lethal amounts of injury and shock that does not necessitate preliminary surgery or external wounding of any kind, or drugs, viz., trauma via the Noble-Collip drum (N-CDT). Repeated exposures to sublethal episodes of tumbling, contusive trauma within this apparatus conditions rats to survive a dose of injury that is 100% lethal for untrained rats. Noteworthy is the fact that tolerance for N-CDT has conferred cross-tolerance for other forms of injury and shock. While studied, the mechanisms behind N-CDT resistance are incompletely known.

Although the search continues for the "lethal factors" of shock that persist despite therapy, i.e., for the mechanis(s) of "irreversibility", the factors underlying induced or "natural" tolerance for injury are of equal importance but are receiving scant attention. Moreover, there are inherent and seemingly insurmountable technical difficulties in both the attempted therapy of dying animals, e.g., animals in irreversible hemorrhagic shock for whom all therapeutic endeavors have thus far failed, and in their study. Thus, the availability of unusually shock-resistant animals, rendered this way by practicable procedures which evoke progressive, shock-attentuating auto-physiologic and/or auto-pharmacologic adjustments, rather than by exogenous polypharmaceutical regimens (some of which have proven to be effective in preventing the development of but not in curing otherwise lethal experimental shock), would permit investigation of the nature of these endogenous adjustments, their loci and mechanisms of action, and would perhaps ultimately provide means for increasing "shock-resistance", without the need for the effective "conditioning" procedure. Consequently, the questions one would seek to answer following the experimental establishment of a shock-resistant state would concern not just a "surviving" animal (chance survivor) rather than a "dying" one, but an animal which was exceptionally tolerant of one form of shock and possibly others. Although investigation of resistance-factors might suffer as much from "critical unknowns" as does the study of the death process, the manifest advantage is that one would be studying how and why a "resistant" animal survives rather than how and why the ordinary animal dies following a standard trauma. Contrasting the performances of "resistant" with "non-resistant" normal animals might reveal the biological agencies that are critical for survival and thus point to rational ways and means for prophylactically increasing tolerance for severe injury and shock. Under conditions of mass disaster, advance opportunities for prophylaxis may be eminently more important than post-traumatic therapy which would be unavoidably delayed. This positive experimental approach to presently irremediable shock, then, places emphasis on the study of the <u>etiology of surviving</u> rather than on the <u>pathogenesis of dying</u>, but embraces and ideally requires the concurrent comparison of these two processes; such comparisons

would involve the search for uncommon denominators in contrast to the common denominator that is frequently mentioned in comparisons of the nature of various forms of fatal shock.

PROGRESS:

Towards these objectives, we have considered possible means for reproducibly accomplishing increased tolerance for otherwise irreversible hemorrhagic shock after the principle of N-CDT-resistance, with some of its patent advantages, but which, unlike it, would additionally permit observation of the animal during its subjection to sublethal or lethal stress. We have, therefore, devised and constructed (Dept. Biophysical Instrumentation, WRAIR) a prototype mechanical apparatus by which we hope to induce controlled hypotension repeatedly in African green monkeys without the need for surgery (obviating incidental infection), or extracorporeal blood loss. The apparatus, a lucite chamber with suitable diaphragms and outlets plus a high capacity vacuum pump, is designed to apply pneumatic suction (negative pressure) to the hind limbs of the monkey, and if necessary, allow the further inclusion of the abdomen to the rib cage.

This apparatus is being tested because similar devices for applying negative pressure to the legs have been successfully used for many hours in young and aged patients undergoing neurosurgical procedures without ensuing untoward local or systemic reactions; induction of controlled hypotension by this means has apparently reduced bleeding (venous ooze) during surgery and thereby provided a dry operating field. The use of these devices in man has apparently been superseded by autonomic nervous system blocking drugs.

The African green monkey is being used because of our prior experience with it in studies of hemorrhagic shock (Einheber, A. and Cerilli, G.J., Am. J. Physiol. 202:1183, 1962), because its hind limb mass relative to body mass is seemingly as large as that of man, and because it is anticipated that the simian primate will approximate the physiological responses of man more closely than the mammalian non-primate.

We intend to test this apparatus, in the anesthetized supine monkey, for its ability to apply sufficient negative pressure to the legs without injury to them, and to produce, thereby, a significant lowering of mean arterial blood pressure to shock levels. If necessary, we further intend to employ varying degrees of head-up tilt to supplement (and thereby reduce the need for excessive) negative pressure in achieving desired levels of hypotension. If we can achieve, by such mechanical means, a controllable and reproducible lowering of the monkey's arterial blood pressure, then we shall attempt to establish an empirically suitable regimen of repeated exposures to sublethal hypotension which upon completion will be demonstrated, by test, to have conferred an increased tolerance of the monkey for actual hemorrhagic hypotension, and for hemorrhagic shock that is otherwise "irreversible" for concurrent control monkeys (Sham-conditioned) which have been handled identically in all ways save for the induced hypotensive episodes.

SUMMARY AND CONCLUSIONS:

No practicable, safe, atraumatic method has yet been devised or attempted for subjecting man or experimental animal to repeated sublethal bouts of hypotension for the purpose of evoking salutary autogenous adjustments that may eventually result in a state of augmented tolerance for severe and prolonged hypotension due to blood loss. Towards this goal, we have devised constructed and are testing a prototype mechanical device for applying controlled pneumatic suction (negative pressure) to the hind limbs of the anesthetized, supine monkey. With this non-surgical, non-exsanguinatory technique, we hope to produce atraumatic, reversible episodes of controlled systemic hypotension, as necessary, to establish a suitable regimen that will consistently provide resistance for hemorrhagic shock that is otherwise "irreversible" to transfusion. If successful, study of the "shock-adapted" monkey may yield important information for formulating rational prophylactic and/or therapeutic approaches to combat shock in man.

LIST OF PUBLICATIONS:

Einheber, A. and Cerilli, G.J.: Hemorrhagic Shock in the Monkey. Am. J. Physiol. 202:1183, 1962.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 802, Combat Surgery

Task 01, Combat Trauma (experimental anesthesia)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Resuscitation
Department of Experimental Surgery
Division of Clinical Surgery

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Lt Col Timothy G. Barila, MC
Capt Daniel B. Nunn, MC

Assistants: Capt Gale E. Thompson, MC*
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (experimental anesthesia)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Lt Col Timothy G. Barila, MC, Capt Daniel B. Nunn, MC, K. E. Woodward, G. Mon, H. Straub

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Experiments testing feasibility of the Army Artificial Heart Pump's design have shown that the pump can be synchronized with the pulse rate of the recipient for extracorporeal circulatory support, and the phase shift of its systole can be accomplished from 0 to 180 plus degrees. The pump's use with an experimental membrane oxygenator proved pulsed flow produces better oxygenation than steady flow.

An electronic analog simulator to handle and relate information and to permit a systems investigation and analysis of the human cardiovascular system is being made. Resonance of the arterial system and other basic considerations permitting wide ranges of flow with minimal changes in force and energy have been suggested to explain, in part, the design of the human cardiovascular system.

Studies using the fluid amplification powering concept to power prototypes of two membrane oxygenators, three respirators and a mechanical closed chest cardiac assistor have been encouraging.

The concept of cardiopulmonary resuscitation by paramedical and non-medical rescuers has been developed. The anesthetic agent halothane was shown to cause no liver damage in experimental animal preparations. Collaborative studies have shown that hydration ameliorates the renal depressant effect of morphine premedication and general anesthesia in the surgical patient. Cerebral blood flow and acid-base changes have also been studied in surgical patients.

BODY OF REPORT

Project No. 3A 0 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (Experimental Anesthesia)

Description:

This report describes current efforts to treat complications of traumatic surgery and shock, to explain some basic mechanisms in such complications, and to prevent them by a continuing program of research and education within the framework of modern concepts of anesthesiology and superior medical practice.

Progress:

Research in, and development of, equipment to maintain the injured or wounded soldier has continued.

Heart Pump: The Army Artificial Heart Pump still is considered to be in various stages of design feasibility of its component parts.

The immediate objective is the development of an extracorporeal pump, controlled by fluid amplification techniques, that can propel blood in human circulatory systems for extended periods.

Functional requirements including pulse synchronization established for the heart pump were realized and demonstrated during FY 63. The pump is capable of synchronizing its delivered pulse from in-phase to 180° plus out-of-phase positions with that of an animal's heart pulse. A Harry Diamond Laboratories (HDL)-designed trigger circuit was used to vary the phase relationship using a standard ECG apparatus.

These tests demonstrated that the animal's arterial pressures could be significantly altered. The addition of a fifth control (stroke volume) enabled the pump to synchronize its pulse with very rapid pulses in animals (or patients). This control will permit the reduction of the number of ventricles to two and still permit the pump to meet most of the flow, pressure and frequency requirements.

Ten pre-production pumps were built and delivered to civilian and military medical centers for field evaluation.

Military-style drawings were prepared and maintained on a current basis. A detailed set of operating instructions were developed to assist the evaluator. A short manual was written to provide the users with fairly complete understanding of the pump's design principles and performance.

Valves: Three types of valves are available - tricuspid, bicuspid and ball. They can be reproduced with consistency. They are statistically equal on a hemolysis basis. Of the three, the ball valves are most reliable, the tricuspids least reliable. Current impressions are favoring the bicuspid for routine use. The ball valve needs improved cage design and reductions in turbulence. Rare early failures of tricuspid valves will probably cause them to be discontinued next year.

Both pump design and production techniques apparently result in a pump whose performance is predictable within narrow limits of acceptance. This does not presume that the pump is an acceptable medical device. Design requirements remain to be validated by users.

Some effort has gone into final pump design. Magnesium or glass bodies have been built reducing weight. They offer corrosion protection as well as portability.

A sub-project not supported directly by U.S. Army Medical Research and Development funds has been encouraged informally. It is related to the heart pump's future application -- an electronic simulation of the human cardiovascular system. A simulator has been built to approximate the arterial pressure pulses by causing a simulated stroke volume efflux to excite a simulated lumped vascular load. It triggers the subsequent flow pulse once arterial pressure has fallen to a pre-set level. This next flow pulse is shaped by the simulator's predictive capabilities to increase the arterial pulse pressure to a pre-determined maximum. It is believed that the human system operates similarly. A more faithful vascular load simulation and a more detailed pressoreceptor control are needed.

Respirators: Two designs for prototype respirators have evolved, using fluid amplification techniques as power sources. One resembles the Medical Equipment Development Laboratory's (MEDL) unit, but is capable of operating either as a volume-controlled or pressure-controlled unit with the inspiratory phase initiated by the subject. It can be capable of field operation with contaminated power gases.

A second design utilizes a bistable fluid amplifier with a capacitor added to provide for the expiratory pause. As a pressure-controlled type, its inspiratory

phase can also be initiated by the subject. In comparison to commercial models, both designs are inefficient at this developmental stage.

Oxygenators: The most promising approach for an extracorporeal oxygenator is to use diffusing membranes. Two design approaches have been taken. The more conventional consists of a series of stacked metal or plastic plates. Fluid amplification offers promise as pulsators for blood boundary layer control. Another advantage is simplicity in maintenance and assembly. All plates can be easily cleaned and may be made of throw-away plastic. The membrane can be installed without heat-sealing, making it possible to evaluate different types of membranes.

A second design resembles a large alveolus. A rubber finger extends into a slightly larger lucite chamber. Between the chamber wall and the rubber finger is the blood to be oxygenated. Pulsing the finger causes better diffusion of respiratory gases.

Resuscitation Concepts: A mechanical closed-chest cardiac massage unit has been developed which offers thepossibility to support injured and diseased heart victims en route to adequate medical care.

Concepts of resuscitation by all levels of the Army Medical Service have been encouraged. All members of the Armed forces should have a working knowledge of respiratory and circulatory resuscitation.

Anesthetic Agents: The relatively new halogenated hydrocarbon (halothane) has been shown to cause no liver damage in animal preparations starved for 48 hours; while the same group of animals die a fulminating liver death with chloroform anesthesia.

Collaborative studies have shown that hydration ameliorates the renal depressant effects of morphine premedication and general anesthesia in the surgical patient. Cerebral blood flow and acid-base balances in the surgical patients have also been studied. Hyperventilation of the patient apparently offers protection against the vasodilation of hypercarbia and anesthesia.

Summary and Conclusions:

Refinements have been made in the design of the Army Artificial Heart Pump effecting improved performance. Ten pre-production pumps were built and delivered to civilian and military medical centers for field evaluation. Experiments with three kinds of valves -- tricuspid, bicuspid and ball -- suggest that

the bicuspid is most practical for routine use. It was demonstrated that the pump can synchronize its delivered pulse from in-phase to 180° plus out-of-phase positions with that of an animal's heart pulse. The addition of a fifth control (stroke volume) enabled the pump to synchronize its delivered pulse with that of an animal having a very rapid pulse. This control also makes it possible to reduce the number of ventricles to two, while maintaining the pump's ability to meet most of the flow, pressure and frequency requirements. Military-style drawings were prepared and kept current. A set of operating instructions and a user's manual were written.

Two design approaches were made for the construction of an extracorporeal oxygenator. Both of these gave promising results and merit further study.

A mechanical closed-chest cardiac massage unit was developed. Preliminary tests suggest that it would support heart patients while en route to adequate medical care.

Experiments with the anesthetic use of halothane show that it caused no liver damage in animal preparations starved for 48 hours. In contrast, the same group of animals died with fulminating livers after chloroform anesthesia. Collaborative studies showed that hydration lessened the renal depressant effects of morphine premedication and general anesthesia in surgical patients. Studies also indicated that hyperventilation protects against the vasodilation of hypercarbia and anesthesia.

Publications:

1. Barila, T.G., Nunn, D.B.: The WRAIR-DOFL Blood Pump. Symposium on Current Surgical Practices, WRGH, April 1962.

2. Nunn, D.B., Doberneck, R.C., Barila, T.G., Dixon, R.B.: Experimental Coronary Arteriovenous Fistula as a Stimulus for Increasing Intercoronary Collaterals. J. of Surg. Res. 4:220-224, 1963.

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4. Nunn, D.B., Barila, T.G., Forsee, J.H., Woodward, K.E.: A New Heart Pump Controlled by a Fluid Amplification System: A Preliminary Report. J. of Thor. and Cardiovas. Surg. 45:585-591, 1963.

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ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 802, COMBAT SURGERY

Task 01, Combat Trauma (Intracapillary thrombi in the etiology of shock, renal failure, and other conditions)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Division of Clinical Surgery

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Colonel Robert M. Hardaway, MC
Captain Dale G. Johnson, MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 A 802 Title: COMBAT SURGERY

Task No. 01 Title: Combat Trauma (Intra-capillary thrombi in the etiology of shock, renal failure, and other conditions)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Col Robert M. Hardaway, MC; Capt Dale G. Johnson, MC; Capt Raymond C. Doberneck, MC; Capt D. C. Drake, MC; D. G. McKay

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Hemorrhagic Shock. Dogs can withstand 4 to 6 hours of 40 mm Hg. mean pressure without mortality if the blood is not traumatized. Even mild trauma to the blood will result in mortality. Prolonged silicone clotting time and a fall in fibrinogen are accurate predictors of death. Shock causes the activation of endogenous heparin probably as a protective mechanism. Trauma administered 48 hours before hemorrhage is highly lethal whereas trauma administered at the same time as hemorrhage is not. Trauma results in a marked rise in fibrinogen and sedimentation rate. Trauma results in marked hemolysis by 48 hours. The results of trauma can be duplicated by the administration of 20 cc of autologous hemolized blood just before hemorrhage. Fibrinolysin is protective against death. Evidence of an episode of intravascular coagulation is associated with irreversibility and death. Monkeys can withstand up to 12 hours of 40 mm Hg. pressure if blood is not restored to them. Under these carefully controlled conditions, shock is not irreversible and there are no blood coagulation changes.

BODY OF REPORT

Project No. 3A 0 12501 A 802 Title: COMBAT SURGERY

Task No. 01 Title: Combat Trauma (Intracapillary thrombi in the etiology of shock, renal failure, and other conditions)

Description:

Hemorrhagic Shock. The role of disseminated intracapillary coagulation in irreversible hemorrhagic shock was studied. It was of immediate interest what role was played by 1) retransfusion of bled blood, 2) trauma, and 3) hemolysis in the intravascular clotting process and in the onset of irreversibility.

Progress:

Hemorrhagic Shock. The clotting mechanism has been studied in hemorrhagic shock in dogs and primates. Under morphine sedation, polyethylene catheters were placed in a femoral artery and a femoral vein of dogs. The arterial catheter was connected to a Sanborn recorder for recording of arterial blood pressure. The other femoral artery was exposed and used to bleed the animal and take blood samples utilizing a new segment of polyethylene tubing for each sample or bleeding. The venous catheter was used to administer blood, Ringer's lactate solution, or medication. The animals were bled through a clinical type of ion exchange resin (for removal of Ca ions) into a clinical type of blood bag until the mean arterial pressure was 40 mm Hg. This pressure was maintained for 4 hours by adding small quantities of decalcified blood or removing small quantities which were discarded. At the end of the 4-hour shock period blood was returned to the animal. Blood samples were taken before hemorrhage, 30 minutes after hemorrhage, and just before retransfusion at the end of the 4-hour shock period. These were tested for the following:

1) silicone clotting time
2) glass clotting time
3) hematocrit
4) sedimentation rate
5) total protein
6) fibrinogen level
7) prothrombin time
8) euglobulin lysis
9) heparin level (special test)
10) platelet count.

The test for heparin was developed by this division and appears to be quite accurate for quantities greater than 6 u/ml. It measures not only heparin but apparently its break-down products as it gives a positive

test even though the anticoagulant action has been destroyed by protamine or by the animal himself. Several variations of this experiment have been done as follows:

1) as described above
2) using a citrate blood bag for collection of the blood (this was also used in the following 3 groups)
3) mallet trauma to one thigh immediately preceding hemorrhage
4) mallet trauma to one thigh 48 hours preceding hemorrhage
5) withdrawal, freezing, and reinjection of 20 cc of blood just before hemorrhage.

In general the results can be described as follows:

1) Basic procedure using an ion exchange resin: Usually results in death which can be significantly prevented by fibrinolysin during shock, or by preheparinization. Last blood sample shows a prolonged silicone clotting time (usually hours), a fibrinogen fall over and above that which can be accounted for by dilution (as determined from plasma protein) and a prolonged prothrombin time.

2) Utilizing a citrate bag and no ion exchange column: Results in survival with only occasional blood clotting changes.

3) Mallet trauma immediately preceding hemorrhage causes no significant alteration.

4) Mallet trauma 48 hours preceding hemorrhage results in a number of changes, several of which take place in the 48 hours waiting period. During this time there is a 100% increase in circulating fibrinogen and an increase in sedimentation rate. Immediately following trauma there is a shortening of silicone clotting time but this changes to a rather prolonged time 48 hours later. There is a mild hemoglobinemia right after trauma. This is markedly increased 48 hours later. The results of hemorrhagic shock are also markedly changed. Mortality is high (90%). In the final blood sample there is a significant fall in fibrinogen over that accounted for by hemodilution. There is a prolonged silicone clotting time (usually hours), a prolonged prothrombin time, and usually the appearance of endogenous heparin.

5) Administration of 20 cc of frozen and thawed endogenous blood preceding hemorrhagic shock gives results similar to trauma 48 hours before including identical hemolysis and hemoglobinemia. Blood coagulation changes are also similar. Heparin and fibrinolysin prevent these changes. Administration of 100 cc of frozen and thawed blood to normal animals gives no ill effects but does result in transient prolonged clotting time (hours) and hemoglobinemia, both of which return to normal within 3 hours.

In another series of experiments monkeys have been subjected to hemorrhagic shock at 40 mm Hg. mean pressure. By means of pre-implanted catheters, and specially constructed frames and boxes, it is possible

to bleed the monkey without anesthesia or even without their knowledge. Recorded arterial blood pressure is somewhat lower under these conditions owing to the lack of mental stress in the animal. Using a careful technique, it has been possible to maintain a blood pressure of 40 mm mean pressure for 12 hours without causing death and without returning any blood. Saline or Ringer's lactate will result in a rise of pressure to normal levels. The animal must be in a recumbent position to withstand the procedure.

Summary and Conclusions:

Hemorrhagic Shock. Conclusions from these experiments are still incomplete but include the following:

1) Dogs can withstand 4 to 6 hours of 40 mm Hg. mean pressure without mortality if the bled blood is not traumatized.

2) Even mild trauma to the blood will result in mortality.

3) Prolonged silicone clotting time and a fall in fibrinogen are accurate predictors of death.

4) Shock causes the activation of endogenous heparin probably as a protective mechanism.

5) Trauma administered 48 hours before hemorrhage is highly lethal whereas trauma administered at the same time as hemorrhage is not.

6) Trauma results in a marked rise in fibrinogen and sedimentation rate.

7) Trauma results in marked hemolysis by 48 hours.

8) The results of trauma can be duplicated by the administration of 20 cc of autologous hemolized blood just before hemorrhage.

9) Fibrinolysin is protective against death.

10) Evidence of an episode of intravascular coagulation is associated with irreversibility and death.

11) Monkeys can withstand up to 12 hours of 40 mm Hg pressure if blood is not restored to them. Under these carefully controlled conditions, shock is not irreversible and there are no blood coagulation changes.

List of Publications:

1. Hardaway, R. M.: A Unified Theory of Shock. Am. Surgeon, 29: 292-298, 1963.

2. Doberneck, R. C., D. G. Johnson and R. M. Hardaway: Blood Volume Adjustments to Shock in Dogs. Studies in Hemorrhagic and Endotoxic Shock. AMA Arch of Surg., 86: 267-271, 1963.

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7. Hardaway, R. M.: Newer Concepts of Shock. Jour. of Occupational Med., 1963. In press.

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11. Hardaway, R. M.: Fibrinolysis and Shock. Revista Latino-Americana de Cirurgia Plastica. In press. (In Spanish). 1963.

12. McKay, D. G. and R. M. Hardaway: Thrombosis of Arterioles, Capillaries and Venules. Experimental Considerations. Monograph edited by Lowell Orbison. Pub. Hoeber. 1963.

13. Hardaway, R. M.: Blood Coagulation Changes after Incompatible Blood, Thrombin, and Shock. Presented at the Conference on Bleeding in the Surgical Patient, New York, N. Y., 2 May 63. To be published. Annals of the N. Y. Acad. of Sciences.

14. Hardaway, R. M. and D. Johnson: Changes in the Blood Clotting Mechanism in Endotoxin Shock. A.M.A. Arch. of Internal Medicine. To be published. 1963.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 802, Combat Surgery

Task 01, Combat Trauma (organ repair and replacement)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Experimental Surgery
Division of Clinical Surgery

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Capt D. G. Kline, MC, Capt Daniel B. Nunn, MC

Assistants: Col George J. Hayes, MC,* B. K. Chun, M. D., Lt Col Thomas J. Whelan, MC, ** Capt Albert N. Martins, MC, USAF***

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (organ repair and replacement)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Capt D. G. Kline, MC, Capt D. B. Nunn, MC, Col G. J. Hayes, MC, B. K. Chun, M. D., Lt Col T. J. Whelan, MC, Capt A. N. Martins, MC, USAF

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

An Experimental Evaluation of the Effect of a Plastic Adhesive, Methyl 2-Cyanoacrylate, on Neural Tissue. Controlled experimental implants of methyl 2-cyanoacrylate on canine peripheral nerves and cerebral cortex and on primate optic chiasms resulted in chronic inflammation, necrosis, demyelination, axonal loss and fibrosis. Care should be exercised if this material is used near neural tissue and further long-term histologic and toxicologic studies seem indicated.

A Comparative Study of the Response of Dog, Monkey, and Chimpanzee Peripheral Nerves to Trauma. The purpose of this study was to find an animal demonstrating a peripheral nerve injury response most analogous to the human. Histologic comparison of the resultant material with the Lyons-Woodhall collection of WW II nerve injuries strongly suggests that the chimpanzee would be a more reliable animal for testing techniques of neural repair.

Experimental Attempts to Improve Peripheral Nerve Repair. Experiments with processed bovine collagen as a cuffing material have been carried out using the chimpanzee as the experimental animal. Collagen cuffs are effective in reducing neuromatous disorganization and are resorbable, thus obviating secondary operations for cuff removal.

Dural Replacement. Modified collagen grafts were used as dural replacements. Partial histologic evaluation of the series suggests that the graft is replaced by living collagen.

Rebound Phenomenon Following Reduction of Intracranial Pressure by Hypertonic Solutions. The use of hypertonic agents to lower intracranial pressure is attended by a rebound in pressure following discontinuation of the agent. These studies were designed to produce a mass lesion and to study the effect of mannitol on the cerebrospinal fluid pressure following removal of the mass.

Autogenous Vein Grafts. This study was performed to further clarify the histologic fate of autogenous vein grafts used to replace segments of arteries. Special attention was given to the morphology of autogenous vein graft endothelium which was examined in en face preparations.

BODY OF REPORT

Project No. 3A 0 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (organ repair and replacement)

Description:

An Experimental Evaluation of the Effect of a Plastic Adhesive, Methyl 2-Cyanoacrylate, on Neural Tissue. The use of methyl 2-cyanoacrylate (Eastman 910 Adhesive) as a "biological adhesive" has attracted widespread surgical interest in the last few years. The adhesive has been used to close arteriotomies, to anastomose bowel, vessels and bone, to apply skin grafts, and to act as a hemostatic agent. The use of this adhesive to repair intracranial vessels and aneurysms has been advocated by some investigators. The purpose of this study was to delineate some of the properties of this adhesive by implanting it on neural tissue and studying its histologic effect.

A Comparative Study of the Response of Dog, Monkey, and Chimpanzee Peripheral Nerves to Trauma. Many species of animals have been used by investigators to explore the problems of peripheral nerve repair. However, methods of repair which have appeared promising in animals have often proved disappointing when tried in the human.

The purpose of this study was to find an animal demonstrating an injury response most comparable to the human. Such an animal would then provide a more reliable experimental model for critically evaluating methods of peripheral nerve repair.

Experimental Attempts to Improve Peripheral Nerve Repair. The placement of cuffs around the repair sites of peripheral nerves has been pursued for many years. The partial success of tantalum foil during WW II and the more recent success of Millipore cuffs by Campbell have rekindled a good deal of interest in this technique.

Dural Replacement. The replacement of dural defects created by trauma or tumor is difficult. Autogenous fascia, synthetic materials such as teflon and dacron, and lyophilized homogenous grafts have been used in the past. The successful use of collagen for experimental peripheral nerve repair has prompted studies designed to evaluate modified collagen as a dural graft.

Rebound Phenomenon Following Reduction of Intracranial Pressure by Hypertonic Solutions. Hypertonic urea and mannitol have been extensively used to decrease intracranial pressure. Experiments in animals and humans infusing these agents to decrease normotensive cerebrospinal fluid pressures to hypotensive levels have shown that a rebound in CSF pressure occurs after termination of the infusion. The proponents of mannitol claim that less rebound of pressure occurs with this agent than with urea or other hypertonic solutions. The purpose of these studies was to produce an intracranial mass lesion, thus raising the cerebrospinal fluid pressure, and to evaluate the effectiveness of mannitol in preventing increased intracranial pressure after removal of the mass.

Autogenous Vein Grafts. This study was performed to further clarify the histologic fate of autogenous vein grafts used to replace segments of arteries. Special attention was given to the morphology of autogenous vein graft endothelium which was examined in en face preparations.

Progress:

An Experimental Evaluation of the Effect of a Plastic Adhesive, Methyl 2-Cyanoacrylate, on Neural Tissue.

a. Peripheral Nerves. Multiple peripheral nerves in twenty dogs were carefully exposed and coated either with saline or Eastman 910 Monomer. Baseline functional thresholds of stimulation were determined in each nerve. At intervals of 2, 6, 8, and 12 weeks, thresholds of conduction were redetermined. Thresholds were unchanged in the saline coated nerves but were elevated in 7 of the 20 adhesive coated specimens. Nerves were studied histologically using H & E, Masson, Bodian, myelin and Nissl's staining techniques. The adhesive coated specimens demonstrated chronic epineural inflammation, tubular demyelination, perineural and intrafasicular inflammatory infiltrates, and axonal damage.

b. Brain. Cortical implants of the adhesive in ten dogs resulted in localized areas of necrosis and chronic inflammation in six of the ten dogs studied. Cultures of these lesions and of the adhesive itself were negative. Saline implants did not result in cortical damage.

c. Optic Chiasm. Frontotemporal craniectomies were carried out in 12 rhesus monkeys. Three drops of adhesive were placed on the optic chiasm in 10 animals while methyl methacrylate was implanted in three control animals. Adhesive implants resulted in localized optic chiasm, hypothalamic, and in one 98-day old specimen, third nerve damage. The methacrylate implants did not result in damage.

A Comparative Study of the Response of Dog, Monkey, and Chimpanzee Peripheral Nerves to Trauma. Multiple nerves of 20 adult dogs, 20 rhesus monkeys, and 12 chimpanzees were subjected to one of three methods of trauma: (1) severance with removal of 1 cm. segment of nerve, (2) crush, (3) severance and primary suture anastomosis.

a. Severance. The canine severed nerves made remarkable attempts to bridge the interstump gap with connective tissue and axons arranged in a predominantly longitudinal fashion. Conduction was restored in one 14 week specimen by this mechanism. Although the monkey nerves made some attempt to bridge the gap, proximal stump axonal disorganization was more prominent. The chimpanzee severed nerves formed bulky areas of disorganization in the proximal stump as early as 2 weeks. There was very little tendency to bridge the gap and then only with connective tissue. Sworls of connective tissue and a disorganized axonal pattern predominated.

b. Crush. Species response to crush injury was quite similar. The continuity of the supporting elements was retained and axonal regeneration occurred in a longitudinal fashion. Thresholds of conduction were restored several weeks earlier in the dogs than in the other species.

c. Suture. Primary suture as executed in this study resulted in neuromas. Endoneurial and perineurial connective tissue proliferation with heavy Schwann cell and fibroblast concentration was most pronounced in the repair area of the chimp. The delay in restitution of functional conduction in the chimp approached that reported in the human (14-24 weeks).

Experimental Attempts to Improve Peripheral Nerve Repair. Using the chimpanzee as the experimental animal the peroneal nerves on both sides were repaired following the experimental model used in previous experiments. The nerve on one side was cuffed with a collagen membrane (supplied by Ethicon, Inc.) The collagen was taken from bovine deep flexor tendons, processed into a thin transparent membrane, and lightly tanned. The cuffed nerves and their controls were biopsied at intervals of 2, 6, 8, 14, 24, and 32 weeks and studied histologically. Comparative study of these two sets of specimens demonstrated the following points: (1) The cuff was resorbable and secondary operation was not necessary. (2) The cuffed nerves had less axonal disorganization, better axonal carry through, and comparatively better functional thresholds of stimulation. (3) A small amount of chronic epineural inflammation unaccompanied by fibrosis was present in the cuffed nerves.

Current studies are designed to evaluate the response of intact nerves to non-tanned and non-tanned and irradiated collagen materials. More sophistocated stimulating and recording instruments will be necessary to complete this phase of the investigation.

Experiences with Acropor (a Dynel microporous membrane on a Nylon substrate) as a cuffing material on repaired baboon nerves have been unsatisfactory to date.

Dural Replacement. A 2 x 2 cm. piece of dura was resected over the frontoparietal lobes of 16 dogs and replaced with modified collagen grafts (made by Ethicon, Inc.) Alternate animals were subjected to left frontal lobectomy in order to test the effectiveness of the graft over a cavity.

Seven animals have been sacrificed to date. In the early specimens the graft was seen to be covered on both sides by connective tissue. Resorption of the collagen occurred slowly and in one 6 month specimen studied, the grafted area was replaced by healthy connective tissue.

Complete histologic evaluation of the entire series will be necessary before further conclusions are justified.

Rebound Phenomenon Following Reduction of Intracranial Pressure by Hypertonic Solutions. The standard technique of sealing balloons in the epidural space was utilized to produce mass lesions. Continuous recordings of cisterna magna pressure, femoral blood pressure, respiration, and pulse were made.

In a control series of animals the epidural balloon was kept inflated for a period of 4 hours maintaining the CSF pressure above 250 mm H_2O. The balloon was then deflated and the CSF pressure maintained for an additional 8 hours. In two other groups of animals, 20% mannitol, 4 Gm/Kg, was begun

before deflating the balloon and after deflating the balloon. Serum and CSF chemistries were obtained on some of these animals.

Analysis of this data is not complete. Further work on this project was halted until better recording equipment was obtained and since this is now available, work will be resumed in the coming year.

Autogenous Vein Grafts. Twenty-six mongrel dogs of both sexes weighing from 12.7 to 29.5 kg. were used for this study. The dogs were anesthetized with sodium pentobarbital (25 mg/kg) administered intravenously. A segment of the left external jugular vein was excised to provide an autogenous vein graft. The abdominal aorta was exposed through a left flank incision and mobilized from the level of the left renal artery to the trifurcation. A composite graft was constructed by anastomosing a 3 cm. segment of the autogenous vein graft to a 3 cm. segment of a crimped, knitted Dacron graft. A composite graft was used for this study in order to determine if autogenous vein graft endothelium could later be demonstrated growing into the Dacron graft. It was reasoned that such a demonstration of growth would prove that the autogenous vein graft endothelium had retained its viability. A 6 cm. segment of abdominal aorta was excised and replaced with the composite graft of vein and Dacron.

All dogs survived the operative procedure and received penicillin, 600,000 units, and Streptomycin 0.5 Gm. IM daily for one week. Three dogs were sacrificed at each of the following periods postoperatively: 1 week, 2 weeks, 1 month, 1½ months, 2 months, 3 months, 3½ months, and 4 months. Two dogs were sacrificed 6 months postoperatively. Ten minutes prior to sacrifice the dogs were given Heparin (1.5 mg/kg) IV to prevent clotting of blood on the endothelial surfaces of the vessels. After sacrifice, the composite graft of autogenous vein and Dacron was removed in continuity with a short segment of the adjacent proximal and distal aorta. A segment of the right external jugular vein was removed from several dogs to provide a control vein specimen. Hautchen preparations, designed to delineate a single layer of endothelial cells for en face examination, were made from the lining of the autogenous vein and Dacron grafts and the segments of adjacent proximal and distal aorta, and of the control vein specimens. Routine histologic sections of the autogenous vein grafts and the control vein specimens were made and stained with (1) hematoxylin and eosin, (2) Masson's trichrome stain for connective tissue, (3) Rinehart-Abul-Haj stain for acid mucopolysaccharides (ground substance), and (4) Verhoeff's elastic stain.

Summary and Conclusions:

An Experimental Evaluation of the Effect of a Plastic Adhesive, Methyl 2-Cyanoacrylate, on Neural Tissue. The use of neural tissue provides a critical experimental model for testing synthetic materials intended for biological use. Eastman 910 Adhesive offers several advantages in that it is very adherent to living tissue and appears to be self-sterilizing. However, it is not innocuous and care must be exercised if it is used near neural tissue.

A Comparative Study of the Response of Dog, Monkey, and Chimpanzee Peripheral Nerves to Trauma. Basic mechanisms of repair are similar but species response to peripheral nerve injury does differ. Comparison of this large collection of material with the Lyons-Woodhall collection of WW II peripheral nerve injuries suggests that the chimpanzee would be a more reliable animal for testing techniques of neural repair. Studies currently in progress will delineate the response of baboons to peripheral nerve trauma.

Experimental Attempts to Improve Peripheral Nerve Repair. The use of protective cuffs around peripheral nerve repairs to reduce constricting adhesions and diminish axonal and tubular disorganization in the rapair area has been pursued for many years. Experiments with processed bovine collagen as a cuffing material have been carried out in our laboratory using the chimpanzee as the experimental animal. Collagen cuffs are effective in reducing neuromatous disorganization and are resorbable, thus obviating secondary operations for cuff removal. However, further experimentation is necessary before this material is ready for clinical trial.

Dural Replacement. Modified collagen grafts were used as dural replacements in a series of 16 dogs. Partial histologic evaluation of the series suggests that the graft is replaced by living collagen. Complete evaluation will be necessary before clinical trial can be begun.

Rebound Phenomenon Following Reduction of Intracranial Pressure by Hypertonic Solutions. The use of most hypertonic agents to lower intracranial pressure is attended by a rebound in pressure following discontinuation of the agent. These studies were designed to experimentally produce a mass lesion and to study the effect of mannitol on the cerebrospinal fluid pressure following removal of the mass.

Autogenous Vein Grafts. This study was performed to further clarify the histologic fate of autogenous vein grafts used to replace segments of arteries. Special attention was given to the morphology of autogenous vein graft endothelium which was examined in en face preparations. The results of the histologic findings may be summarized as follows:

1. Endothelial growth from the vein grafts into the Dacron was demonstrated in all specimens in which the Dacron graft was incompletely lined with endothelium.
2. The endothelium of the autogenous vein grafts retained its normal morphology.
3. The thickness of the grafted vein wall became thicker at two weeks and one month postoperatively, and later gradually thinned out, but never assumed its original thickness.
4. The elastic fibers of the vein wall appeared separated but their total amount appeared unchanged.
5. The acid mucopolysaccharides (ground substance) increased throughout the series, particularly in the earlier groups.

List of Publications:

1. Kline, David G., Hayes, George J. An Experimental Evaluation of the Effect of a Plastic Adhesive on Neural Tissue. J. of Neurosurgery.

2. Kline, David G., Hayes, George J. The Use of a Resorbable Cuff in Peripheral Nerve Repair. To be published.
3. Kline, David G., Hayes, George J., Morse, Arthur A. A Comparative Study of Species Response to Peripheral Nerve Injury. I. Severance. To be published.
4. Nunn, Daniel B., Chun, B. K., Whelan, T. J., Martins, Albert N. Autogenous Veins as Arterial Substitites: A Study of Their Histologic Fate With Special Attention to Endothelium. To be published.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 802, COMBAT SURGERY

Task 01	Combat Trauma (Metabolic and nutritional problems associated with injury)
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C. Department of Surgical Metabolism and Pathology Division of Basic Surgical Research
Period Covered by Report:	1 July 1962 through 30 June 1963
Principal Investigators:	Edwin J. Pulaski, Col, MC Costan W. Berard, Capt, MC Stephen C. Woodward, Capt, MC H. Kenneth Sleeman, Ph. D. Clarence E. Emery, Jr., B. S. Hyman Rosen, Ph. D.* Erving F. Geever, M. D.*
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Reports Control Symbol:	MEDDR-288
Security Classification:	UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 802	Title: COMBAT SURGERY
Task 01	Title: Combat Trauma (Metabolic and nutritional problems associated with injury)
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington, D. C.
Period Covered by Report:	1 July 1962 through 30 June 1963
Authors:	Edwin J. Pulaski, Col, MC, Costan W. Berard, Capt, MC, Stephen C. Woodward, Capt, MC, H. Kenneth Sleeman, Ph. D., Clarence E. Emery, Jr., B. S., H. Rosen, Ph. D., and E. F. Geever, M. D.
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

The Department of Surgical Metabolism and Pathology has continued to use physiologic, biochemical and histopathologic methods to broaden understanding of the metabolic and nutritional problems associated with injury. Studies were conducted on the effects of stress on the metabolism of tyrosine in guinea pigs, the effect of deuterium oxide on rat collagen, wound healing and metabolism, and the metabolic effects of sulfonamides with particular reference to the thyroid gland.

BODY OF REPORT

Project No. 3A 0 12501 A 802 Title: COMBAT SURGERY

Task 01 Title: Combat Trauma (Metabolic and nutritional problems associated with injury)

Description:

The Department of Surgical Metabolism and Pathology has continued to use physiologic, biochemical and histopathologic methods to broaden understanding of the metabolic and nutritional problems associated with injury. Studies were conducted on the effects of stress on the metabolism of tyrosine in guinea pigs, the effect of deuterium oxide on rat collagen, wound healing and metabolism, and the metabolic effects of sulfonamides with particular reference to the thyroid gland.

Progress:

(1) Effects of stress (burns) on the metabolism of tyrosine in guinea pigs. Previous progress reports have recounted that guinea pigs deficient in vitamin C excreted large amounts of urinary phenylhydroxy-carboxylic acids when challenged with an oral tyrosine load test. This was not found in animals given adequate vitamin C. The phenomenon was observed also in animals subjected to severe burns even when the dietary vitamin C level was adequate. Further experiments suggested also that a difference existed in response to the tyrosine load test between animals burned at temperatures of 73° C and 85° C.

Twenty four Walter Reed strain guinea pigs were divided into groups of 12 burned at 85° C, 8 burned at 73° C, and 4 unburned controls. The animals were maintained at 78±2 degrees, food and water intake measured, and 2 mg. daily of vitamin C given intragastrically. The maximum secretion of urinary tyrosine metabolites was found previously to occur on the sixth day post-burn with guinea pigs burned at 73° C. Tyrosine load tests (0.75 mg/g body wt.) were given on the 3rd, 6th and 9th day post-burn, to exclude the possibility of different times of maximum urinary tyrosine metabolites. The urines were collected daily and analyzed for phenylhydroxy-carboxylic acids (U.V. absorption) and for tyrosine (chemical).

The results observed in this experiment revealed surprisingly little differences between the animals burned at 85° C and 73° C, and the control animals. The food and water intake followed expected patterns, decreased immediately after burns and returned to normal in 24 hours and all animals maintained body weight. The urinary excretion of tyrosine was high after each load test. The amounts excreted

by the burned animals were only slightly higher than the controls. Impaired tyrosine metabolism, as shown by large increases in urinary levels of p-hydroxy-phenylpyruvic acid following tyrosine load test, was not found. The highest levels of phenylhydroxy-carboxylic acids were found only on the sixth day post-burn, but the amounts excreted by the burned animals were not significantly higher than the control animals. The amounts of phenylhydroxy-carboxylic acids excreted were highest in the 73° C burn.

(2) The effect of deuterium oxide on rat collagen, wound healing, and metabolism. Deuterium oxide has long been known to participate in many of the in vitro and in vivo reactions normally associated with water but, because of its differing mass and charge densities, to confer unique characteristics upon the phenomena in which it is substituted for H_2O. Hydrogen-bonding, for example, is an important structure-stabilizing feature of proteins and substitution of deuterium for hydrogen in protein systems has shed considerable light on the significance of hydrogen-bonding for the properties of proteins. In an earlier progress report it was stated that this department was using deuterium oxide to elucidate further the parameters of collagen synthesis and metabolic response to injury in the rat. The details of the first metabolic experiment, using 15% D_2O in drinking water, were reported two years ago. Last year's progress report contained data on the fresh and fixed tensile strengths of 27 and 47 wounds of animals on 75% H_2O: 25% D_2O compared to controls on 100% H_2O. These studies have now been finalized, including the metabolic parameters, and although it is impossible to present the data in detail the primary findings are as follows:

(a) Rats allowed to drink 25% D_2O ad lib as their only fluid intake achieved an eventual equilibrium body concentration of about 15%. The difference between intake and equilibrium percentage is almost certainly attributable to water of oxidation of food intake.

(b) Dorsal skin incisions healed abnormally in rats on 25% D_2O. Their fresh breaking strengths were about 40% less those of controls. Formalin fixation corrected the deficiency. Histologic examination of the wounds showed focal porosity due to decreased number, caliber, and density of the collagen fibers as compared with the controls. There was no apparent abnormality in ground substance or cellular population of the wounds.

(c) The implanted sponges were normal histologically and in their amounts of bound hydroxyproline.

(d) Tail tendon collagen dissolved in acetic acid was normal with respect to chromatographic fractionation, shrink temperature, and

optical rotation, indicating that the abnormalities induced by D_2O depend on the entire fibrous structure, not on the collagen macromolecule alone.

(e) Although the "deuterated" rats showed no gross pathologic abnormalities, they weighed 15% less than controls at the end of the experiment (67 days) although their food intakes had been identical; they had drunk 20% more D_2O-H_2O than the controls had drunk H_2O; their response to the stress of operation was more severe than the controls in terms of urinary loss of nitrogen, sodium, and potassium; spermatogenesis was depressed in the deuterated rats.

(f) Uptake of deuterium by various tissues was 6% for liver, tail tendons and Achilles tendon, and 8% for testes. Extracted skin collagen had 5-6% deuterium.

(g) Although adrenal hypertrophy was not observed in these experiments, it was considered possible that the effects of D_2O might result from chronic hyperadrenalism. An attempt was, therefore, made to reproduce these findings by ACTH administration. Although a 40% adrenal hypertrophy (by weight) resulted, there were no abnormalities in wound healing.

We conclude from this work that the deleterious effects of D_2O on wound healing are due mainly or entirely to the solvation effects of the D_2O on developing collagen, that direct incorporation of deuterium into the collagen macromolecule plays little part, and that nutritional and endocrinological factors are also of minor importance.

(3) The metabolic effects of sulfonamides, with particular reference to the thyroid gland. Since the introduction of the sulfonamides over 20 years ago, there has accumulated an impressive body of evidence that these drugs can affect markedly the metabolism of the host quite aside from their influence upon the resident microflora. One of the earliest documented effects in the experimental animal was interference with thyroid function and consequent goiter formation. This effect is seen in varying dosage with all sulfonamides and has also been reported with sulfonylureas, para-aminobenzoic acid, and para-amino-salicylic acid. In the human, clinical goiter is observed only in patients on long-term, high-dosage therapy for conditions such as ulcerative colitis or tuberculosis but measurements of protein-bound iodine, radioiodine uptake, and basal metabolic rate have demonstrated that these drugs can interfere with thyroid function rapidly and in low dosage. The nature of this interference has been a subject of continuing interest but the pathogenesis remains obscure. Since the sulfonamides are frequently used in association with both injury and a necessity for optimal wound healing, this

department has conducted studies on the pathophysiology and prevention of this effect of the sulfonamides.

Using a 2% sulfaguanidine diet in the rat as the experimental model, it was shown that rats so treated develop goiter rapidly (within one week) and that the goiter increases in size rapidly for two months and then little if at all thereafter out to four months, the longest time period studied. Such rats have a 15-20% lower voluntary food consumption and gain only about half as much weight in 4 months as their normal controls. The pituitary gland is rich in "thyroidectomy" cells but the adrenals show no histologic abnormality. The salivary glands, which have been claimed to require a trophic thyroid hormone influence, show no involution or other change. Studies of the pancreas, kidneys, knee joints(and metaphyses) and reproductive organs of such animals are not yet complete and will be reported later.

The goitrogenicity of the sulfonamides has been attributed by some workers to their structural similarity to tyrosine (the natural substrate for thyroxine synthesis) and their tyrosine-like great affinity for iodine. If such were the case, it would seem possible to prevent the goiter effect by giving an animal on sulfa drug supplemental quantities of tyrosine or iodine. It was found that l-tyrosine given orally in equimolar dosage with sulfaguanidine was totally ineffective in this regard. Since l-tyrosine is too insoluble for parenteral administration, glycyl-l-tyrosine was used, again with the tyrosine moiety equimolar with the orally administered sulfa drug; this preparation was likewise ineffective. A third preparation, tyrosine ethyl ester-HCl was then tried. In two successive experiments, this drug, when orally administered in equimolar quantity to animals on a 2% sulfa diet, showed its ability partially to prevent and/or to reverse the goitrogenic action of the sulfa drug. Thyroids of animals thus treated were significantly smaller than those of animals on sulfa drug alone and, in one experiment, were not significantly larger than those of control animals on a stock diet free of any drug. These thyroids were still histologically abnormal, however, and whether administration of the drug in higher dosage would have resulted in totally normal thyroids is still unknown. Supportive to the impression that this drug effect actually represented a sulfa reversal was the finding that such animals had a normal or even supernormal food intake instead of the lower food intake seen in animals on only sulfa drug.

The effect of supplemental iodide was investigated in two experiments employing diets calculated to give each rat an intake sufficient to saturate all or a part of his daily sulfonamide dose. On low dosage iodide, the sulfa drug effect was potentiated. At high dosage, there was a significant reduction in goiter size but the high iodide diet

was poorly consumed and relatively toxic; such animals gained almost no weight over a period of 28 days. It is thus uncertain whether the high-dose iodide effect on the goiter is attributable to nonspecific toxicity or to a direct relationship to sulfa-thyroid interaction.

In long-term (8 and 16 weeks) experiments, animals on sulfa drug were compared with surgically thyroidectomized litter mates. It was found that both groups had, relative to controls, a diminished food consumption and weight gain of essentially identical magnitude. This seemed to corroborate the impression that these effects of the sulfa drug were attributable to its interference with thyroid metabolism. It was, therefore, totally unanticipated to find that sulfa drug administration to a thyroidectomized animal is not "invisible" but rather is extremely toxic and in about one-third of the animals lethal with 4 months. The surviving animals, who with either sulfa or thyroidectomy alone would have had about 80% of normal weight gain, show instead absolutely no gain from their original weight of 175 grams. This devastating effect of sulfonamide in a thyroidectomized animal has not to our knowledge been noted previously and certainly seems to warrant further investigation.

(4) Synthesis and administration of iodinated sulfa-drug analogues. As stated above, the "antithyroid" activity of sulfaguanidine has been postulated to result from the formation of molecular compound with iodine or else by an iodine substitution reaction (Fawceth and Kirkwood, J.B.C., 204: 787, 1953). If this theory is true, the iodinated compounds of sulfaguanidine, mono-iodosulfaguanidine (MIS) and diiodosulfaguanidine (DIS), should possess little or no antithyroid activity. Therefore, MIS and DIS were synthesized in our laboratory in order to test this theory.

Mono-iodosulfaguanidine and DIS were prepared by iodinating sulfaguanidine with iodine monochloride (ICl) (Heimkamp, et al., Cancer Res., 20: 1495, 1960). Sulfaguanidine was treated with an excess of ICl and reacted for one hour at 37° C. The maximum yield of MIS was produced by allowing the reaction mixture to stand for 3 to 4 hours at room temperature, then neutralizing with 20% NaOH to pH 7. The MIS precipitated above pH 1. The solution was cooled and the precipitate was collected on a Buchner funnel. The DIS was prepared by allowing the reaction mixture to stand 18 hours at room temperature. The less soluble DIS precipitated and was removed by filtration. The solution was then neutralized with 20% NaOH as above, and the MIS collected. The theoretical yield was about 80%. The compounds were recrystallized from hot ethanol-H_2O or acetone-H_2O. MIS melted at 282° C with decomposition; DIS at 268° C - 270° also with decomposition. The purity of the preparation was determined by nitrogen and iodine analyses.

These drugs have since been administered to rats in a preliminary metabolic experiment to determine the percentage absorption and degree of esterification of each *in vivo*. Urines and stools were collected and four successive 24 hour specimens are currently being analyzed. It is intended that these drugs will be tested for their goitrogenicity, general metabolic acceptance, and antimicrobial activity relative to the parent sulfonamide.

(5) Chromatographic analysis of stable iodine-containing compounds of the thyroid. Since the administration of sulfaguanidine results in the enlargement and hyperplasia of the thyroid gland and interferes with its synthesis of thyroxine, methods were devised to ascertain the effects of sulfaguanidine on the intermediates of thyroxine synthesis.

The method developed is sufficiently sensitive to detect the iodinated compounds from a single rat thyroid. The thyroid is removed and homogenized immediately with 0.9% saline. The insoluble material is removed by centrifugation, and the supernatant solution decanted. Concentrated hydrochloric acid equal to 1/3 the volume is added and the solution is hydrolyzed for 4 hours at 110° C. The hydrolysate is cooled and extracted with 2 volumes of butanol. The butanol extract is washed once with 1/2 volume of water and then evaporated to dryness. The residue is dissolved in 1 part of 2% diethanolamine in 50% propylene glycol and 9 parts 50% ethanol and chromatographed overnight on Whatman #1 paper pretreated with 0.067M phosphate buffer pH 6.0. The solvent is the upper layer of a butanol-acetic acid-water mixture (4:1:5).

The chromatogram, after drying, is sprayed with 1.0N sulfuric acid to neutralize the phosphate buffer. It is then sprayed with a solution containing 2 volumes of 2% ceric sulfate and 1 volume of 0.2N arsenious acid. The iodine containing compounds appear as a white area on a yellow background. This spray is sensitive to fractions of micrograms of iodide and in addition is specific for iodinated aromatic compounds containing a free phenolic hydroxyl group. In addition, the ceric sulfate, completely absorbs ultraviolet light. Therefore, when the chromatograms are viewed under ultraviolet light (360 mμ), the iodinated compounds, which allow the transmission of light, appear as light spots on a black background. A permanent record of the chromatogram may be made by preparing a photograph under ultraviolet light.

The described chromatographic method will be used in conjunction with *in vivo* I^{131} incorporation experiments to study further the inhibitory effects of drugs on thyroid function. By adding I^{131} radioautochromatography to the method here described for chemical analysis

of stable compounds, it should be possible to determine on a single rat thyroid gland both the percentage of each of the iodinated precursors present and their relative rates of turnover. Such combined information is not obtainable by any technique devised heretofore.

Summary and Conclusions:

Studies have been conducted, employing physiologic, biochemical, and histopathologic methods, on the metabolic and nutritional problems associated with injury. It was found that guinea pigs burned at either 73° C or 85° C manifest abnormalities in tyrosine metabolism even on an adequate daily intake of ascorbic acid; these abnormalities, however, were minor, highly variable, and not consistently different from findings in unburned animals.

Deuterium oxide has been used to demonstrate the influence of biochemical milieu on the synthesis and function of collagen in vivo. Such animals demonstrated physical and histologic abnormalities in wound healing and biochemical alterations in the metabolic response to injury.

Progress has also been made in defining the effects of the sulfonamides on the metabolism of the host, in particular upon thyroid function. It was shown that the goitrogenicity of these drugs could be partially antagonized by a tyrosine ester. A dosage of sulfa drug which is well-tolerated by a normal animal is extremely toxic and sometimes lethal for thyroidectomized animals. This work has involved the development of a chromatographic procedure for determining the stable intermediates in thyroxine synthesis in a single rat thyroid gland.

List of Publications:

1. Rosen, H., Levenson, S. M., Geever, E. F., and Berard, C. W.: The effect of deuterium oxide on rat collagen, wound healing, and metabolism. (Submitted for publication)

2. Pulaski, E. J.: Septicemia and bacteremia. In TRAUMATIC MEDICINE AND SURGERY FOR THE ATTORNEY, Vol. 9 - Selected Infections. Butterworth, Inc., Washington, D. C. (In press)

3. Pulaski, E. J.: Chapt. 3, Infection. In CHRISTOPHER'S TEXTBOOK OF SURGERY, 8th ed., W. B. Saunders Co., Philadelphia. (In press)

4. Herrmann, J. B.: Thermal injuries and their treatment in a disaster situation. J. Am. Assn. Ind. Nurses. (In press)

5. Rosen, H., Berard, C. W., and Levenson, S. M.: Simplified procedure for automatic amino acid analysis. Anal. Biochem., 4: 213, 1962.

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6. Sleeman, H. K.: A serological study of a trichinosis epidemic in Sweden in 1961. Acta path. et microbiol. scandinav. (in press)

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 802, Combat Surgery

Task 01, Combat Trauma (metabolic and nutritional problems associated with injury)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Experimental Surgery
Division of Clinical Surgery

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Capt Raymond C. Doberneck, MC

Assistants: Capt Daniel B. Nunn, MC
B. K. Chun, M. D.
Capt D. G. Johnson, MC
Capt A. S. Morse, MC
Capt D. G. Kline, MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (metabolic and nutritional problems associated with injury)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Capt Raymond C. Doberneck, MC, Capt Daniel B. Nunn, MC, B. K. Chun, M. D., Capt D. G. Johnson, MC, Capt A. S. Morse, MC, Capt D. G. Kline, MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Portacaval Shunting and Hemosiderin Deposition in the Liver. Hepatic hemosiderin deposition consistently followed diversion of all portal flow into the inferior vena cava, infrequently followed shunting of all but duodenal flow and did not occur after shunting of only duodenal flow. After complete portal diversion, weight loss and reduction of red cell mass with storage of unused iron rather than increased iron absorption accounted for hepatic hemosiderin deposition.

BODY OF REPORT

Project No. 3A 0 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (metabolic and nutritional problems associated with injury

Description:

Portacaval Shunting and Hemosiderin Deposition in the Liver. Hemosiderin deposition in the liver of cirrhotics after portacaval shunting has been reported. Whether such deposition is causally related to the underlying disease or the presence of the shunt is not clear but an increased iron absorption has been suggested. The purpose of these experiments is to determine (1) whether the shunt alone may be responsible for the siderosis and (2) identification of the mechanism involved in the iron deposition.

Nearly all healthy dogs subjected to total diversion of the portal flow into the inferior vena cava developed hepatic hemosiderin deposits. Fewer dogs with diversion of all but duodenal flow and no dog with diversion of only duodenal flow into the cava developed such hemosiderin deposits. Absorption of iron was not increased by portacaval shunting nor were bilirubin and thymol turbidity elevated. BSP retention increased immediately after shunting and did not change thereafter while body weight and red cell mass were drastically reduced.

Summary and Conclusions:

Portacaval Shunting and Hemosiderin Deposition in the Liver. These studies suggested that portacaval shunting may produce hepatic hemosiderin deposition in the healthy canine liver and that the frequency of such siderosis is related to the size of the shunted branch. The data suggested that due to the loss of weight and red cell mass, a large amount of unused iron was stored in the liver as hemosiderin.

List of Publications:

1. Doberneck, R. C., Nunn, D. B., Johnson, D. G. and Chun, B. K. Alteration of Iron Metabolism After Portacaval Shunting in Dogs. Arch. Surg. In press.

BODY OF REPORT

Project No. 3A 0 12501 A 802 Title: Combat Surgery

Task No. 01 Title: Combat Trauma (metabolic and nutritional problems associated with injury

Description:

Portacaval Shunting and Hemosiderin Deposition in the Liver. Hemosiderin deposition in the liver of cirrhotics after portacaval shunting has been reported. Whether such deposition is causally related to the underlying disease or the presence of the shunt is not clear but an increased iron absorption has been suggested. The purpose of these experiments is to determine (1) whether the shunt alone may be responsible for the siderosis and (2) identification of the mechanism involved in the iron deposition.

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1. Doberneck, R. C., Nunn, D. B., Johnson, D. G. and Chun, B. K. Alteration of Iron Metabolism After Portacaval Shunting in Dogs. Arch. Surg. In press.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 802 COMBAT SURGERY

Task 02, Blood & Expanders (Revision of field transfusion service)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Hematology
Division of Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Colonel William H. Crosby, MC

Assistants: Major Marcel E. Conrad, MC
Lt Col Edward Jahnke, MC *
Lt Col G. W. Fisher, MC **
Captain Roger A. Brald, MC
Captain Simeon Pollack, MC
Captain Richard Kaufman, MC
Pearl Anderson, Ph.D.
Mr. James W. Eichelberger
Mr. Harold Williams #
MSgt Allen Young
MSgt Lewis E. H. Allen
Sp5 John Butkiewics

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

* WRAH, Thoracic Surgery Service

** WRAH, Thoracic Surgery Service

\# WRAH, Cancer Chemotherapy Section

ABSTRACT

Project No. 3A 0 12501 A 802 — Title: COMBAT SURGERY

Task No. 02 — Title: Blood & Expanders (Revision of field transfusion service)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors:
Colonel William H. Crosby, MC
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Sp5 John Butkiewicz

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. The blood donor panel 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 absorption of iron means are sought to increase production of blood in normal blood donors.

2. A program for testing plasma volume expanders has been initiated. A solution of hydralyzed keratin (chicken feathers) was toxic when given to dogs. Dextran was found to cause a reduction in platelet factor 3 activity in vitro even when there was no prolongation of bleeding time.

3. In collaboration with the Thoracic Surgery Service, Walter Reed General Hospital, a study has been established which is leading to the substitution of a low molecular weight dextran solution for whole blood to prime the pump-oxygenator in open-heart surgery.

BODY OF REPORT

Project No. 3A 0 12501 A 802 Title: COMBAT SURGERY

Task No. 02 Title: Blood & Expanders (Revision of field transfusion service)

Description: A continuing study to improve quality and safety of the Army's blood transfusion service.

Progress:

1. Kinetics of iron transport in dog duodenum have been investigated. Data are compatible with rate limiting reaction proximal to transfer of iron to plasma.

The relationship of iron absorption to reducing agents has been elucidated - reducing agents increase iron absorption beyond their intraluminal action. Compounds known to increase iron absorption have been hypothesized to influence iron absorption through the generation of DPNH.

Pilot experiments failed to find an iron-absorption-regulating factor in iron-loaded rat plasma.

2. Preliminary characterization, antigenicity and toxicity studies have been conducted with a keratin hydrolysate obtained from chicken feathers. Paper chromatography, paper and moving boundary electrophoresis and ultra centrifugation indicated that the material consisted of at least 22 peptide and amino acid fragments, with the sulfur probably all in the form of 8 to 10 cysteic acid peptides and the basic groups mainly in one large peptide fragment. Approximately 70 per cent of the material had a molecular weight in the 70,000 range. The hydrolysate was not antigenic as indicated by precipitin antibody and parrine cutaneous anaphylaxsis tests. Acute in vivo toxicity was investigated in dogs by sterile intravenous infusions of 1.5 to 5 per cent saline solutions. Consistent findings in all animals tested were a transient thrombocytopenia and leukopenia, hemoconcentration, oliguria, and postinfusion lethargy lasting for several days. Vital signs showed only slight alterations during testing and numerous chemical studies were unremarkable. Bleeding times remained normal. There were no deaths. Several animals developed cyanosis due to methemoglobinemia. All of the solutions tested contained oxidizing agents other than peroxides. It was possible to remove these by repeated washings with a dilute acidic solution; however, the hematologic effects remained unaltered. Autopsy and histopathologic findings in sacrificed animals were unrevealing.

Twenty intravenous infusions of standard dextran (MW 75,000) and/or low molecular weight dextran (MW 40,000) produced a transient marked deficiency of platelet factor 3 activity in 11 of 12 normal subjects tested. Three intravenous infusions of 5 per cent albumin solutions had no effect on platelet function. The decrease in factor 3 activity was related to both the concentration and molecular weight of the infused dextran. The platelet defect was correctable by sonic disruption indicating that the platelets were not intrinsically deficient in factor 3, but that dextran prevented its release. This was confirmed by in vitro incubation studies. In 5 of 7 subjects who developed prolongation of the bleeding time or positive tourniquet tests, there was a direct relationship to the lowest platelet factor 3 activity. There was no direct relationship of factor 3 deficiency to hemodilution, total platelet counts or platelet adhesiveness. Further studies to determine the clinical significance of these findings are in progress.

Vacuum pressure and sterility studies have been performed on a small number of units of 6 per cent standard dextran solutions manufactured prior to 1954 and obtained from Army stock piles. All solutions had a moderate amount of particles and loss of vacuum pressure. The rubber stoppers flaked when penetrated. Sterility studies of the dextran solutions, fluid path of the recipient set, and airway needles were negative.

The effect of PVP and a new Japanese plasma volume expander, Alginon, prepared from seaweed on platelets has been studied in vitro. Incubation of these solutions with normal platelets produced a marked deficiency in platelet factor 3 activity. The defect was corrected by sonic disruption of the platelets. Clinical testing of Alginon is under consideration.

3. Intravascular aggregation of red cells occurs during normothermic perfusions with open-heart pump oxygenators and is increased by hypothermia. Recent reports have indicated that infusions of low molecular weight dextran (LMWD) could prevent or minimize intravascular aggregation of red cells, postoperative anemia, and microscopic infarcts of vital organs. LMWD (average molecular weight of 41,000) is extremely effective as a "flow improver" and increases perfusions of vital organs by decreasing the viscosity and increasing the fluidity of blood. At the present time LMWD is being utilized as a priming solution in pump oxygenators at the Bethesda Naval Medical Center and ten University Hospitals. Recently LMWD has been utilized as a partial priming solution (3 gm./Kg. body wt.) in the pump oxygenators at Walter Reed General Hospital. Whole blood requirements for open-heart surgery procedures have been significantly reduced especially in adult patients. An extensive study of the hematologic effects of dextran-heparinized blood as compared to total heparinized blood as a priming solution are underway. Particular emphasis is being directed at platelet and red blood cell destruction, heparin and fibrinogen levels, fibrinolysis, and protamine neutralization of heparin.

Summary and Conclusions:

1. Control of absorption of iron by the intestine appears to reside in the ability of the epithelial cell to accept or transport iron.

2. Hydrolyzed keratin has proved not to be tolerated as a plasma volume expander.

3. A solution of small molecule dextran proves an adequate substitute for whole blood for priming the pump oxygenator in open-heart surgery.

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2. Crosby, W. H.: Blood Transfusion. Traumatic Medicine and Surgery for the Attorney, Vol. 9. Butterworths, London. (In press).

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7. Crosby, W. H.: The misuse of blood transfusion. Rakt Dann 1: 45, 1962.

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9. Pollack, S., Balcerzak, S. P. and Crosby, W. H.: Transferrin and the absorption of iron. Blood 21: 33, 1963.

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12. Barry, K. G., Hunter, R. H., Davis, T. E. and Crosby, W. H.: Acute uric acid nephropathy. Treatment with mannitol diuresis and peritoneal dialysis. Arch. Int. Med. 111: 452, 1963.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803, Military Internal Medicine

Task 01, Internal Medicine (Instrument Development for Cardiac Research)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Cardiorespiratory Diseases
Division of Medicine

Period Covered by Report: 1 July 1962 to 30 June 1963

Principal Investigator: Merlin Davis*

Assistants: Donald E. Gregg, Ph.D., M.D.
Edward M. Khouri

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

* National Bureau of Standards

ABSTRACT

Project 3A 0 12501 A 803, Military Internal Medicine

Task 01, Internal Medicine (Instrument Development for Cardiac Research)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 to 30 June 1963

Authors: Merlin Davis (National Bureau of Standards)
Donald E. Gregg, Ph.D., M.D., Edward M. Khouri

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Tests continue on the adequacy of the 5 micromanometers supplied by the National Bureau of Standards.

BODY OF REPORT

Project 3A 0 12501 A 803, Military Internal Medicine

Task 01, Internal Medicine (Instrument Development for Cardiac Research)

Description:

Tests continue on the adequacy of the 5 micromanometers supplied by the National Bureau of Standards.

Summary:

Final design of a new micromanometer with improved characteristics is being evaluated.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803 MILITARY INTERNAL MEDICINE

Task 01, Internal Medicine (Functions and disorders of the spleen)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Hematology
Division of Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Colonel William H. Crosby, MC

Assistants: Dr. Ferdinand Ruiz *
Dr. Romano Airo **
Dr. Nicolas Arias ***

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

* USPHS Postdoctoral Fellow

** Research Associate, The George Washington University

*** Fellow in Hematology, Lilly International Fellowship

ABSTRACT

Project No. 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 01 Title: Internal Medicine (Functions and disorders of the spleen)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Colonel William H. Crosby, MC
Dr. Ferdinand Ruiz
Dr. Romano Airo
Dr. Nicolas Arias

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. Raw bovine spleen fed to rats caused a drop in platelets and leukocytes.

2. The postsplenectomy anemia which develops in dogs is evidently not related to the reservoir function of the spleen.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 01 Title: Internal Medicine (Functions and disorders of the spleen)

Description:

The relation of the spleen to the concentration of blood cells has been investigated.

Progress:

1. After splenectomy, rats develop lasting thrombocytosis and leukocytosis of moderate degree. When such animals were placed on a diet of raw spleen (beef or dog) the platelet and white cell counts were promptly depressed and remained so until the diet was changed back to regular laboratory chow. This pattern was repeated several times. Raw kidney, liver or stomach had no such effect, nor did cooked spleen. One group of animals was maintained on raw spleen for 2 months without overt signs of toxicity and without developing anemia, except for 10 per cent which showed signs of mild iron deficiency. There was, however, no escape of the leukocyte or platelet counts. They remained depressed.

Control animals, with spleens intact, also developed leukopenia and thrombocytopenia on the diet of raw spleen.

2. The red cell volume of the dog varies between sleeping and waking. Asleep as much as a third of the red cells may be sequestered in the spleen, yet the bone marrow does not react to this "anemia." Removal of the spleen results in a gradual onset of anemia due to insufficient production of red cells. The red cell mass becomes as small as that of the sleeping dog. However, the anemia does not appear to be related to the reservoir function of the spleen. Dogs' spleens in situ were tightly wrapped in nylon mesh to prevent swelling of the organ and sequestration of red cells. In four months the animals did not develop anemia. After splenectomy the anemia appears as usual.

Summary and Conclusions:

Bovine spleen evidently contains a factor which inhibits production of platelets and leukocytes in rats. Dog spleen may be the source of some factor which is necessary to maintain a normal red cell mass.

List of Publications:

1. Crosby, W. H.: Editorial. Is hypersplenism a dead issue? Blood 20: 94, 1962.

2. Crosby, W. H. and Ruiz, F.: Evidence of a myeloinhibitory factor in the spleen. Blood 20: 793, 1962.

3. Crosby, W. H.: Hypersplenism. Ann. Rev. Med. 13: 127, 1962

ANNUAL PROGRESS REPORT

Project 3A O 12501 A 803 MILITARY INTERNAL MEDICINE

Task No. 01, Internal Medicine (The bone marrow's function and its reaction to injury)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Hematology
Division of Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Colonel William H. Crosby, MC

Assistant: Dr. Johannes Blom *

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 803 — Title: MILITARY INTERNAL MEDICINE

Task No. 01 — Title: Internal Medicine (The bone marrow's function and its reaction to injury)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Colonel William H. Crosby, MC
Dr. Johannes Blom

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

The destruction of marrow by x-irradiation has been studied in 22 patients by serial sampling of the marrow cavity that was exposed to therapeutic irradiation. In 14 patients who were not studied during the course of irradiation, the marrow was studied after a varying but minimal interval of 3 months. Similar studies are in progress in dogs.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 01 Title: Internal Medicine
(The bone marrow's function and its reaction to injury)

Description:

An investigation of the rate and pattern of recovery of bone marrow after irradiation in humans and dogs.

Progress:

In 22 patients the destruction phase and the recovery phase of the marrow after therapeutic irradiation could be studied. In these patients and in 14 patients who were studied only on the recovery plan or thereafter it was sometimes difficult or impossible to obtain marrow. Marked hypoplasia was found even as long as one or more years after the course of irradiation.

Presently dogs are receiving irradiation to one side of the thorax. Marrow obtained from an irradiated site will be injected into the marrow cavity of the irradiated ribs 3 months after completion of the course of irradiation and then serial resections of these ribs will be done to observe the effect of the injected marrow.

Summary and Conclusions:

The destruction of marrow by x-irradiation is studied by serial sampling of the sternal cavity in patients receiving therapeutic irradiation of the mediastinum. After 2,000 r. - 3,000 r. there is usually complete or partial recovery. After larger doses, usually complete hypoplasia. The effect of autologous marrow injected into the irradiated marrow cavity is presently under study in dogs.

List of Publications:

None.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803 MILITARY INTERNAL MEDICINE

Task 01, Internal Medicine (Blood and blood disorders)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Hematology
Division of Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Colonel William H. Crosby, MC

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Mr. James Eichelberger, Jr.
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Reports Control Symbol: MEDDH-288

Security Classifications: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 803 — Title: MILITARY INTERNAL MEDICINE

Task No. 01 — Title: Internal Medicine (Blood and blood disorders)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Colonel William H. Crosby, MC
Captain Harvey J. Weiss, MC
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Captain Richard Kaufman, MC
Mr. James Eichelberger, Jr.
Ruth Brennan
Elizabeth Houchin

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. The bleeding disease in epidemic hemorrhagic fever in Thailand was probably a result of thrombocytopenia.

2. There is evidence that the megakaryocytes which are present normally in the lung originate in the bone marrow.

3. Studies of abnormality of platelet factor 3 activity reveal that it may occur as an acquired or an hereditary fault. In some of the hereditary cases the lesion can be corrected temporarily by corticosteroids.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 01 Title: Internal Medicine (Blood and blood disorders)

Description:

This task encompasses the study of the functions and disorders of the blood plasma factors and formed elements.

Progress:

1. In the autumn of 1962 Captain Harvey Weiss went to Bangkok to study the hemorrhagic disease occurring in children with epidemic hemorrhagic fever. In 27 children with hemorrhagic fever, a modest amount of purpura was seen in about one-third. This was probably due to thrombocytopenia. The bone marrow was similar to that seen in ITP. Abnormal prothrombin activity, probably related to liver disease, was found in the 5 patients with evidence of central nervous system involvement. Prolongation of the silicone clotting time and abnormalities in the thromboplastin generation test are, to a certain extent, unexplained.

2. A modified Blalock procedure was performed on dogs so that the left lung received only arterial blood from the left heart and the right lung receives all the venous return from the right heart. Dogs were killed one, two and four weeks postoperatively and lung sections obtained. All shunts were patent at autopsy. (Dogs with heart worms were discarded.)

Morphologically, neither lung is normal; an interstitial infiltrate is observed which appears equally severe, apparently, in either lung. But there is a marked preponderance of megakaryocytes in the venous-perfused lung - from 50-80 times that seen in the arterial-perfused lung - where megakaryocytes are virtually absent. This suggests that megakaryocytes from the bone marrow are carried in the venous circulation to the lung. This may be the way platelets are delivered to the blood.

3. Twelve patients with undiagnosed bleeding disorders were found to have a platelet factor 3 deficiency using a method previously reported. These twelve cases bring to a total of twenty the number of patients that have been diagnosed as idiopathic thrombocytopathia.

Nine patients with factor 3 deficiency were treated with corticosteroids. In every case the factor 3 became normal in 5 to 14 days. Three of the patients underwent surgery with a minimal amount of bleeding.

A family with factor 3 deficiency is currently being studied. Six members in four generations have been found to be abnormal. Four of these six have histories of bleeding. Two have had no abnormal bleeding. The abnormal cases are all females. The data of this family and two others suggest that idiopathic thrombocytopathia is inherited as a Mendelian dominant.

Summary and Conclusions:

1. The bleeding disease in epidemic hemorrhagic fever in Thailand was probably a result of thrombocytopenia.

2. There is evidence that the megakaryocytes which are present normally in the lung originate in the bone marrow.

3. Studies of abnormality of platelet factor 3 activity reveal that it may occur as an acquired or an hereditary fault. In some of the hereditary cases the lesion can be corrected themporarily by corticosteroids.

List of Publications:

1. Weiss, H. J. and Eichelberger, J. W.: The detection of platelet defects in patients with mild bleeding disorders. Amer. J. Med. 32: 872, 1962.

2. Weiss, H. J. and Eichelberger, J. W.: Secondary thrombocytopathia. Platelet factor 3 in various disease states. (Submitted to Arch. Int. Med.)

3. Weiss, H. J. with technical assistance of Eichelberger, J. W.: Further observations on thrombocytopathia. Family studies. The effect of corticosteroids. Evidence of the presence of an abnormal serum factor. (Submitted to Blood).

4. Wheby, M. S., Conrad, M. E., Hedberg, S. E. and Crosby, W. H.: The role of bile in the control of iron absorption. Gastroenterology 42: 319, 1962.

5. Weiss, H. J.: Hereditary elliptocytosis with hemolytic anemia. Report of six cases, including hemolytic disease of the newborn, megaloblastic anemia of pregnancy and a family study. Amer. J. Med. (in press).

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803 — MILITARY INTERNAL MEDICINE

Task 01, Internal Medicine — (Effects of physical agents on skin and its permeability)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Dermatology
Division of Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: D. Joseph Demis, Major, MC
James G. Zimmer, Captain, MC

Assistant: Walter G. Larsen, Captain, MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task 01, Title: Internal Medicine (Effects of physical agents on skin and its permeability)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: D. Joseph Demis, Major, MC
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Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

Therapeutic evaluation of the antimetabolite 6-thioguanine was continued in various systemic and cutaneous diseases, with favorable results and little or no evidence of toxicity.

A project in Panama on the effect of a tropical environment on the normal skin flora and on cutaneous infection was carried out jointly by this department and a university contract group.

A study on the metabolic effect of grisiofulvin and candicidin on dermatopathic fungi revealed that candicidin, a topical antibiotic, had a profound inhibitory effect on respiration, griseofulvin, an orally effective antibiotic, had none.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: INTERNAL MEDICINE

Task No. 01 Title: Effects of physical agents on skin and its permeability

Description:

These studies are directed toward evaluating the action and effect of newer agents on cutaneous diseases and establishing the nature and incidence of cutaneous infections in unacclimatized troops in the tropics.

Progress:

The cautious evaluation of the antimetabolite 6-thioguanine in various systemic and cutaneous diseases was continued. The diseases considered most likely to respond to this treatment include those with an established or possible immunologic pathogenetic mechanism and of course those diseases manifested by proliferation of the reticulo-endothelial system. Small numbers of patients were studied and the most favorable responses were seen in those with collagen-vascular diseases, including systemic lupus erythematosus and scleroderma, and with plasmacytosis, psoriasis, atopic dermatitis, and Waldenstrom's hyperglobulinemic purpura. Two patients with the latter condition, the most recently studied, had excellent clinical remissions during therapy with disappearance of purpura, edema, and arthralgia. When therapy was stopped the symptoms returned. Despite the clinical remission during therapy, gamma globulin concentration, bentonite flocculation titre, and platelet factor III remained abnormal.

The department sponsored and assisted a civilian research team from the Department of Dermatology, University of Miami School of Medicine, in Project Swampfox II. The purpose of the study was an evaluation of skin disease under field conditions in a group of soldiers brought from a temperate to a tropical environment. The men were testing Transportation Corps vehicles in the jungles of the Republic of Panama. Initial baseline studies, including cultures, skin pH measurements, and ultraviolet fluorescence examinations, were obtained at Fort Eustis, Virginia, and compared with measurements made later in Panama. Preliminary findings indicate a significant change in the flora of the skin occurred shortly after arrival in the tropics.

A comparison was made between the effects of Griseofulvin, an orally effective fungistatic antibiotic, and those of Candicidin, a topically applied polythene antibiotic, on the respiration of Trichophyton mentagrophytes, and the respiration and anaerobic glycolysis of Saccharomyces cerevisiae. Griseofulvin had no effect on the respiration of the dermato-

phyte or on the respiration or anaerobic glycolysis of the yeast in experiments up to 6 hours. In contrast, Candicidin inhibited the respiration of T. mentagrophytes within 1-2 hours and had a very marked inhibitory effect on respiration and anaerobic glycolysis in S. cerevisiae within 20-30 minutes. This inhibition in yeast could be reversed by the addition of potassium or ammonium ions.

Summary and Conclusions:

The antimetabolite 6-thioguanine was found to be of value in the treatment of systemic lupus erythematosus, scleroderma, plasmacytosis, psoriasis, atopic dermatitis, and Waldenstrom's hyperglobulinemic purpura, and significant toxic effects were not noted with the range and duration of dosage used.

An evaluation was completed of changes in skin flora and the incidence and nature of skin infections in troops entering the tropics.

The respiration of dermatopathic fungi was found to be inhibited by Candicidin while Griseofulvin was found to have no effect.

List of Publications:

1. Demis, D. J., Eisen, B. W., Howie, M.D., Crosby, W.H. and Brown, C.S.: The use of 6-thioguanine in dermatology. A preliminary report. J.A.M.A. 180: 583, 1962. (Abstract).

2. Weiss, M.J., Demis, D.J., Elgart, M.L., Brown, C.S. and Crosby, W.H.: Treatment of two cases of hyperglobulinemic purpura with thioguanine. N. Eng. J. Med. 268: 753, 1963.

3. Demis, D. J., Brown, C.S. and Crosby, W.H.: Thioguanine in the treatment of certain autoimmune, immunologic and related diseases. N. Eng. J. Med. (In press).

4. Larsen, W. G. and Demis, D. J.: Metabolic studies of the effect of griseofulvin and candicidin on fungi. J. Invest. Derm. (In press).

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803 MILITARY INTERNAL MEDICINE

Task 01, Internal Medicine (Capillary and lymphatic circulation of the skin)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Dermatology
Division of Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: D. Joseph Demis, Major, MC
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 01 Title: Internal Medicine (Capillary and lymphatic circulation of the skin)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: D. Joseph Demis, Major, MC
James G. Zimmer, Captain, MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

A study of the pharmacologic effect of various vasoactive compounds on cutaneous blood vessels, using capillary microscopy and the newly adapted technic of television cinephotomicrography, has clarified certain aspects of the local action of epinephrine, norepinephrine, angiotensin, serotonin, and cholinergics. Cutaneous capillary counts and morphology were compared in a number of systemic and cutaneous diseases with the conclusion that while definite morphologic changes occur locally in many skin lesions, the systemic diseases studied do not significantly alter capillary counts or change the morphology diagnostically. A distinctive response of the skin vasculature was observed during the healing of manyy experimental and spontaneous lesions, and suggests that a capillary-attracting factor is produced in injured tissue.

Studies of histamine metabolism in patients with mastocytosis (mast cell tumors) were continued, and the effect of alpha-methyl dopa on symptomatology was evaluated with the conclusion that its lack of effect is due to its failure to inhibit the specific histidine decarboxylase of mast cells. The significance of heparin, presumably produced by the mast cells, was evaluated both clinically and biochemically in mastocytosis, with the conclusion that though it is present in human mast cells it is rarely of clinical significance in this disease, and was not excreted in measurable amounts by the mastocytosis patients.

BODY OF REPORT

Project No. 3A 0 12501 A 803 **Title: INTERNAL MEDICINE**

Task No. 01 **Title: Capillary and lymphatic circulation of the skin**

Description:

These studies of the physiology, pharmacology, and pathology of the cutaneous microcirculation have been undertaken for the general purpose of elucidating normal and abnormal vascular processes as related to the skin, and with the objective of evaluation of the clinical and research applicability of the techniques used. The research on mastocytosis is ultimately directed at the elucidation of the normal and abnormal functions of mast cells, particularly in connection with histamine and heparin, and their relationship to allergies and urticarias in general.

Progress:

A successful method for obtaining a motion picture record of blood flow in the superficial vessels in the skin of human subjects was developed. A monitored television camera circuit is used with simultaneous visual and sound tape recording which can later be transferred to standard movie film. Magnifications up to several thousand power can be achieved with reflected, low intensity, cool lighting. Individual red blood cells flowing in a single capillary loop can be distinguished and measurements of capillary diameter and blood flow rates are easily made. The technique is applicable to the study of the physiology and pharmacology of the cutaneous microcirculation.

Visual observation, capillary microscopy, and the newly adapted method of television cinephotomicrography have been applied to the study of some aspects of the physiology and pharmacology of the human cutaneous microcirculation. Certain vascular phenomena have been elucidated and the following conclusions can be drawn:

1. Vasomotion, resulting from precapillary constriction and dilatation with secondary changes in capillary blood flow, occurs in some, but apparently not all, human cutaneous capillaries.

2. There is no evidence of intrinsic contractility of the cutaneous capillaries, and all changes in caliber and blood flow observed in them are secondary to changes in pre- and post-capillary muscular vessels.

3. Epinephrine and norepinephrine act locally as venoconstrictors as well as arterial constrictors in human skin, with epinephrine apparently having the more potent effect.

4. Angiotensin II, known to be a potent precapillary constrictor, has little or no effect on cutaneous veins, and causes a less profound blanch than do the catecholamines.

5. Mecholyl and related cholinergic agents are potent vasodilators, increasing flow rates in cutaneous capillaries and abolishing vasomotion.

6. Serotonin, a known venoconstrictor, causes an unusual cyanotic response in human skin. This apparently results from constriction of larger cutaneous veins with resulting stasis in the subpapillary plexus. The cyanosis is enhanced by admixture with epinephrine or norepinephrine as a result of the synergistic effects of these agents.

An evaluation of forearm and nailfold capillary counts and morphology was undertaken using capillary microscopy. Normal individuals were compared with patients having a variety of diseases, including arteriosclerosis, peripheral arterial insufficiency, acute and chronic glomerulonephritis, hypertension, rheumatoid arthritis, idiopathic thrombocytopenic purpura, systemic lupus erythematosus, psoriasis, ichthyosis, gout and hyperuricemia, hyperlipemia, Reiter's syndrome, and several purpuric conditions. No significant variation from normal count or consistent morphological abnormality was noted in any of the conditions studied. However, capillary microscopy does reveal definite, though not always pathognomonic, changes in many cutaneous lesions, such as psoriasis, discoid lupus erythematosus, necrobiosis lipoidica, dermatomyositis, Raynaud's disease, secondary lues, etc.

The morphological changes in the microcirculation of human skin during healing of burns and other lesions were studied with capillaroscopy. In all the experimental and accidental burns, abrasions, and lacerations studied, in addition to the anticipated revascularization by the deeper dermal vessels, a distinctive pattern of response by the surrounding papillary capillaries was consistently observed. This change appears to be a general phenomenon in the healing of any lesion of human skin. The characteristic orientation of these vessels toward the injured area suggests that a potent capillary attracting factor may be produced within traumatized tissue.

The metabolism of histamine by patients with cutaneous mastocytosis (abnormal proliferation of tissue mast cells) has been under continued study. Homogenates of skin lesions were found to contain an active histidine decarboxylase; no evidence for the decarboxylation of 5-hydroxytryptophan or dihydroxyphenylalamine was obtained. Increased urinary excretion of histamine by mastocytosis patients has been consistently observed. Chromatographic analysis and autoradiography of the urine of five patients with mastocytosis have permitted identification and estimation of increased quantities of the specific histamine metabolite 1,4-methyl imidazole acetic

acid. Studies with intradermally administered histamine have demonstrated plasma clearance and urinary excretion of histamine and the histamine metabolites, 1,4-methyl imidazole acetic acid and free imidazole acetic acid, to be comparable. The principle histamine metabolite was 1,4-methyl imidazole acetic acid. From these studies it has been concluded that the histaminuria of mastocytosis results from simple overproduction of histamine. In clinical trial of a known aromatic amino acid decarboxylase inhibitor, alpha-methyl dopa, the drug failed to decrease either histamine excretion of the symptoms associated with increased circulating histamine. The failure is attributable to the inability of alpha-methyl dopa to inhibit the conversion of histidine to histamine which in mastocytosis depends on a specific histidine decarboxylase.

A quantitative study was undertaken of mucopolysaccharide excretion in 15 patients with mastocytosis. Uronic acid determinations showed no increase in 24 hour mucopolysaccharide excretion when compared with normals studied in our laboratory and reported in the literature. Qualitative study using chromatography and electrophoresis revealed only normal urinary mucopolysaccharides with no evidence of heparin excretion. Analysis of one mastocytoma lesion by electrophoresis revealed heparin but no other mucopolysaccharides. While mast cells are known to contain heparin, it is concluded from these studies, clinical evaluation of 35 patients, and a review of the literature that heparin only rarely plays an important part in mastocytosis and that there is as yet no conclusive evidence that mast cells produce other mucopolysaccharides.

Summary and Conclusions:

A new technic, television cinephotomicrography, was adapted and found valuable in the study of the physiology and pharmacology of the human cutaneous microcirculation. This and the standard techniques of capillary microscopy and gross observation were utilized in the study of vasoactive compounds with resulting elucidation of various phenomena including the local venoconstrictor actions of the catecholamines and serotonin. A study of cutaneous capillary counts and morphology revealed no consistent generalized variations in systemic disease, but definite, though not always specific, abnormalities occur in skin lesions. A distinctive response of the cutaneous vasculature in healing burns and other lesions was observed which suggests the production of a capillary attracting factor by injured tissue.

Increased excretion of histamine and its metabolites was consistently observed in mastocytosis patients. Those patients with the histamine induced mastocytosis syndrome did not respond to a non-specific decarboxylase inhibitor, suggesting that histamine is formed by a specific histidine decarboxylase in human mastocytosis. These patients rarely show evidence of increased circulating heparin, and in no case was a measurable

amount of heparin excreted in their urine. Since Mastocytoma tissue contains heparin, these findings indicate that heparin is not necessarily released with histamine from human mast cells.

List of Publications:

1. Demis, D.J., Zimmer, J.G., Verhonick, P.J. and Catalano, P.M.: The pharmacology of human skin. I. Epinephrine and norepinephrine; catecholamine-seretonin combinations. J. Invest. Derm. 39: 419, 1962.(Abstract).

2. Zimmer, J.G. and Demis, D.J.: Studies on the microcirculation of the skin in disease. J. Invest. Derm. 39: 501, 1962. J.A.M.A. 180: 583, 1962. (Abstract).

3. Demis, D.J. and Zimmer, J.G.: Carcinoid flush. Lancet 2: 360, 1962.

4. Zimmer, J.G. and Demis, D.J.: Burns and other skin lesions: microcirculatory responses in man during healing. Science (In press).

5. Zimmer, J.G., Curtis, R.W., Grubby, N.C., and Demis, D.J.: Television cinephotomicrography in the study of the human cutaneous microcirculation. Angiology (In press).

6. Zimmer, J.G. and Demis, D.J.: The study of the physiology and pharmacology of the human cutaneous microcirculation using capillary microscopy and television cinematography. Anat. Rec. 145: 376, 1963. (Abstract).

7. Demis, D.J.: The mastocytosis syndrome: clinical and biochemical studies. Ann. Int. Med. 56: 684, 1962. (Abstract).

8. Demis, D.J.: Urinary excretion of histamine and 1,4-methyl-imidazole acetic acid by patients with mastocytosis. Excerpta Medica Int. Cong. Ser. 48: 695, 1962. (Abstract).

9. Demis, D.J.: Abnormalities of histamine-metabolism in mastocytosis with particular reference to increased urinary excretion of 1,4-methyl imidazole acetic acid. Excerpta Medica Int. Cong. Ser. 52: 284, 1962. (Abstract).

10. Demis, D.J. and Zimmer, J.G.: Histaminuria in mastocytosis; the effect of alpha-methyl dopa on urinary excretion of free histamine. Arch. Int. Med. 111: 309, 1963.

11. Zimmer, J.G., McAllister, B.M. and Demis, D.J.: Mucopolysaccharides in mast cells and mastocytosis. J. Invest. Derm. (In press).

12. Demis, D.J.: The mastocytosis syndrome: clinical and biochemical studies. Ann. Int. Med. (In press, Aug. 1964).

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803, Military Internal Medicine

Task 01, Internal Medicine (Experimental Arterial and Heart Disease)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Cardiorespiratory Diseases
Division of Medicine

Period Covered by Report: 1 July 1962 to 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 803, Military Internal Medicine

Task No. 01, Internal Medicine (Experimental Arterial and Heart Disease)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 to 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

The control of regional blood flow and metabolism has been studied in the unanesthetized resting and/or active dog by means of chronically implanted flowmeters and special tubes in the aorta and coronary sinus for blood pressure and blood sampling. During reactive hyperemia, the oxygen debt of the left myocardium is overpaid although not to the extent that the flow debt is overpaid. Mild to moderate exercise does not greatly alter the cardiac output, left coronary flow and coronary oxygen usage per heart beat, but in strenuous exercise the coronary flow and oxygen usage per heart beat increase greatly, and the stroke cardiac output moderately. During excitement and stellate ganglion stimulation, left coronary flow and oxygen usage per heart beat rise greatly, and the stroke cardiac output moderately. No evidence has been found for reflex coronary vasoconstriction. Rapid and large blood transfusions cause only slight increases in blood pressure, heart rate and cardiac output. Preliminary experiments on hypovolemic shock in chronic dogs indicate the value of a compensated blood pH in improving myocardial performance. Studies have been started to test the accuracy of methods used to estimate the coronary flow in human subjects.

BODY OF REPORT

Project 3A 0 12501 A 803, Military Internal Medicine

Task 01, Internal Medicine (Experimental Arterial and Heart 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 every day life.

Progress:

As a prerequisite to such a study, it was necessary to develop a standardized animal preparation. This included development of miniature electromagnetic flowmeters for chronic implantation on the aorta and regional arteries, and of techniques for chronic implantation of special tubes in the aorta and coronary sinus so as to permit continuous blood pressure recording and blood sampling for hemodynamic and metabolic studies in the unanesthetized state. This preparation has been rather successful, allowing studies up to 8 weeks postoperative. It has been used in all the chronic studies reported herein.

1. Development of instruments and methods for cardiovascular research.

a. A considerable number of miniature electromagnetic flowmeters have been made and are now available for chronic implantation. Miniature electromagnetic flowmeters of the cannulating type have also been developed and found very useful for quantitating regional blood flow in acute experiments.

b. The electromagnetic flowmeter although a dependable instrument, still does not allow one to obtain a reference zero electronically. To overcome this difficulty, a pneumatic occlusive cuff has been designed and tested which makes it possible to obtain zero flow under all conditions.

c. A modified technique for implantation of an indwelling plastic tube in the coronary sinus makes it possible to obtain blood samples repeatedly for periods of up to 8 weeks.

d. A method has been developed for the quantitative isolation and estimation of adenosine, inosine, and hypoxanthine from blood and plasma.

e. Efforts to estimate the in vivo intracellular pH have been delayed because of synthesis difficulties encountered by the only commercial supplier of dimethyl oxazolidinedione.

f. Evaluation of the carbon monoxide-Clark electrode method for measuring the oxygen content of whole blood has been completed. The reproducibility of the procedure was outside an acceptable range.

g. The pricing schedule follow-through to an engineering survey and proposal submitted by Emertron for the construction of an instant tissue-fixing apparatus employing high frequency electromagnetic radiation never materialized. The company ignored our communication in this matter.

6. Effects of rapid blood transfusions on cardiodynamics. Transfusions into the dog of one-fourth to one-third its calculated blood volume in 20-30 minutes have been made in resting chronic dogs. Preliminary experiments indicate rapid adjustment, for there occurs only a slight increase in mean blood pressure and heart rate, and no change in cardiac stroke volume.

7. Cardiac metabolism and dynamics in canine hemorrhagic shock. Dogs 2 weeks postoperative, with a chronically indwelling aortic electromagnetic flowmeter and plastic cannula in the pulmonary artery, aorta, and coronary sinus were studied daily for postoperative recovery trends. Measurements of cardiac output, blood gases, pH, and total body oxygen consumption were recorded. After stabilization was reached (usually in 10 days), the above parameters were measured during a period of hemorrhagic shock. The effects of alterations in ventilating rhythm and gas mixtures are being evaluated to judge myocardial performance and to establish the value of a compensated blood pH during hypovolemic shock.

8. Evaluation of methods for measuring coronary blood flow in man. Methods have been developed in different laboratories for estimating coronary flow in human subjects. These are being used in many clinics to determine normal coronary flow and the extent of coronary insufficiency induced by coronary artery disease. These include the nitrous oxide technique and the method of isotope injection and monitoring over the precordium its rate of disappearance. Their accuracy has not been established. Because of the great practical importance of such observations, and because the different methods have given different results, comparisons are underway in the normal conscious dog of the coronary flow determined by these methods, with the coronary flow determined by a reference method using the chronically implanted electromagnetic flowmeter. Before such comparisons have meaning, it is necessary to know the distribution of flow among the coronary arteries in an acute preparation. Accordingly, preliminary experiments have been done in the open-chest dog using "bloody" or the cannulating type of electromagnetic flowmeter. These early experiments indicate that the relative flow in the left coronary artery, septal artery, and right coronary artery approximate 80, 10, and 15 per cent, respectively.

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 flowmeters and special tubes in the aorta and coronary sinus for blood pressure and blood sampling. During reactive hyperemia, the oxygen debt of the left myocardium is overpaid, although not to the extent that the flow debt is overpaid. Mild to moderate exercise does not greatly alter the cardiac output, left coronary flow and coronary oxygen usage per heart beat, but in strenuous exercise the coronary flow and oxygen usage per heart beat increase greatly, and the stroke cardiac output moderately. During excitement and stellate ganglion stimulation, left coronary flow and oxygen usage per heart beat rise greatly, and the stroke cardiac output moderately. No evidence has been found for reflex coronary vasoconstriction. Rapid and

large blood transfusions cause only slight increases in blood pressure, heart rate and cardiac output. Preliminary experiments on hypovolemic shock in chronic dogs indicate the value of a compensated blood pH in improving myocardial performance. Studies have been started to test the accuracy of methods used to estimate the coronary flow in human subjects.

List of Publications:

1. Gregg, Donald E., E. M. Khouri, C. R. Rayford, L. Granata and A. Huvos. The systolic component of coronary arterial inflow in the active unanesthetized dog. Proc. 22nd International Congress of Physiological Sciences, Leiden, Holland, Sept. 1962 (abstract).

2. Gregg, Donald E. Homeostasis of the arterial circulation. In "Man's Dependence on the Earthly Atmosphere" (ed. K. E. Schaeffer). The Macmillan Co., New York, 1962, p. 216.

3. Gregg, Donald E. Physiology of the coronary circulation. Ohio State Med. J. 58:1005, 1962.

4. Gregg, Donald E. The coronary collateral circulation in the etiology of myocardial infarction. In "The Etiology of Myocardial Infarction" (ed. T. N. James and J. W. Keyes). Little, Brown & Co., Boston, 1963, p. 361.

5. Gregg, Donald E. Factors normally affecting coronary blood flow. In "Coronary Heart Disease" (ed. Wm. Likoff and J. H. Moyer). Grune & Stratton, New York, 1963.

6. Khouri, E. M., and D. E. Gregg. Miniature electromagnetic flowmeter applicable to coronary arteries. J. Appl. Physiol. 18:224, 1963.

7. Gregg, Donald E. Physiology of the coronary circulation. George E. Brown Memorial Lecture. Circulation June 1963.

8. Gregg, Donald E., and Lloyd C. Fisher. Blood Supply to the Heart. In "Handbook of Physiology" Sec. 2, Vol. II, Ch. 46. American Physiological Society, Washington, D. C., 1963.

9. Rayford, C. R., A. Huvos, and D. E. Gregg. Chronic catheterization of the coronary sinus. Proc. Soc. Exp. Biol. Med. (in press).

10. Gregg, Donald E. Coronary flow measured by electromagnetic flowmeters. American Heart Association Symposium on the Coronary Circulation, Chicago, 1962. American Heart Journal (in press).

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ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803 MILITARY INTERNAL MEDICINE

Task 01 Internal Medicine (Gastrointestinal physiology)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D.C.

Department of Gastroenterology
Division of Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Lt Colonel Norman M. Scott,Jr., MC
Major Marcel E. Conrad, MC
Captain Lewis R. Weintraub, MC

Assistants: Mrs. Betty A. Merrill, DAC
Mr. Arthur L. Foy, DAC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project 3A O 12501 A 803 Title MILITARY INTERNAL MEDICINE

Task No. 01 Title Internal Medicine (Gastro-intestinal physiology)

Reporting installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D.C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Lt Colonel Norman M. Scott,Jr.,MC
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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 has 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.

Anemia, circulating plasma iron concentration, plasma transferin levels and size of the body iron store have been excluded as the primary stimulus to iron absorption. The role of the rate of plasma turnover of iron is being investigated.

Abnormal absorption of iron from the gut has been demonstrated in various hematologic disorders, cirrhosis of the liver, and starvation.

Iron is not absorbed from dietary materials in the same proportion as from an oral dose of iron salts. However, absorption of dietary iron increases when the animal becomes iron deficient.

Methods for chemical analysis of iron have been developed for determination of total iron in animal carcasses, various organs and excrement. Methods of quantifying non-heme iron, ferritin and hemosiderin have been improved.

A ferritin-containing inclusion body has been demonstrated in the intestinal epithelium of normal, iron-replete humans.

BODY OF REPORT

Project No. 3A O 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 01 Title: Internal Medicine (Gastro-intestinal physiology)

Description: Study of the absorptive process in normal and abnormal conditions of the small intestine.

Progress:

Iron deficient humans absorbed more of a test dose of radioiron$_{59}$ than normal, iron replete subjects. Further, iron$_{59}$ was detected in stools or iron deficient volunteers for only 3-6 days after the test dose whereas it was passed in the feces of normal humans for 6-15 days. The cause for this delay is thought to be due to sequestration of iron by intestinal epithelial cells until they were desquamated from the villus at the end of their lifespan. Radioautographs of duodenum and jejunum obtained from normal, iron-replete rats at intervals after an oral dose of iron$_{59}$ showed loss of labeled columnar epithelial cells which progressed from the base to the tips of the villi over a period of 40 hours. This approximates the turnover time of villus epithelium in the rat. Iron deficient and iron-loaded animals showed little radioiron within epithelial cells at any time. Thus, in iron deficiency, iron is not retained by villous cells because it passes into the body; in iron loaded animals, the mucosa accepts little iron from the intestinal lumen. We believe the factor controlling absorption by the epithelial cells was their own intrinsic iron content. Radioautographs of rat intestine after intravenous administration of radioiron$_{59}$ showed tagging of intestinal epithelial cells of normal and iron-loaded animals. This may provide the mechanism for limiting absorption of iron from the gut lumen and a means of ridding the body of excess iron.

The search continues to identify the factor controlling absorption of iron from the intestine. Iron deficiency was induced in a group of normal volunteers by phlebotomy. Increased absorption of iron persisted after hemoglobin, plasma iron concentration and the plasma iron binding capacity had returned to pre-phlebotomy levels.

Removal of two-thirds of the total liver iron of the rat was performed by partial hepatectomy. Following this procedure absorption of radioactive iron as measured by the small animal liquid scintillation counter was not increased. Removal of a similar quantity of iron by phlebotomy resulted in a significant increase in absorption of iron. Thus, under the present experimental conditions the size of the liver iron store does not seem to be the controlling factor of absorption of iron.

The role of bone marrow activity in regulating absorption of iron was investigated. It has been demonstrated in man and animals that there is a four to five day lag period following acute phlebotomy before iron absorption is increased. In animals, if re-transfusion of an equal amount of blood takes place within the first three days, the stimulus to iron absorption is suppressed. Investigation of iron kinetics in humans during this five day period was performed in an attempt to elucidate the primary stimulus to iron absorption.

We have noted a fall in the plasma iron on the fourth to fifth day following phlebotomy. At the same time there is an increase in the disappearance rate of radioactive iron from the plasma. The fall in plasma iron is transient, lasting one to five days, followed by a return to normal levels. However, at this time the plasma disappearance rate of iron and iron absorption are still increased. Thus, the clearance rate of iron from the plasma may be the factor influencing intestinal absorption in an attempt to maintain homeostasis. Further studies are in progress to estimate the amount of iron in the intestinal mucosa during the initial period following phlebotomy.

Patients with an increased rate of erythropoiesis absorb increased quantities of iron from the gut despite their state of iron repletion. It seems unimportant whether erythropoiesis is effective (hereditary spherocytosis, polycythemia) or ineffective (pyridoxine deficiency, thallesemia). Increased absorption of iron was observed in cirrhotics with an apparently normal rate of erythropoiesis but an increased turnover of iron. Further studies are necessary to delineate the relationship of plasma iron turnover and iron absorption.

To date, the majority of the experimental work in iron absorption has been performed with the use of iron salts as the test dose. However, iron in the normal diet is primarily in the form of porphyrins. Thus, it is important to determine the absorption of iron in the latter state. Experimental work has been performed using Fe^{59} hemoglobin solution as the test dose and rats as the experimental animal. It has been shown that iron in the porphyrin form is not as effectively absorbed as the ferrous salt in iron replete rats. Absorption of porphyrin iron is increased in the iron deficient rat but not to the same magnitude as the salt form. Ascorbic acid does not increase absorption of hemoglobin iron as contrasted to its marked effect in the ferrous salt. Further work in humans with the use of the human whole-body radioactivity detector is planned to confirm the above results.

It has also been shown that the ferrous and ferric form of iron salts are absorbed from the gut in similar quantities.

Chemical methods for quantification of iron in various organs, excrement and total carcasses of animals have been developed. Chemical methods of separating ferritin and hemosiderin in tissue specimens have been improved. These determinations are being used to investigate iron kinetics in animals in various states of depletion and repletion by permitting measurement of the proportion of tracer $iron_{59}$ and cold iron in organs and carcasses of experimental animals.

A ferritin-containing inclusion body has been demonstrated in the apical cytoplasm of normal human small intestinal epithelium.

Summary and Conclusions:

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 has 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.

Anemia, circulating plasma iron concentration, plasma transferin levels and size of the body iron store have been excluded as the primary stimulus to iron absorption. The role of the rate of plasma turnover of iron is being investigated.

Abnormal absorption of iron from the gut has been demonstrated in various hematologic disorders, cirrhosis of the liver, and starvation.

Iron is not absorbed from dietary materials in the same proportion as from an oral dose of iron salts. However, absorption of dietary iron increases when the animal becomes iron deficient.

Methods for chemical analysis of iron have been developed for determination of total iron in animal carcasses, various organs and excrement. Methods of quantifying non-heme iron, ferritin, and hemosiderin have been improved.

A ferritin-containing inclusion body has been demonstrated in the intestinal epithelium of normal, iron-replete humans.

List of Publications:

1. Conrad, M.E., & Crosby, W.H.: The natural history of iron deficiency induced by phlebotomy. Blood. 20: 173, 1962.

2. Conrad, M.E., Berman, A., & Crosby, W.H.: Iron kinetics in Laennec's cirrhosis. Gastroenterology. 43: 385. 1962.

3. Forrester, R.H., Conrad, M.E., & Crosby, W.H.: Measurement of total body $iron_{59}$ in animals using whole-body liquid scintillation detectors. Proc. Soc. Exper. Biol. & Med. 13: 115, 1962.

4. Conrad, M.E., van Hoek, R., & Crosby, W.H.: Observations on the natural history of iron deficiency and the gastrointestinal absorption of iron. Proc VIIIth Internat. Cong. of Internat. Soc. of Hematol. Tokyo, Pan Pacific Press, 1962, 1151.

5. Weintraub, L.R., Conrad, M.E., & Crosby, W.H.: Role of hepatic stores in iron absorption. Clin. Res. 11: 201, 1963 (Abstract).

6. Conrad, M.E., & Crosby, W.H.: Intestinal mechanisms controlling iron absorption. J.Clin. Invest., 1963 (Abstract).

7. Crosby, W.H., Conrad, M.E., & Wheby, W.H.: The rate of iron accumulation in iron storage disease. Blood. (In press).

8. Conrad, M.E., & Crosby, W.H.: Intestinal mucosal mechanisms controlling iron absorption. Blood. (In press).

9. Hartman, R.S., Conrad, M.E., Hartman, R.E., Joy, R.J.T., & Crosby, W.H.: Ferritin-containing bodies in human small intestinal epithelium. Blood. (In press).

10. Wheby, M.S., & Crosby, W.H.: The gastrointestinal tract and iron absorption. Blood. (In press).

FINAL REPORT

Project 3A 0 12501 A 803, Military Internal Medicine

Task 01, Internal Medicine (The Influence of Bed Bath Procedures on Skin Condition)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Nursing

Period Covered by Report: 15 February 1961 through 30 June 1963

Principal Investigator: Major Phyllis J. Verhonick, ANC

Assistants: Captain Lois A. Johns, ANC
Captain Eleanore F. Sullivan, ANC

Consultant: Major D. Joseph Demis, MC

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine (The Influence of Bed Bath Procedures on Skin Condition)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 15 February 1961 through 30 June 1963

Authors: Major Phyllis J. Verhonick, ANC, Captain Lois A. Johns, ANC, and Captain Eleanore F. Sullivan, ANC

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

An experiment concerning the effect of bed bathing techniques on the skin condition of patients during hospitalization has been completed by a team of nurse investigators. This research was designed to test the hypothesis that the use of a specific nonionic emulsifier in giving complete or partial bed baths does not make a significant difference in the skin condition of patients. A total of 62 patients were bathed for periods ranging from 14 to 21 consecutive days by nurse investigators. One-half of the body (control) was bathed with soap and water. The other half (experimental) was bathed with a specific nonionic emulsifier and water. A "blind approach" was used to make as objective an observation as possible by not disclosing experimental and control sides of body to observers. Keratin was stripped from the skin by placing cellulose tape on the lateral surfaces of the ankle and exposed to a densitometer for an objective registration of the amount of epithelium three times throughout the study. Medical judgements were made each week by a dermatologist who served as a consultant to the nursing research team. Gross examination of observations suggested that differences were apparent between the subjective ratings for degree of roughness of the experimental and control extremities. Comparisons of the densitometer readings made by the analysis of variance technic between the use of two specific methods for bathing, two methods of skin massage after bathing, and three various age groups did not show significant differences in the degree of scaliness or roughness. Replication of this study should be done by other nurse investigators.

SCOPE OF REPORT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine (The Influence of Bed Bath Procedures on Skin Condition.)

Description:

An experiment designed to investigate the influence of bed bathing procedures on the condition of the skin of selected patients has been completed by a team of nurse researchers. The research was designed to evaluate the effects of the following nursing measures on the skin: (1) conventional soap and water bathing; (2) back massage with alcohol and lotions following bathing; and (3) the use of wash cloths as compared to cellulose sponges for bathing. The hypothesis tested was that the use of a specific nonionic emulsifier in giving complete or partial baths does not make a significant difference in the skin condition of selected patients.

The patient's comfort is a primary nursing concern in the administration of care. Hospitalized patients may complain of dry, itchy skin, particularly on the elbows, knees and back where friction with bed linen occurs. The skin of elderly patients often shows overt signs of dryness after daily bathing during a period of hospitalization of 10 days or longer. It was the premise of the investigators that conventional bed bathing procedures with soap and water may contribute toward an increase in dryness of the skin.

Patients selected from orthopedic, neurosurgical and general medical and surgical wards at Walter Reed General Hospital were bathed for approximately 21 consecutive days by the nurse investigators. One-half of the body (control) was bathed with soap and water. The other half of the body (experimental) was bathed with a specific nonionic emulsifier and water. The portions of the body which were control and experimental were randomly designated by the investigator bathing the patient and this knowledge was not disclosed to other investigators or observers. A "blind approach" was used in making observations in that the observer was not aware of the experimental and control designations. One-half of the sample was bathed with wash cloths and the other half was bathed with cellulose sponges. The sample was again subdivided into thirds so that one-third received an alcohol rub to the back and lower extremities following bathing; one-third received a similar rub with massage lotion; and the remaining patients received no massage or applications following bathing.

A rating scale was devised to record observed estimates of the degree of roughness and scaliness of the skin. A translucent plastic slide was also used to scrape lightly the lateral surfaces of the patients' ankles. The amount of epithelium collected on the slide was judged by nurse observers to estimate the amount of scaliness which was in turn recorded on the rating scale.

On the first, tenth and last day of the study a one-time keratin stripping was done by placing a strip of cellulose tape on the anterior surface of the ankles of the patients. The tape was removed and mounted on a microscopic slide for examination and measurement of light passage through the thickness of the epithelium with a densitometer.

Progress:

Data were collected on a total of 62 patients throughout an entire year so that possible seasonal influences on the condition of the skin could be taken into consideration. Initially a pilot study was done to ascertain the optimum length of time daily bathing was necessary before changes in the condition of the skin were observed. Skin changes did not begin to appear until between the fourteenth and twenty-first days of bathing. During the pilot phase the data collection forms were devised as well as the method and frequency of observations decided upon. An illustration of the data-collecting form is shown in Figure 1, as follows:

Figure 1. Data-collecting Form for Observations of Skin Condition

OBSERVATION SCALE - BATH STUDY

Date _____ Observer _____

Patient		Rough	Scale	Remarks
	L			
	R			
	L			
	R			
	L			

Code: ROUGH
0 - negative
1 - slight (smooth)
3 - moderate (fine emery)
5 - marked
7 - severe (coarse emery)

Code: SCALES
approx. no. scraped on slide
0 - none to 10
1 - 10 to 50
3 - 50 to 100
5 - 100 or more

Code: Subjective remarks:
ITCHING
0 - no complaint
1-occas. TID-QID
3 - Q4H - Q2h
5 - Constantly

Remarks: Note changes in tone, color, condition

To eliminate the possibility of bias, the sample of patients was divided into three age groups for study; one-sixth were 35 to 40 years of age; two-thirds were 41 to 69 years of age; and one-sixth were 70 years of age or older. Also the patients in the sample were divided almost equally between male and female. Daily observations were made and recorded by the investigator bathing the patient. Once each week throughout the study independent observations were made by other members of the Department of Nursing to examine the skin for amount of dryness and scaling. The dermatology consultant examined the condition of the patient's skin each week. Keratin strippings were done on the first, tenth, and final day of the study.

Gross examination of observations for roughness and scaliness made by nurses and the physician suggested differences existed between the experimental and control extremities. These subjective data were analyzed by the t-test and significant differences at the .01 level were shown for the second and third week's observation for the physician and nurse respectively. One of the problems encountered in this type of research in clinical nursing is the lack of objective instruments for measuring observations. Therefore, conclusive statements concerning differences were not made on these subjective data.

The densitometer registrations, still a relatively crude measure, were compared by use of the analysis of variance technic. Comparisons were made between (1) the use of a wash cloth and a sponge; (2) between methods of after-care, alcohol rub, dermassage, and no after care, and (3) between the two extreme age groups. The following tables show the results of the analysis of variance tests:

Table I. Analysis of Variance Between Methods of Bathing: Wash cloths and Cellulose Sponge

VS	DF	SS	MS	F	
Between Methods	1	.00129	.00129	1.05	NS
Between Rx* within Method	2	.00434	.00217	1.76	NS
Between Exper. vs Cont.	1	-.00177	-.00177	-0.29	NS
Interaction between Methods and Rx	1	.00611	.00611		
Between Individuals Within Methods	53	.08600	.00162	1.32	NS
Residual (error term)	53	.06506	.00123		
N=110 Total	109	.16103			

*Rx - Experimental and Control

Table II. Analysis of Variance Between Care After Bathing.

VS	DF	SS	MS	F	
Between after Care	2	.00010	.00005	0.2	NS
Between Rx* Within After care	3	.00458	.00153	1.22	NS
Between Exper. & Cont.	1	-.00153	-.00153	-0.5	NS
Interaction Between After Care and Rx	2	.00612	.00306		
Between Individuals Within Treatments	52	.08719	.00168	1.34	NS
Residual (error term)	52	.06482	.00125		
N=110 Totals	109	.16434			

*Rx - Experimental and Control

Table III. Analysis of Variance Between Extreme Age Groups*

VS	DF	SS	MS	F	
Between Age Groups	1	.00001	.00001	0.00008	NS
Between Rx** Within Ages	2	.00530	.00265	2.09	NS
Between Rx	1	-.00839	-.00839	-0.61	NS
Interaction Between Age & Rx	1	.01369	.01369		
Between Individuals Within Ages	18	.01210	.00067	0.53	NS
Residual	18	.02290	.00127		
N=40 Totals	39	.04561			

*Age Groups; 35-40 years and 70 years or older
**Rx - Experimental and Control

As the foregoing tables illustrate analysis of the densitometer registrations did not show statistical differences between methods of bathing, skin care following bathing [redacted] gross examination revealed rather obvious difference it might be assumed that the densitometer readings did not reflect true differences. For example, the amount of keritan obtained from the skin on the cellulose tape could not be accurately compared with the amount of oil which may have been residual on the skin even 24 hours after bathing. It is recommended that for further study more refined measurements be devised if possible to determine the amount of moisture present in the epithelium.

Summary and Conclusions:

An experimental investigation to test the hypothesis that the use of a specific nonionic emulsifier in giving complete and partial bed baths does not make a significant difference in the skin condition of selected patients has been completed. One-half of the body was bathed with soap and water (control) and the other half was bathed with a

specific nonionic emulsifier and water (experimental). A total of 62 patients have been bathed. The "blind approach" was used to make independent observations of roughness and scaliness on the experimental and control sides of the body.

Epithelium was stripped from the skin by placing cellulose tape on the lateral surfaces of the ankles on the control and experimental sides three times during the bathing period. These strippings have been exposed to a densitometer for a more objective measure by registration of the amount of light passing through the epithelium.

Analysis of data indicated differences in the amount of roughness and scaliness between the experimental and control sides of the body upon gross examination. Analysis of variance technics did not show significant differences to exist between the experimental and control sides of the body when variations in methods of bathing, skin care after bathing and in age groups were made. Because observation does show the tendency toward dryness of the skin after daily bathing for a period of 21 consecutive days, particularly in elder patients, we support the thesis that patients are bathed too frequently during hospitalization. It is recommended this research be replicated by other nurse investigators to substantiate findings. Replication of this study could provide documentation that hospitalized patients are bathed too frequently or if daily bathing is practiced some method of keeping the skin from becoming dry should be incorporated into the bathing procedure.

List of Publications:

Two reports for the nursing literature are in preparation.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803, Military Internal Medicine

Task 01, Internal Medicine (Nursing Measures Which Contribute to Development, Prevention, Care, and Treatment of Pressure Areas and Decubiti)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Nursing

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Major Phyllis J. Verhonick, ANC

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine (Nursing Measures Which Contribute to Development, Prevention, Care and Treatment of Pressure Areas and Decubiti)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Author: Major Phyllis J. Verhonick, AMC

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

Systematic investigation of the effectiveness of various nursing measures on decubitus ulcers and pressure areas is being continued. To date the sample includes a total of 79 patients with decubitus ulcers. Observations of patient progress are made in terms of effectiveness of selected measures recommended for healing. The clinical study will continue until a larger number of patients are included in the sample. Results obtained from the sample thus far are not definitive but successes have been obtained with selected measures tried to date.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine (Nursing Measures Which Contribute to Development, Prevention, Care and Treatment of Pressure Areas and Decubiti)

Description:

The clinical investigation of the effectiveness of selected nursing measures employed in the care of patients with decubitus ulcers and pressure areas is being continued until larger numbers of patients are included in the study. During a four-year period a total of 79 patients have been observed in this investigation. Of this total approximately 80% of the patients were hospitalized and the remaining 20% of the patients were either in their homes or in a nursing home.

Through the systematic collection of data nursing plans of care are evaluated for their effect on the rate of healing, comfort to the patient, and simplicity of administration. Careful observations are made on each patient once each week. A data-collecting form, "Criterion-Measure for Decubitus Observations" is used to record the size of the lesion, color changes, skin tone, amount and type of drainage, general skin condition and the presence and type of infectious organisms. Because there are many variables which may have an influence on formation of decubitus ulcers, a supplementary data-collecting sheet is also necessary. This form, "Nursing Diagnosis Check-List," is employed to record type of skin care, activity, diet and appetite, body support, laboratory findings, cardinal signs, and additional information that may be related to decubitus ulcers. Individual plans of nursing care initiated for patients are based on the size, location and condition of the lesion, as well as the general condition of the patient. The nursing plan of care which is considered within the prescribed medical regimen is discussed with the attending physician before beginning the specific nursing measure.

Comparisons are made between various nursing measures applied to patients within selected age groups and selected gross diagnostic categories.

Progress:

Numerous variables may have an influence on the formation of decubitus ulcers as well as influencing the progress of wound healing. In order to evaluate the rate of healing at least three weekly observations are necessary. Often, patients who develop decubitus ulcers are so debilitated that they expire before the effectiveness of the nursing measure can be established. The biological variation occurring in patients does not permit a tightly controlled study; therefore data are

descriptive in nature. The following Tables I and II show the sample by age and the types of measures used to date.

Table I. Distribution of Patients by Age Group

Age Group	Number of Patients	Percent
0-19 years	4	5.1
20-39 years	23	29.1
40-69 years	19	24.0
70 years or over	33	41.7
Total	79	99.9

Various nursing measures are used, on the basis of their effectiveness, comfort-giving qualities and the ease with which they can be administered. They are compared only within each age group and gross diagnostic category and not between groups and categories. Consideration of these two dimensions in making comparisons dictates a much larger sample of patients. Measures tried to date are listed as follows:

Table II. Distribution by Nursing Measures Used

Nursing Measure Used	Frequency	Percent
Drying Sprays	15	15.5
50% Glucose	12	12.4
Alternating Pressure Mattress*	10	10.3
Granulated Sugar	10	10.3
Unclipped Sheep Skin*	9	9.3
Karaya Gum Powder	9	9.3
Orange Peel	8	8.2
Cornstarch*	7	7.2
Adhesive Foam Rubber	7	7.2
Ointments	5	5.2
Heat Lamp	3	3.1
Foam Rubber Squares*	1	1.0
Sawdust bed	1	1.0
Total	97**	100.0

*Primarily Preventive Measures

**Application to more than one lesion on the same patient

Because of this variation in age and diagnostic category, conclusive evidence cannot as yet be furnished concerning the effectiveness of selected nursing measures. Inaccessibility of local clinical facilities for collection of data has interfered with the numbers of patients included in the study during the past year. It is anticipated that other clinical facilities may be explored for the purpose of including greater numbers of patients in the study in the future.

Summary and Conclusions:

The clinical investigation of the effectiveness of nursing measures employed in the care of patients with decubitus ulcers and pressure areas is being continued. A total of 79 patients have been included in the study during a period of four years. Systematic collection of data on two forms serves as a guide for the investigator to evaluate selected plans of nursing care for their effectiveness in healing lesions, giving comfort to the patient, and for their ease of administration.

Definitive results are not possible at the present because larger numbers of patients are required. It is anticipated that additional clinical facilities will be explored for data collection so that the sample size may be increased.

List of Publications:

Demis, D. Joseph, Zimmer, James G., Vermonick, Phyllis J. and Catalano, Philip M. "The Pharmacology of Human Skin: I Epinephrine and Norepinephrine; Catecholamine-Serotonin Combinations." The Journal of Investigative Dermatology, 39: 419-429. November 1962.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803, Military Internal Medicine

Task 01, Internal Medicine (Factors of Nursing in the Continuity of Medical Services in the Army)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Nursing

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Lt Colonel Harriet M. Werley, ANC

Assistants: Major Phyllis J. Verhonick, ANC and Captain Elenore F. Sullivan, ANC

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine (Factors of Nursing in the Continuity of Medical Services in the Army)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Lt Colonel Harriet E. Werley, ANC, Major Phyllis J. Verhonick, ANC and Captain Elenore F. Sullivan, ANC

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

This is a study of the contributions the nurse makes toward the continuity of care within the Army Medical Service. It is felt that the nurse has a role and functions which influence the continuity of care at all junctures in the total cycle of care from outpatient visits and hospital admission to clinic and home care service and possible readmission to the hospital.

Questionnaire, interview, and direct observation will be employed to gain perceptions from patients, nurses and physicians at all junctures in the cycle of care. At a later date experimentation will be initiated to study variables influencing the continuity of patient care.

Three questionnaires have been completed and will be mailed to Army health nurses, nurse administrators and a random sample of Army Nurse Corps officers to gain an insight into their perceptions of continuity of care, the components of care and the contribution the hospital care nurse and the Army health nurse make toward continuity of care.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine (Factors of Nursing in the Continuity of Medical Services in the Army)

Description:

The mission of the Army Medical Service and its respective component parts is to maintain the health of the Army and conserve its fighting strength. It is therefore fitting to explore systematically in what way one large segment of the Medical Service officer component, the Army Nurse Corps, makes its contribution to this overall medical mission.

The basic assumption underlying this research is that the nurse has a role and functions which influence the continuity of care at all junctures in the total cycle of care as the patient makes the transition from outpatient or clinic visits to admission to the hospital, to discharge, to referral and home care visits, to return outpatient visits, and possible readmission. The overall objectives of the study include the following: (1) to identify nursing contributions to the continuity of medical and nursing care provided the military man and his dependents in selected groups or with selected conditions; (2) to identify, compare and contrast the components of "health" nursing/ "sick" nursing; (3) to assess the need for a redefinition of the concept of military nursing, or for a shift in the focus of nursing, to include to a greater degree the positive health aspects of the medical load as it is moving more to clinic type or outpatient care; (4) to devise measures, such as patient response indices, which may reflect what is being accomplished through the nursing process.

A questionnaire survey will be completed to elicit information from groups of Army Nurse Corps officers concerning perception of continuity of care generally, continuity of care within the Army Medical Service, components of nursing care, and the contribution the hospital care nurse and the Army health nurse make towards continuity of care. At a later period in the study, questionnaire, interview and observation techniques will be used to gain more specific data relating to continuity of care. Patients, physicians, and nurses at all junctures in the cycle of care will be included in the study sample. It is planned that data collected from these survey-type approaches may be later used as a basis for devising instruments and techniques for further testing of concepts in experimental situations.

Progress:

Three separate questionnaires have been designed to gain information from the total number of Army health nurses, nurse administrators, and a random sample of the remaining Army Nurse Corps officers within the continental United States and in overseas commands.

The three questionnaires designed last year were given to three Army Nurse Corps officers stationed at the Walter Reed Army Medical Center to test for the reliability of the questions as well as to ascertain an idea of the type of answers to be received. As a result of this reliability check, all three questionnaires were reviewed and revised in the light of the suggestions offered by the respondents and Department of Nursing staff.

Consultations were sought from an authority on public health nursing and a psychometrician to examine the validity of the revised questionnaires. These consultations also gave guidance for selecting the samples of the Army Nurse Corps officers to participate in the study.

Samples will be taken from rosters of Army Nurse Corps officers in the continental United States as well as in the overseas commands. In addition to the total number of Army health nurses and nurse administrators a 15 percent random sample will be selected from the remaining Army Nurse Corps officers.

The final drafts of the questionnaires have been received from the U. S. Government Printing Office and are being prepared for mailing.

Progress in working on this subtask has been delayed by three factors: the transfer of the principal investigator to an overseas assignment; the appointment of the assistant investigator to Chief, Department of Nursing with an increased workload and responsibilities; and the assignment of an assistant investigator to the study who has needed time to become acquainted with all aspects of the project.

Summary and Conclusions:

The three questionnaires designed last year were given to three Army Nurse Corps officers to check the reliability of the questions. The suggestions and recommendations offered were taken into consideration for revision of the questionnaires. Consultations were sought from a psychometrician and an authority in public health nursing to attempt to establish validity of the revised questionnaires.

The three questionnaires have been returned from the Government Printing Office and will be mailed to selected members of the Army Nurse Corps in the near future.

Following this initial pilot survey, the study will be continued in the patient-setting in the hospital, out-patient clinics and home-care services.

List of Publications: None

FINAL REPORT

Project 3A 0 12501 A 803, Military Internal Medicine

Task 01, Internal Medicine (Nursing Measures in Irradiation Therapy)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Nursing

Period Covered by Report: 26 June 1962 through 30 June 1963

Principal Investigator: Captain Lois A. Johns, ANC

Report Control Symbol: MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine (Nursing Measures in Irradiation Therapy)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 26 June 1962 through 30 June 1963

Author: Captain Lois A. Johns, ANC

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

An experiment was planned to test the hypothesis that anorexia and/or vomiting may be significantly decreased by the the manipulation of selected diets in patients receiving irradiation therapy. Selected dietary contents and patterns were planned to be used in conjunction with various selected anti-emetic medications. It is assumed findings from the study might be generalized to situations where ionizing radiation has been received. Preliminary exploration for a beginning phase was accomplished. Because of the inaccessability of available clinical facilities and transfer of the principal investigator, the study will be discontinued.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine (Nursing Measures in Irradiation Therapy)

Description:

An experiment had been planned to be carried out by a nurse investigator. The objective of this research was to test the hypothesis that anorexia and/or vomiting might be significantly decreased by the manipulation of selected diets in patients receiving irradiation therapy. Patients chosen for inclusion in the study were to have met the following criteria: to receive irradiation therapy for the first time, to receive irradiation therapy to the torso, not to receive any chemotherapy in conjunction with irradiation therapy, and to exhibit varying degrees of anorexia and/or vomiting. Three dietary patterns with inherent variations in the dietary content were planned to be administered. Also, three variations of anti-emetic medication patterns planned for use included a standard anti-emetic, a placebo, and no medication. Data concerning eating patterns, amount of food eaten, time and number of emesis were to have been collected on data sheets by the investigator and hospital personnel. The investigator planned for additional data relating to other factors to be secured, i.e., area, type, and sequence of irradiation; amount of daily dose of irradiation; length of daily therapy, and diagnosis. It was assumed findings from the planned study could be generalized to situations where ionizing radiation has been received.

Progress:

Preliminary plans for a pilot phase of the study have been under way. Informal exploration for use of a hospital ward and a preliminary review of patients receiving irradiation therapy at Walter Reed General Hospital has been done. Discussion was held with representatives of a reputable drug company concerning the provision of a standard anti-emetic and a placebo capsule which they would supply. However, due to the inaccessibility of adjacent clinical facilities for the planned study, no further progress has been made. As the investigator is being reassigned, this is submitted as a final report.

Summary and Conclusion:

An experiment was designed to test the hypothesis that manipulation of dietary content and feeding patterns would significantly be related to the occurrence of anorexia and vomiting in selected patients receiving irradiation therapy. Preliminary steps for a pilot phase were completed but the use of available clinical facilities for data collection was discouraged. With this in view plus the transfer of the principal investigator, the experiment was not carried through.

List of Publications: None

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803, Military Internal Medicine

Task 01, Internal Medicine (Relationship of Recorded Nursing Observation to Patient Care)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Nursing

Period Covered by Report: 25 September 1962 through 30 June 1963

Principal Investigator: Major Leanora M. Moseley, ANC

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine (Relationship of Recorded Nursing Observations to Patient Care)

Reporting Installations: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 25 September 1962 through 30 June 1963

Author: Major Leanora M. Moseley, ANC

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

A study of recorded nursing observations and their relationship to patient care was initiated. Nurses have a responsibility for assisting members of the medical team in the early diagnosis of complications of disease in patients and in the early recognition of physical and emotional changes in patients' condition. Early recognition of symptoms of complications of diseases and changes in patients' condition should enable the medical team to begin prompt, effective therapy, thus avoiding prolonged hospitalization and excessive discomfort and expense for patients.

Patients' records were reviewed from both medical and surgical services to discover if the same types of general information were recorded by nurses on both services. This information was obtained in order to ascertain if a single guide for recording by nurses should be developed for universal use. The type of information recorded was found to be the same.

A questionnaire was designed and distributed to members of the medical team to ascertain which members read nurses' notes and for what reasons. The information obtained from these questionnaires will be used as a basis for designing the guide for recording nursing observations to be tested at a later date.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine (Relationship of Recorded Nursing Observations to Patient Care)

Description:

A retrospective study revealed that there are not sufficient data in nurses' notes to indicate a change, if any occurs, in the natural history of a complicated disease (ulcerative colitis) in a patient. This study also revealed that symptoms of complications occurring in patients with this disease cannot be detected from nurses' recorded observations. If complications and changes cannot be detected from recorded observations when a disease is as complicated as ulcerative colitis, can we assume that this is equally true when a disease is less complicated?

The purpose of this study is to ascertain if the content of nurses' notes will be more meaningful to members of the medical team if nurses' observations are recorded systematically. The basic assumption is that members of the medical team use nurses' notes to assist in planning patient care.

The overall objectives of the study include the following: (1) to assess the value of a systematic guide in recording nursing observations; (2) to ascertain if systematic recording of observations influences the content of nurses' notes; and (3) to ascertain if there is a relationship between content of nurses' notes and their use by the medical team.

In recent years, nurses have discovered that they have legal as well as moral and professional responsibilities to record their observations. Legislation states that reporting and recording is an undisputed, independent area of professional nursing. The recognition of this area as a function of nursing is so clear that physicians have a legal right to rely upon the facts contained in nursing records relating to any physical and emotional changes in the patients' condition. This function encompasses the recording of facts relating to supervision of patient care, observation of signs and symptoms, and reactions to any medical or nursing procedures and techniques upon which the course of further care depends.

Military nurses have a responsibility to teach and supervise non-professional personnel to make and record accurate observations in selected situations. If the non-professional personnel make and record inaccurate observations, serious errors may be made in the treatment of patients throughout the chain of evacuation.

Progress:

Records from both medical and surgical services were reviewed to ascertain if nurses recorded the same general types of information on both services. It was discovered that the information found on records from both services was generally the same, and showed no indication that the nurse had made any observation of the patient. The medical specialties from which these records were selected were: general medicine, general surgery, neurology, neurosurgery, orthopedics, and gynecology. The nurses' notes were also compared with the doctors' progress notes to ascertain if any observation recorded by the nurse might indicate a change in treatment or diagnosis. There was no indication that this was true on any of the services.

A questionnaire was designed to discover what members of the medical team read nurses' notes and for what reasons. One hundred questionnaires were distributed to physicians, nurses, and allied scientists. Sixty-five percent of these questionnaires were returned. The information gained from this survey is being used as a basis for designing the guide which is to be tested.

The guide is to be tested on an experimental ward in a military hospital. The observations recorded on this experimental ward will be compared with observations recorded on a control ward where the recording of observations will be done in the usual manner.

Summary and Conclusions:

A study of recorded nursing observations and their relationship to patient care was initiated. Two surveys were conducted for the purpose of: (1) ascertaining if nurses record the same type of general information on medical and surgical patients, and (2) gaining information to use as a basis for designing a guide for recording nursing observations.

A guide for the systematic recording of nursing observations is being designed and is to be tested in a military hospital at a later date.

List of Publications: None

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803, Military Internal Medicine

Task 01, Internal Medicine (Nursing Care of Patients Confined Within Isolator Systems)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Germfree Research,
Division of Basic Surgical Research

and

Department of Nursing

Period Covered by Report: 9 October through 30 June 1963

Principal Investigators: Major Maria L. La Conte, ANC and
Major Miriam K. Ginsberg, ANC

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine (Nursing Care of Patients Confined Within Isolator Systems)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 9 October 1962 through 30 June 1963

Authors: Major Maria L. LaConte, ANC and Major Miriam K. Ginsberg, ANC

Reports Control Symbol: MEDDH-288

Plastic, germfree animal isolators were thought to have possibilities for isolation of patients. A prototype of a closed system was constructed to test this theory. It was composed of four parts: a flexible, clear plastic enclosure or tent; means for manipulation through the tent walls; a continuous filtered air supply; and a pass-through lock. A study was undertaken: to ascertain the feasibility of using a prototype for isolation care of patients; to describe the nursing care of patients confined within the enclosure; and to test, modify, and adapt the equipment as indicated. It was renamed "Regulated Environment for Safety" (RES).

Three clinical trials were undertaken. The findings to date are as follows: (1) The RES system was generally accepted by all hospital personnel who inspected and studied it; (2) The three patients demonstrated no adverse reactions during or after their stay in the RES tent; (3) Multiple and extensive modifications of nursing procedures were necessary in order that nursing care in the RES tent could become feasible and could support medical therapy; (4) Disinfection and subsequent housekeeping of the tent presented problems that are still only partially resolved; (5) Bacteriological testing of the system is underway; (6) Oxygen and carbon dioxide content, and temperature and humidity inside the tent are to be analyzed in the near future.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 01 Title: Internal Medicine (Nursing Care of Patients Confined Within Isolator Systems)

Description:

Isolation of patients to prevent cross-infection is a fundamental principle of medical practice. Isolation is almost always ordered by physicians for patients with communicable diseases. Isolation technics are also used for the protection of those patients whose defenses against infection are lowered by injury, disease or medical therapy. Included are patients with extensive body burns, acute leukemia, uremia, whole body x-irradiation, those being treated with steroids and those undergoing organ homotransplantation. The search for improved methods of isolation care for these types of patients is a continuing process. A completely closed, clean environment is considered the ideal.

In this regard, medical investigators involved in germfree animal research at Walter Reed Army Institute of Research considered the possibility of adapting for clinical use, the flexible plastic animal isolators which have proved successful in the rearing of germfree animals. Subsequently, a full-sized isolator encompassing an individual patient care unit was built for the purpose of testing this proposal. However, when time approached for placing a sick patient into the system, many nursing and medical problems became evident. In an attempt to overcome the apparent difficulties in effective patient care, this study was undertaken.

The objectives of this investigation are as follows: to ascertain the feasibility of using a closed system for care of patients in need of isolation; to describe the nursing care of patients confined within the enclosure; and to test, modify and adapt the equipment as indicated.

Progress:

The investigators felt that the name of the system should be changed from "Plastic Patient Isolator" to "Regulated Environment for Safety" (RES). This was done in order to avoid using such terms as "isolator" and "isolation" because of their psychological connotation. Moreover, RES tent seems to imply a restful, soothing atmosphere which the investigators believe will be more acceptable to both personnel and prospective patients.

Components of the RES System:

The RES system is composed of the following four parts: a flexible, clear plastic enclosure or tent; means for manipulation through the tent walls; a continuous filtered air supply; and a pass-through lock.

1. The RES Tent: The tent used is constructed of clear, vinyl, plastic material of approximately 10 mil thickness and measures approximately 3 x 4 x 6 feet in overall dimensions. It was made to fit over the bed springs so that the mattress could be placed inside. Attached on either side are jackets for manipulatory purposes. Built into one wall near the head-end is an extension designed to fit over a bed side table. The supporting structure is made of one inch aluminum pipes secured to the head and foot of the bed allowing semi-mobility of the unit. The pass-through lock is attached at the foot end of the tent, and the source of continuous filtered air is attached to the head end of the tent. (See figure 1).

2. Means for Manipulation: Prior to the beginning of clinical trials, the jackets were the only means for manipulation. The body portion was constructed from opaque vinyl material, whereas the hood and face pieces were of clear vinyl and plexiglass respectively. (See figure 2). A standard size glove which could be worn by all personnel was attached. During the clinical trials armports, equipped with clear plastic sleeves with gloves attached, were added to the tent as an additional means for manipulation.

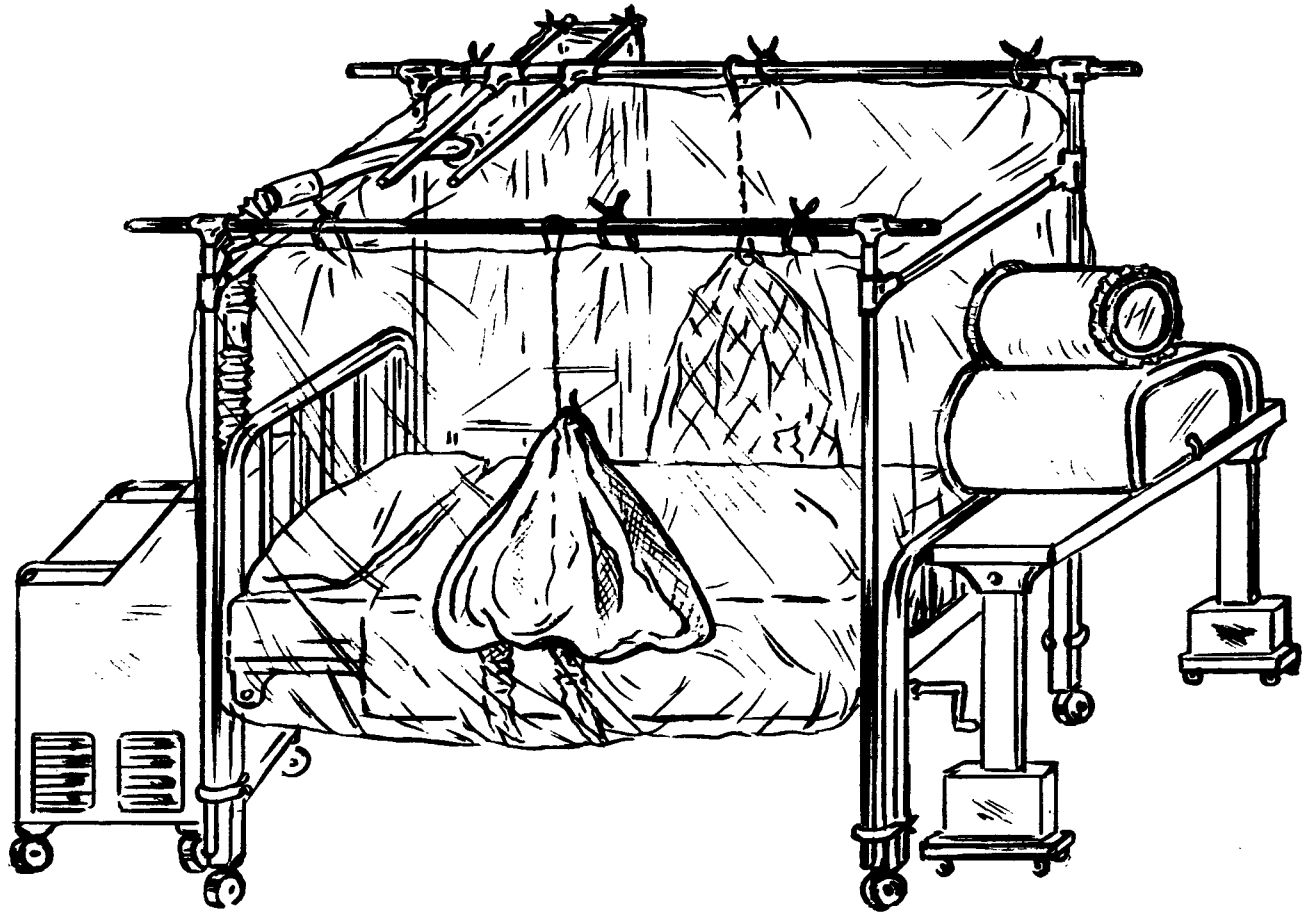
3. A Continuous Filtered Air Supply: Two types of motor-driven air supplies were used. During the preclinical phase, a turbo-compressor which delivered fifty cubic feet of air per minute was tried. Although this unit had sufficient pressure to force air through an absolute filter, its motor was extremely noisy, a clinically undesirable feature, therefore, it was not used in clinical trials. Instead, an oxygen tent motor (without employing oxygen) was used. A series of three air-conditioning filters was interspersed in the air inlet tubing to remove large dust particles since this motor did not possess sufficient power to force air through an absolute filter.

4. The Pass-through Lock: At the beginning of clinical trials, the lock was of double door, one-chamber construction and measured approximately 20 x 20 x 30 inches. It was fitted with ultraviolet lights and a sliding tray on the floor of the chamber. A second chamber was improvised so that food trays and used bed pans would not be passed through the same lock. It was built out of plastic, plywood, and oilcloth, measured 12 x 12 x 18 inches and was fastened to the top of the first chamber.

Preclinical Phase:

Familiarization with the RES system was the objective of the preclinical phase of testing. Nursing technics and procedures which are relatively easy to perform outside of the RES tent proved most difficult when performance was attempted within the system. It was during this

Fig. 1



The RES System

Fig. 2

Jacket for manipulation through RES tent wall.

phase of testing that the turbo-compressor air supply was found to be too noisy for a hospital environment, and the decision was made to replace it with the readily available, quiet oxygen-tent motor.

The problem of glove changes, within the RES tent while the system was in use, was overcome by two modifications. Hard plastic cuffs were attached to the wrist end of the sleeves of the jackets (and the armports at a later date). The cuffs of the gloves were stretched firmly over the plastic cuffs and secured in place with rubberbands. A technic was developed whereby one glove could be replaced with a new one without contamination of the interior of the tent. The original surgeon's glove was replaced with the stronger kitchen glove in order to reduce the risk of tears and the need for glove changes.

The placement of the pass-through lock was determined during this period of testing also. It was found that the lock was more convenient to the operators, regardless of where they were working, when the lock was attached to the foot of the tent. Furthermore, this location seemed to cause the least obstruction to the patient's view.

Sterility of the interior of the tent and lock remained problematic throughout the preclinical phase. Methods of sterilization that were effective inside of the vacant tent were either potentially toxic for a patient, or mechanically too difficult to perform. Disinfection seemed to be more practical. Wescodyne solution was selected and used throughout the subsequent tests.

After becoming completely familiar with the mechanics, the investigators then attempted to experience the system from the patient's standpoint by spending varying periods of their time inside of the tent. They experienced no respiratory distress; moreover, they found the temperature and air flow inside of the tent to be comfortable. They had no feelings of claustrophobia. Therefore, the project was considered ready for clinical trials.

Clinical Phase:

Despite certain inadequacies of the equipment on hand, clinical trials involving hospitalized patients were initiated in January 1963 at Walter Reed General Hospital in a large two bed room on Ward 25. Three clinical trials were made in order to study acceptance of the system by patients and personnel, the equipment in use, and to some extent, psychological reactions of the patient. A two week lag was encountered between the second and third trials due to a seasonal flu epidemic in the Washington, D. C. area.

Patients for these clinical trials were selected by the investigators after consultation with the attending physicians. All of the patients were given an explanation of the study and asked to volunteer for varying periods of time. The patients were as follows:

Patients	Age	Race	Sex	Diagnosis	Med. Treat.	Ambulatory*	Days in RES Sys.
1	25	W	M	Awaiting Med. Board Action	None	Yes	2
2	22	W	M	Awaiting Med. Board Action & Sinusitis, Mild	Gargles Nosedrops	Yes	3
3	30	W	M	Segmental Fract. of Rt Femur-Healed	None	No	14

*Ability to ambulate if not restricted in the RES System.

Diversional therapy was provided by television and radio which the patients could operate themselves through the plastic tent wall. In addition, the patients were allowed reading and writing materials.

Results of Clinical Trials:

1. Acceptance: All three patients unanimously stated that they slept more soundly in the RES tent, were comfortable at all times, and experienced no feelings of isolation or ostracism. They all expressed that being in the RES system gave them a feeling of security which they had not experienced outside. They were vitally interested in the purpose and the mechanics of the system and attempted to participate in its improvement. Since there is no contraindication to visitors as in other forms of isolation or barrier care, visitors to the patient and the system were encouraged. One patient held hands with his wife with only the plastic tent separating them. He even attempted to kiss her this way -- but found this to be unsatisfactory.

The size and construction of the RES tent precludes even moderate activity such as "dangling" of the patient's feet over the side of the bed. In its present form, the system appears to be limited to care for strict bed patients.

General acceptance of the system by hospital personnel was excellent. Physicians, nurses, ancillary personnel, one chaplain, and several ambulatory patients came to see the system. Everyone asked many questions and all appeared convinced of its usefullness. The reception and wholehearted support offered by the hospital physicians and nurses directly involved in the project were extremely gratifying to the investigators.

2. Convenience, Comfort and Safety: Insufficient storage space within

the enclosure presented special problems. For example, there was no place to store the over bed tray which is used to support the patient's food tray, nor was there room to store the patient's personal articles such as shaving equipment, toothpaste, and the like. Plastic boxes had to be attached to the tent wall, near the head of the bed, in order to store small items. A shelf was constructed under the lock, for storage of the over bed tray when it was not in use.

Certain medical procedures require tubing and other equipment to be passed from inside the tent to the outside or vice versa, without contamination. These include: stethoscope tubing, intravenous therapy tubing, the patient's call-bell cord, suction tubing, and electronic monitoring equipment, etc. After experimenting with different methods, this problem was partially solved. Specifications for such entrances should be incorporated in future RES system construction.

Getting in and out of the jackets proved inconvenient and slow. In order to prevent the possibility of accidentally puncturing the tent with sharp objects, for instance, military insignia, and hair pins, a cloth gown and cap were worn over the normal hospital uniform. The cap was also worn for sanitary purposes explained later in this report. In addition, the operator was required to wear a special "collar" through which he received air for breathing while in the jacket. Despite the length of time required for preparation and entry, once in the jacket, the operator had exceptional mobility and could work effectively.

The investigators felt that quick-entry devices were necessary to facilitate care. Therefore, armports equipped with shoulder length sleeves and gloves were attached to the RES tent near the pass-through lock. These attachments eliminated the necessity of getting into the jacket each time an article was introduced or removed via the pass-through lock. Similar armports are needed near the head of the RES tent for performance of quick action procedures, such as suctioning a tracheostomized patient, assisting a vomiting patient, etc. Whether the jackets should be eliminated and replaced with sleeve-glove attachments cannot be answered at present. Nevertheless, it can be stated without reservation that jackets as the only means of manipulation will not suffice.

As mentioned previously, the gloves that were used were of a standard size, kitchen type glove. Persons with large hands encountered no particular difficulties other than becoming accustomed to performing nursing procedures with gloves on. On the other hand, persons with small hands (particularly true of female personnel) were extremely frustrated with the oversized gloves and had difficulty working effectively. This problem was partially resolved by wearing a second pair of small-sized gloves over the gloves attached to the jackets. Backrubs, which were anticipated to be a problem with gloved hands were then relatively simple to do. In addition, these backrubs were as acceptable to the patient as they were without gloves, so long as the gloves were kept well lubricated with

backrub lotion. Nevertheless, the gloves continued to present serious difficulties for fine finger movements. Further investigation is necessary regarding types of gloves.

The pass-through lock in its current form is inadequate for further testing. A new prototype lock is being constructed and should be available for testing about the end of May, 1963.

In the beginning of this study, nursing procedures were generally awkward and difficult to perform within the NES tent. After modifications were made, the practice of nursing care became feasible and able to support medical therapy.

3. Sanitation: Sanitation of the jackets between wearers was attempted by cleansing the face piece and hood with alcohol after each use. By wearing a washable cap and gown, the operators lessened the chance of accidentally transferring to the next attendant unrecognized infectious agents. The effectiveness of these procedures was not studied bacteriologically and investigation must be made to insure safety of personnel if the jackets are to remain in the system.

Disinfection of the tent with Wescodyne solution was a messy procedure and resulted in some discoloration and streaking of the plastic. Hand prints and cosmetic marks were quite obvious on the tent, and yet were difficult to remove. Housekeeping inside of the NES tent presented an unexpected problem. A combination of crumbs, dried skin, loose hair, and dust have defied usual cleaning measures. The collection of odors inside the tent seemed to be absorbed by the plastic material and at times became offensive to the patient. This problem was partially resolved by periodically spraying the standard hospital room deodorant into the air supply.

4. Environmental Problems: The oxygen tent motor provided continuous, fresh, cool air with a relatively noiseless operation. During the clinical trials, temperatures inside of the tent were consistently two to five degrees higher than outside of the tent and were controlled by regulating the temperature of the room. Oxygen and carbon dioxide content of the air in the tent will be analyzed, although there was no evidence of carbon dioxide build-up. Humidity determination is also planned. A relatively noiseless, prototype air unit with the capacity to force air through an absolute filter has been ordered. The amount of dirt on the air-conditioned filters at the end of the clinical trials attests to the necessity of absolute filtration of air.

5. Bacteriological Tests: The degree of bacterial contamination of the inside of the NES tent, and an evaluation of the degree of safety within the system as compared to the surrounding area, are planned for the coming months. Bacteriological testing has begun; however, definitive findings are dependent on the improved lock and air units now on order.

6. Psychological Reactions: Psychological testing is beyond the scope of this study. Fortunately, none of the patients in the 3 clinical trials showed any untoward reactions during or after their stay in the NES tent. However, these experiences should not preclude psychological studies at some later time.

Summary and Conclusions:

The objectives of this investigation are: to ascertain the feasibility of using a closed system of care of patients in need of isolation; to describe the nursing care of patients confined within the enclosure; and to test, modify, and adapt the equipment as indicated.

After becoming familiar with the equipment in the laboratory, a series of three clinical trials was made. Three patients volunteered to stay in the isolator two, three and fourteen days respectively. No untoward reactions occurred. The experiences showed the investigators what modifications of the equipment were essential, and allowed them an opportunity to provide actual nursing care within the NES system. Bacteriological testing of the system is planned for the coming months.

List of Publications: None

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803 MILITARY INTERNAL MEDICINE

Task 02, Metabolism and Nutrition (Interrelationships of parathyroid gland activity and calcium and phosphorus metabolism)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Metabolism
Division of Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Lt. Colonel Kevin G. Barry, MC

Assistants: Major James P. Knochel, MC
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Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 02 Title: Metabolism and Nutrition (Interrelationships of parathyroid gland activity and calcium and phosphorus metabolism)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Lt. Colonel Kevin G. Barry, MC
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The diagnosis of hyperparathyroidism is being made with greater frequency due to continuing efforts to improve diagnostic tests. During the past year, analysis of results has been performed and the findings published.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 02 Title: Metabolism and Nutrition (Interrelationships of parathyroid gland activity and calcium and phosphorus metabolism)

Description: Metabolic abnormalities of calcium and phosphorus metabolism compatible with but not diagnostic of hyperparathyroidism remain a problem. Generally, the decision to operate commits the surgeon to prolonged and extensive search for a parathyroid lesion. It is desirable to develop diagnostic techniques which will do away with unnecessary surgery and permit all patients with hyperparathyroidism primary the benefit of surgery.

Progress:

1. Hyperparathyroidism. The clinical experience of the Department of Metabolism with hyperparathyroidism has been recently tabulated, evaluated, and published. The experience indicates an increased awareness and frequency of diagnosis of the disease with a successful surgical outcome.

2. The Effect of Calcium Infusion on Phosphate Clearance by the Kidneys. Occasionally, phosphate clearance will be increased associated with high normal or slightly elevated serum calcium levels. It was postulated that in patients with biologically elevated levels of serum calcium not due to hyperparathyroidism the infusion of calcium would decrease significantly the phosphate clearance when compared with clearance obtained prior to the calcium infusion. The techniques of the test have been worked out and the test has been evaluated in several patients. The results suggest that the test is an additional supportive diagnostic tool.

3. The Effect of Diuresis on Renal Clearance of Phosphorus. The clinical value of phosphorus clearance is not as great as was hoped because of variability of clearance in a given individual despite use of standard methods. It was postulated that during the first hour

of diuresis following water loading phosphorus clearance might be higher than during the second and third hours of diuresis due to changing gradients within the kidney. For this reason, ten normals were studied in whom the first, second and third hours of diuresis consisting of a urine flow of over 5 ml. per minute were compared. It was found that there was a significant elevation of clearance of phosphorus during the first hour as compared with the second and third hours which were comparable in their values. This work suggests that clearances should not be initiated until the urine flow of over 5 ml. per minute has been established for at least 60 minutes. This procedure may further standardize the test and increase its clinical value.

4. Study of the effect of cercadian rhythm on phosphorus excretion is under way. Comparison of effect of hyperparathyroidism on this rhythm is being compared with its effect on renal clearance of phosphorus.

Summary and Conclusions:

The diagnosis of hyperparathyroidism is being made with greater frequency due to continuing efforts to improve diagnostic tests. Use of changes of the cercadian rhythm in the diagnosis is being compared to value of standard clearance of phosphorus and to date does not appear to add to the diagnostic accuracy.

Publications:

1. Kyle, L. H., Beisel, W. R., and Canary, J. J.: Evaluation of the Relative Value of Diagnostic Tests in Hyperparathyroidism. Ann. Int. Med. 57: 957, 1962.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803 MILITARY INTERNAL MEDICINE

Task 02, Metabolism and Nutrition (Acute renal injury and failure)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Metabolism
Division of Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Lt. Colonel Kevin G. Barry, MC

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Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

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ABSTRACT

Project No. 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 02 Title: Metabolism and Nutrition (Acute renal injury and failure)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Lt. Colonel Kevin G. Barry, MC
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Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

The ability of hypotonic mannitol infusion to maintain urinary flow, renal perfusion and glomerular filtration rate even during anesthesia and surgery has permitted trial of solute diuresis as a method of protecting renal function during anesthesia and surgery in humans. Studies to date confirm the belief that patients should be hydrated and diuresis established prior to and during surgery. A new peritoneal cannula for acute and chronic peritoneal dialysis has undergone extensive testing during the past year and appears to be an important technical improvement. Intensive application and study of peritoneal dialysis to define its value and limitations continues. Correlative animal studies in the treatment and prevention of acute renal failure continue.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 02 Title: Metabolism and Nutrition (Acute renal injury and failure)

Description: The goal of this study is to reduce the incidence of acute renal failure and to decrease the moribidity and mortality in patients already shut down to a minimum. Parallel studies in humans and animals are being performed.

Progress:

1. Pathogenesis of Acute Renal Failure. Prospective studies in patients with normal preoperative renal function subjected to halothane anesthesia and surgery indicate that depression of renal function which invariably occurred in association with oliguria in control patients did not occur when a solute diuresis and hydration with one-third normal saline was established and maintained during anesthesia and surgery. Moderate depression of renal function occurred despite solute diuresis only when the concentrations of halothane used were above 2-1/2 per cent.

Current studies of older patients with cardiorenal abnormalities subjected to anesthesia and surgery are being performed. The effect of traditional dehydration is being compared with solute diuresis and mannitol diuresis in these patients. It is notable that since prophylactic mannitol diuresis during abdominal aortic aneurysmectomy has become standard in WRGH that not one instance of postoperative renal shutdown has occurred in this group. We would have expected four to five shut-downs in this interval of time prior to the use of mannitol.

2. Renal Protective Effect of Osmotic Diuresis During Anesthesia and Surgery in Patients with Chronic Renal Insufficiency. Eight urologic patients with severe chronic depression of renal function who required surgery on the upper genitourinary tract were protected by hydration using one-third normal saline and mannitol diuresis during anesthesia and surgery. Further depression of already depressed renal function was effectively prevented in these patients. Some patients had previously undergone similar surgery without the

protection of mannitol and had experienced further acute depression of renal function during anesthesia and surgery.

3. Urinary Bladder Catheterization. The questions of danger and benefit of catheterization of the urinary bladder are not settled. Studies performed in the department require frequent catheterization of the bladder for varying intervals. Studies to date indicate that, in patients without genitourinary infection, bladder catheterization properly performed is a safe procedure. Studies in patients with acute renal failure and other genitourinary disease indicate that intermittent irrigation of the urinary bladder using an inlying catheter has merit and overcomes the tendency for infection associated with stasis to occur.

4. Treatment of Acute Exacerbations of Chronic Renal Failure. More patients are being transferred to the department for artificial dialysis to overcome what has been diagnosed as acute exacerbation of chronic renal disease. We have found in many instances correction of hydration, acid base, and electrolyte balance utilizing intravenous therapy frequently restores the patient to a stable level of chronic renal insufficiency. When this therapeutic maneuver is unsuccessful, peritoneal dialysis has been effective. Hemodialysis in these patients has not been required. In some patients, clinical deterioration has been due to inevitable advance of renal disease. In these patients, studies of chronic peritoneal dialysis have been performed and have expanded knowledge of the abnormalities associated with prolonged inability to form urine and how to counteract them.

5. Methods of Dialysis. Peritoneal dialysis has become the standard method of therapy for patients with acute renal failure in Walter Reed General Hospital. Hemodialysis utilizing the Twin-coil has been used in only two patients during the past year. Hemodialysis has been required because of multiple intra-abdominal injuries which made peritoneal dialysis impossible.

6. The Prevention and Therapy of Acute Renal Failure by the Infusion of the Osmotic Diuretic Mannitol. Continuing studies at Walter Reed have validated previous work. Some important changes in use of mannitol for this purpose include the more liberal administration of water and sodium and the administration of mannitol in 20-gram acute doses in order to present the kidneys with a bolus for maximum acute effect. This replaces chronic titration with

mannitol which permitted more of the drug to reach the extracellular, extravascular space. Reports received from centers throughout the country and overseas have been confirmatory.

7. Studies of Acute Uric Acid Nephropathy. The effect of hyperuricemia on renal function has been studied in five patients. In three patients showing the characteristic evidence of renal shutdown associated with hyperuricemia, mannitol was effective in producing and sustaining a diuresis and restoring serum uric acid and blood urea nitrogen levels to normal. In one other patient responsive to mannitol for several hours, shutdown occurred and peritoneal dialysis was required. It was found that urinary alkalinization was not achieved in this patient and re-emphasized the importance of alkalinization even when mannitol is used.

8. Treatment of Intoxication with Weak Acids by Peritoneal Dialysis Incorporating THAM in the Dialysis Solution. The buffer tris(hydroxymethyl) aminomethane (THAM) was incorporated in peritoneal dialysis solution to bring the pH from 5 to 10. The solution maintained its buffering capacity for several hours and more than doubled the removal rate of phenobarbital and seconal when peritoneal dialysis was used in therapy of experimentally intoxicated dogs. Currently, the efficacy of intraperitoneal THAM in the treatment of hyperuricemia is under investigation. This work performed by Major Knochel is believed to represent a major therapeutic advance.

9. Studies of Other Exogenous and Endogenous Intoxications. Studies in humans indicate that osmotic diuresis utilizing mannitol and alkali is an effective therapy for preventing renal damage in response to and hastening the renal excretion of toxins including uric acid, hemoglobin, barbiturates, and other exogenous toxins. Peritoneal dialysis has also been shown effective for these patients. Comparison of various diuretic regimens and peritoneal dialysis in the treatment of intoxications is being performed in humans and animals. In addition, clearances of commonly used drugs by peritoneal dialysis is being initiated. This is an important area in which few studies have been performed and information is urgently required. Pilot studies indicate that peritoneal dialysis does not extract radioactively labelled digitoxin from the body. Studies of antibiotic removal are under way.

10. The Effect of Purine Antagonist on the Nephrotic Syndrome. Trial of 6-Thioguamine has been accomplished in six patients with varying grades of glomeruloenphritis associated with the nephrotic syndrome. In no instance was there evidence of significant beneficial effect. Further investigations will be restricted to patients unresponsive to prolonged therapy with other agents.

11. Hemorrhagic-Pulmonary Renal Syndrome. Three patients who presented with pulmonary hemorrhage, severe anemia, and severe nephritis were studied. In each case the severe nephritis progressed to complete renal failure and, despite peritoneal dialysis, death. Pathologically, the pulmonary changes were very similar to idiopathic pulmonary hemosiderosis with heavy, bloody lung organs and with massive exudation of blood into the alveoli, thickened alevolar walls and many hemosiderin laden macrophages on microscopic. The kidneys were characterized by proliferation of the parietal epithelium of Bowman's Capsule with crescent formation, extravasation of red cells and protein into dilated tubules and lymphocytic interstitial infiltration. The glomerular changes progressed to hyalinized replacement of the glomerulus in some areas.

12. Renal Biopsy in Infectious Hepatitis. Renal functional abnormalities have been observed in a variety of acute infectious diseases while autopsy studies have revealed mild associated renal histologic changes. The relationship of these acute functional and morphologic changes to chronic renal disease and chronic proteinuria remains speculative. During the past year, renal biopsy was performed on 20 patients hospitalized in Korea during the acute stage of infectious hepatitis and repeated in 8 during either exacerbation or convalescence.

The correlation of renal function abnormalities with changes in renal histology by light microscopy and electron microscopy has been recently tabulated and presented in a preliminary report.

Summary and Conclusions: Continued prospective studies of the pathogenesis of acute post-traumatic renal failure in humans have confirmed the importance of hydration and maintenance of a solute diuresis during anesthesia and surgery in these patients. This method of management is a radical departure from what has become traditional procedure but is being accepted not only at WRGH but by other institutions throughout the country and overseas. These studies

continue to follow a logical evolution of experimental and clinical import.

A peritoneal cannula has been devised for acute and chronic use which markedly improves the technical performance of peritoneal dialysis. Peritoneal dialysis has almost totally replaced hemodialysis in the conventional treatment of acute renal failure. The value and limitation of this method of therapy and adoption for field medical use continues.

Publications:

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9. Barry, K. G., Shambaugh, G. E., Goler, D., and Matthews, F. E.: A Self-Retaining Peritoneal Cannula (SPC) for Intermittent Peritoneal Dialysis. Clin. Res. 11: 237, 1963 (Abstract).

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12. Pollak, V. E., Schwartz, F. D., and Pirani, C. L.: Nephritis in Systemic Lupus Erythematosus. Clin. Res. 11: 250, 1963 (Abstract).

13. Seitzman, D. M., Mazze, R. I., Schwartz, F. D., and Barry, K. G.: Mannitol Diuresis: A Method of Renal Protection During Surgery. Accepted for publication by Jour. Urology.

14. Barry, K. G., Shambaugh, G. E., and Goler, D.: A New Flexible Cannula and Seal to Provide Prolonged Access to Peritoneal Cavity for Dialysis, Drainage, and Other Procedures. Accepted for publication by Jour. Urology.

15. Mazze, R. I., Schwartz, F. D., Slocum, H., and Barry, K. G.: Renal Function During Anesthesia and Surgery: I. The Effects of Halothane Anesthesia. Accepted for publication by Anesthesiology.

16. Barry, K. G., Shambaugh, G. E., Goler, D., and Matthews, F. E.: A New Flexible Cannula and Seal to Provide Prolonged Access to the Peritoneal Cavity for Dialysis. Presented at American Society for Artificial Internal Organs and accepted for publication.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803 MILITARY INTERNAL MEDICINE

Task 02, Metabolism and Nutrition (Metabolic and nutritional problems associated with injury)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Metabolism
Division of Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Lt. Colonel Kevin G. Barry, MC

Assistants: Major James P. Knochel, MC
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Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

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ABSTRACT

Project 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 02 Title: Metabolism and Nutrition (Metabolic and nutritional problems associated with injury)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Lt. Colonel Kevin G. Barry, MC
Major James P. Knochel, MC
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Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

The extensive use of peritoneal dialysis within the department for the treatment of acute renal failure has led us to consider the possibility of using the peritoneal membrane as an absorptive surface for administered antibiotics, nutrition, fluid, solute, and other drugs. If the peritoneal membrane can be used as an absorptive surface, a method would be available to markedly simplify the management of mass casualties and patients for whom intravenous therapy is difficult.

Abnormalities of fluid compartmentalization rather than in total body water appear to be of increasing importance in traumatic shock and surgery. A method for simultaneous determination of the three major fluid compartments of body water has been developed for man and animals. This technique will be applied to patients undergoing cardio-pulmonary by-pass procedures for cardiac surgery and, on a lesser scale, to other problems of critical illness. Presently available techniques should allow better understanding not only of

fluid shifts within the body but also acute shifts of electrolyte and non-electrolyte solute responsible for deaths in patients subjected to acute injury, trauma, or illness.

The Steroid Laboratory continues to make strides in the development of techniques for fractionation of various steroid groups. The gas chromatograph is being used productively, especially in the field of ketosteroid and pregnanetriol fractionation. This developmental work should make it possible not only for our own laboratory but other laboratories to unravel problems of steroid biosynthesis in response to various types of injury.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 02 Title: Metabolism and Nutrition (Metabolic and nutritional problems associated with injury)

Description:

This task includes: (A) Study of the absorption from the peritoneal cavity of fluid, solute, and nutritive substances across the serosal membranes. (B) Fluid and electrolyte shifts within the body in response to acute stress are poorly defined and more poorly understood. Frequently, these shifts will in themselves precipitate death. Perhaps, as often, these shifts lead to lethal toxicity of administered drugs within the body similar to the lethal toxicity of digitalis when hyperkalemia is suddenly combated. Our aim in this area is to delineate these shifts, look for indices to forecast them, and, finally, to prevent and combat them. (C) 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. Absorption of Antibiotics from the Peritoneal Cavity. Rabbits were used for control study of the absorption of penicillin, streptomycin, and chloromycetin administered in therapeutic dosages intraperitoneally. Blood level sampling in response to acute and chronic dosage was obtained. The animals were sacrificed at varying periods after antibiotic administration and no evidence of peritoneal adhesions or other anatomic toxicity was noted. The blood levels of antibiotics have not yet been determined but are being processed.

2. Absorption of Glucose and Water Across the Peritoneal Membrane. Pilot studies have been initiated and the evidence to date, while too meager to draw definite conclusions, indicate that absorption of glucose across the peritoneum is rapid and perhaps practical for therapy. Absorption of water given as hypotonic solution also appears to be much slower than expected. These studies in humans will be continued until sufficient evidence is

accumulated to permit definite statements as to the feasibility of this route of administration of fluid and glucose.

3. Body Fluid Determinations by Multiple Isotopic Dilution Techniques. Simultaneous injection of Cr^{51}, H^3, and S^{35} have not been as reliable as anticipated in the study of patients before and after surgery. Further animal studies have been initiated to seek out the facets of the problem.

4. Metabolic Edema. Studies of its mechanism(s) which appear to be related to capillary permeability and defective adrenal steroid biosynthesis are in progress. Publications of the results obtained thus far are in preparation.

5. Acute and Chronic Fluid Retention. Experience with the osmotic diuretic mannitol has indicated that it is effective and safe and should form the basis for therapy especially in patients with refractory fluid retention. Over 50 patients have been treated. Measurements of renal clearances and clinical response indicate that renal perfusion and glomerular filtration rate is usually depressed in these patients, thereby limiting the effectiveness of diuretic agents which act at a tubular level. Mannitol increases renal perfusion and glomerular filtration rate in addition to causing an osmotic diuresis. During mannitol infusion, therefore, sufficient fluid is delivered to tubular sites of reabsorption for tubular blocking diuretics to have an enhanced effect.

6. Adrenal Cortical Suppression and Stimulation. Studies on the standardized test of adrenal cortical function utilizing ACTH for stimulation and decadron for suppression are continuing in patients with Cushing's Syndrome. Two patients with Cushing's Syndrome have been studied and both stimulated with ACTH and appropriately suppressed with 8 mg. of Decadron. Of interest was a female patient with marked hirsutism and virilism who had marked elevation of urinary 17-ketosteroids which decreased 50 per cent of control values after Decadron suppression. At surgery, this patient was found to have an arrhenoblastoma. The androgen output of the ovary characteristically does not decrease with Decadron administration.

7. Pregnanetriol Determinations. Pregnanetriol determinations continue to be utilized in the study of patients with both adrenal and ovarian abnormalities.

8. Total Urinary Corticoid Spectrum. The simultaneous measurement of the 17-hydroxycorticosteroids, 17-ketosteroids and pregnanetriol from a steroid gum is being utilized in the study of patients with Cushing's Syndrome, arrhenoblastoma of the ovary and adrenal function in Klinefelter's patients. This method is quite useful in these studies.

9. Gas Chromatography Studies of Steroids. Gas liquid chromatography is being utilized in the measurement of urinary steroids. Methods have been worked out for the separation and quantitation of urinary pregnanetriols. In addition, methods have been worked out for the separation and fractionation of the 17-ketosteroids. Androsterone, etiocholanolone, dehydroepiandrosterone and epiandrosterone are converted into their tri-methylsilyl ethers, then introduced into a QF-1 column of the gas liquid chromatograph and are separated and quantitated. Work is continuing on the production of tri-methylsilyl ethers of urinary 17-ketosteroids and separation and quantitation with gas liquid chromatography in normals, patients with Klinefelter's Syndrome and in a patient with an arrhenoblastoma of the ovary.

10. The Use of SU-4885 in Endocrine Patients. Studies are continuing utilizing SU-4885 in the study of pituitary function in patients with Klinefelter's Syndrome, Cushing's Syndrome, and ovarian arrhenoblastoma. This drug continues to be a useful agent in the assessment of pituitary function.

11. Endocrine Evaluation of Patients with Klinefelter's Syndrome. Complete endocrine evaluation of patients with Klinefelter's Syndrome is continuing. These studies include buccal smears, karyograms, pituitary and adrenal function, testicular function and thyroid function. The majority of patients have chromatin positive buccal smears, XXY sex chromosome pattern, marked decrease in excretion of urinary 17-ketosteroids probably representing decreased gonadal and adrenal function with normal glucocorticoid excretion. These patients generally have increased urinary gonadotrophins and azoospermia. Of interest is the abnormality of thyroid function. Characteristically, nearly all of the 12 patients studied have a decreased I-131 uptake and low BMR's. The PBI's are borderline low in some patients and normal in others. The T-3 red cell uptakes are normal in all. The secretion rate of I-131 from the thyroid gland is normal in the four patients tested and results are pending in two others. The

thiocyanate test is normal in four patients tested and we are awaiting the results in two others. Clinically, these patients are euthyroid. The possibility exists that these patients have an iodide-trapping defect or an abnormal TSH which is mild. Special studies for definition of the defect are being carried out.

12. Thyroid Binding Globulin Decrease in a Patient with Multiple Endocrine Adenoma. A patient with hyperparathyroidism, an enlarged sella turcica, normal pituitary adrenal, thyroid and testicular function was noted to be euthyroid but had low PBI's and high T^3 red cell uptake. The PBI's ranged from 1.5 to 2.0 mg. per 100 ml. and T^3 red cell uptakes over 32 per cent. Dr. Robbins at the National Institutes of Health found a decreased thyroid binding globulin in this patient. The possibility exist that the abnormal thyroid binding is related to the excess parathormone secretion and the hypercalcemia. These studies will be repeated after parathyroid surgery.

13. Adrenal Carcinoma. Studies in adrenal carcinoma have been completed and published.

Summary and Conclusions:

Methods for the study of fluid compartmentalization have been developed utilizing isotopic dilution techniques and will soon be applied to the study of fluid shifts in acute injury, trauma or illness. Other studies are continuing in metabolic edema, fractionation of urinary steroids by gas chromatography, adrenocortical response to stress, Klinefelter's Syndrome, virilizing adrenal hyperplasia, adrenal suppression and stimulation, diabetes insipidus, hyperparathyroidism, abnormalities of growth hormone secretion, hypopituitarism, inappropriate ADH secretion, and response to SU-4885.

Major studies continue in dynamics of the peritoneal membrane regarding fluid and electrolyte transfer and the application of peritoneal dialysis for acute and chronic exacerbated renal failure, severe fluid and electrolyte derangements, acute endogenous or exogenous poisoning and absorption and excretion of antibiotics and digoxin by this route.

Investigation of the therapeutic and diagnostic efficacy of the osmotic diuretic mannitol in various types of fluid retention, poisoning and its application in patients with chronic renal disease undergoing major surgical procedures continues.

Publications:

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ANNUAL PROGRESS REPORT

Project: 3A O 12501 A 803, Military Internal Medicine

Task: 02 — Metabolism and Nutrition (Investigation and Analytical Determination of Drugs and Compounds of Toxicological Importance)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Clinical Chemistry
Division of Biochemistry

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Leo Kazyak
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Assistant: Gerald Houlihan, Pfc

Reports Control Symbol: MEDDH-288

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ABSTRACT

Project No: 3A O12501 A 803 **Title:** Military Internal Medicine

Task No: 02 **Title:** Metabolism and Nutrition (Investigation and analytical determination of drugs and compounds of toxicological importance).

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Applications of gas chromatography to analytical toxicology have not only proven successful but have exceeded expectations. In addition to the many drugs which can be isolated and detected by this technique new procedures have been developed for the analysis of volatile compounds. These procedures provide results within a few minutes which are specific, quantitative, and independent of specimen size. No special preparation of the specimen is necessary and the analysis may be repeated without depleting the specimen.

BODY OF REPORT

Project No: 3A O 12501 A 803 **Title:** Military Internal Medicine

Task: 02 **Title:** Metabolism and Nutrition (Investigation and analytical determination of drugs and compounds of toxicological importance).

Description: The versatility of gas chromatography has proven especially suitable for analytical toxicology. For screening suspect materials, it is rapid, specific and sensitive. Elaborate preparation of specimens is unnecessary since solvent extracts of blood, urine or tissue may be chromatographed directly after the initial volume of solvent has been reduced to concentrate the solute. Barbiturates, sympathomimetic amines, alkaloids, local anesthetics, and tranquilisers can be isolated and identified. Newly developed procedures have further simplified the analysis of volatiles by taking advantage of the partial pressure of volatile materials in equilibrium with the air above the surface of a liquid specimen and injecting an aliquot of the equilibrated vapor into the analytical system. This procedure enables rapid identification without altering or destroying the liquid specimen.

Progress:

1. New Techniques: For the analysis of volatile substances, such as alcohol in blood or urine, the sample is transferred to a 50 or 100 ml erlenmeyer flask fitted with a rubber diaphragm stopper. A small gage needle is inserted in the stopper to provide a port for inside pressure equilibration with atmospheric pressure. In accordance with Henry's law, the partial pressure in the gas space in the container above the liquid (head space) is the partial vapor pressure of the dissolved volatile in the solution. Analysis of an aliquot of this gas space represents a measure of the concentration of the volatile substance in the solution; therefore, an aliquot of the head-space gas is injected into the column and chromatographed. The Argon B-ionization (Sr^{90}) detector must be used since the thermal conductivity detector is not sufficiently sensitive for this purpose. Ethanol and methanol may be detected and quantitated within 3-5 minutes and within 10 minutes most common volatiles such as carbon tetrachloride, chloroform, ethylene glycol, chloral hydrate, etc. can be determined by this method. Most of the application to date has been to blood alcohol analysis, but further study of recovery of other volatiles from tissues and body fluids will be undertaken to extend this technique for the analysis of post mortem specimens with emphasis toward those specimens which have become somewhat autolysed.

2. New Drugs: Gas chromatographic analysis of drugs in biological specimens continues to be investigated in relation to new commercial compounds. These investigations are being made at several column temperatures and pressures to reduce the possibility of missing any of the compounds which can be determined by this technique. These data provide a profile of the compound as it reacts to temperature and pressure changes on a specific column substrate. In parallel with the compilation of ultraviolet spectral data, retention data on compounds being separated on the gas chromatograph are being collected also for distribution with the spectroscopy information.

3. Drug Distribution Studies: Some preliminary preparative gas chromatography investigations have been unproductive for the isolation and identification of drug metabolites. Equipment modifications have been undertaken and further studies have been planned, which may prove useful in following the fate and metabolism of drugs in humans and animals.

Studies on chloroquine deposition in the eye have continued and the drug has been found in corneal scrapings from patients on sustained chloroquine therapy. These findings are supported by a recent report by a British scientist who observed visual impairment of an individual being treated with chloroquine. Evidence of deposition of the drug in the cornea was presented. In addition to the data being collected on humans, animal experiments have been planned to improve present analytical techniques as well as to better evaluate the significance of the clinical data in relation to chloroquine levels in the various body fluids.

Summary and Conclusion: In analytical toxicology it is no longer practical or expedient to devise an individual test for each new potential poison introduced to the public. Indications are that gas chromatography may very well enable rapid screening of biological specimens for most of the poisons that are likely to be encountered in toxicological analyses. Data have been collected on approximately 100 compounds which include many not previously reported analytically. Many possibilities in gas chromatographic techniques remain to be investigated. The most interesting of these is the electron capture principle which has been applied to the detection of pesticide residues. Alleged sensitivity of this device is reported to be one hundred to one thousand times greater than the argon ionization detector. With such improvements in detector systems and continuing introduction of new column substrate materials, the use of advanced techniques such as the "head space" gas method for volatile poisons offers great promise of keeping abreast of the analytical requirements for the newer families of potentially toxic agents.

List of Publications:

1. L. Kasyak and E. C. Knoblock

An Application of Gas Chromatography to Analytical Toxicology. Accepted for publication by Anal Chem.

2. S. K. Abul-Haj, R. A. Ewald and L. Kasyak

Fatal Mushroom Poisoning: Report of a Case Confirmed by Toxicological Analysis of Tissue. Accepted for publication by The New England Journal of Medicine.

3. L. Kasyak and E. C. Knoblock

Gas Chromatography In Analytical Toxicology. Proceedings of the Third International Meeting in Forensic Immunology, Medicine, Pathology and Toxicology to be published in Science, Law, and Medicine.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 803, MILITARY INTERNAL MEDICINE

Task No. 02, Metabolism and Nutrition (Methods for the chemical analysis of foods)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Veterinary Subsistence Testing Laboratory
Department of Veterinary Microbiology
Division of Veterinary Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Ernest S. Windham, M.S.
Captain Alan D. Stevens, VC

Assistant: Pfc Quock S. Hom

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project No. 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 02 Title: Metabolism and Nutrition (Methods for the chemical analysis of foods)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 to 30 June 1963

Authors: Ernest S. Windham, M.S.
Captain Alan D. Stevens, VC
Pfc Quock S. Hom

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

1. The feasibility of chemical preservation of ice cream samples to be analyzed for fat and total solids was studied. A solution containing 2% merthiolate plus 1.4% sodium borate added in the proportion of 1 ml per 100 g ice cream was found to be a suitable preservative and did not affect adversely the results of fat and solids analysis.

2. A search for suitable reference standard solutions for cryoscopic tests on milk was continued. A solution of potassium acid pthalate (National Bureau of Standard standard sample) was studied for possible use as a reference standard in place of currently used NBS sucrose solutions, because of its greater stability. This compound appears promising for this purpose.

3. Gerber Fat Test equipment and methods for fat determination of dairy products, meats and cheeses, were evaluated to determine if these methods are as accurate and more rapid than those presently used in the Army Medical Laboratories. Results have been satisfactory for chocolate milks, homogenized milk and cream, and butter milk. However, these methods had limitations for determination of fat in whipping cream, ice creams, and meats. Further study is needed before use of these methods by Army Medical Laboratories is recommended.

4. A commercially available instrument for rapid determination of moisture content of diverse food products was evaluated. It was found to be unsatisfactory for testing fluid products. Moreover, it had limitations with regard to time necessary to conduct tests and the number of samples that could be handled. The instrument does not appear to be suitable for use in the conventional laboratory. It may have some usefulness for screening tests under field conditions.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: MILITARY INTERNAL MEDICINE

Task No. 02 Title: Metabolism and Nutrition (Methods for the chemical analysis of foods)

Description:

1. The shipment of ice cream samples in the frozen state from distant inspection stations to Army Medical Laboratories has posed problems of refrigeration and packaging. In many instances samples were spoiled or unfit for analysis upon arrival. A suitable preservative which would hold an ice cream sample in satisfactory condition for several days and still not interfere with analysis for fat and solids was sought.

2. Previous studies have shown the need for better freezing point standards for the cryoscopes used to determine adulteration of milk with water. The present sucrose solutions used as standards are subject to deterioration and do not freeze in the area of maximum interest, -0.550 to -0.500°C. The usefulness of other standard solutions was therefore investigated.

3. Evaluation of equipment and methods for fat and moisture analysis of foods was conducted to determine if recent advances in commercially available equipment is adaptable and suitable for use in the subsistence testing performed by Army Medical Laboratories.

Progress:

1. Studies were conducted on preserving ice creams with 1 ml of 2% thimerosal added per 100 g of sample and on the methods of preparing these samples in the laboratory for analysis. It was known from previous studies that thimerosal would prevent microbial spoilage of ice cream, however, it was frequently difficult to obtain a representative portion for analysis because of separation of lipids or proteins in the preserved samples. Methods for obtaining a homogenous mixture of preserved ice cream samples in which separation of organic compounds had occurred were examined. It was found that simple shaking or a combination of heating and shaking would not homogenize all samples. An Osterizer Blendor was tried, primarily because the container in which the blending was done could also be used for shipping, thus preventing loss of fat due to transferring of samples to blending cups. However, the heat generated by machine blending caused leakage of sample through the gasket seal and blending assembly. Satisfactory blending, allowing accurate determination of fat, could be accomplished by blending samples for 2 to 4 minutes in a Waring Blendor employing cups of appropriate size.

After determining the conditions necessary for handling the ice cream samples, 36 samples were exchanged between this laboratory and the Veterinary Division, 4th U. S. Army Medical Laboratory. Good agreement (within 0.1%) was obtained between laboratories, even though many of the preserved samples were badly churned and/or precipitated when received. It was shown through bacterial counts that this preservative is bacteriostatic rather than germicidal. Samples preserved with thimerosal could be stored under adverse temperatures (e.g. 37°C) for at least seven days before spoilage was evident. On the basis of findings, a procedure for preservation and preparation of ice cream samples for fat and total solids analysis was outlined and recommended for use by army laboratories.

2. The potential usefulness of solutions of potassium acid pthalate (the National Bureau of Standards primary standard for acidimetry) as a primary standard for freezing point determinations of milk were studied. The use of KH pthalate over present sucrose and sodium chloride standards has several obvious advantages. It can be easily weighed to provide accurate solutions freezing in the range of interest, it is not hygroscopic, and not readily subject to microbial contamination. Solutions prepared three months previously show no signs of change. Two standard solutions of potassium acid pthalate have been studied--2.5000 g of the salt per 100.000 g of water (F.P.=-0.424°) and 3.1000 g of the salt to 100.000 g of water (F.P.=-0.522°). Additional tests will determine if reproducible freezing point determinations are found with solutions of different lots of potassium acid pthalate.

3. Two commonly employed laboratory methods for the rapid determination of butterfat in dairy products are the Babcock and Gerber tests. The latter has been used more commonly in Europe. The Babcock test is the procedure usually employed in army laboratories. The Babcock test, and modifications thereof, are frequently found to be unsatisfactory for the accurate determination of fat content of homogenized milk and cream, chocolate drinks, ice cream and cheese. With these products, the Gerber test is reported to provide more satisfactory determinations. A series of tests was instituted with Gerber equipment to determine its applicability for use in army laboratories. Butterfat determinations were made on a variety of products. The reference test was the standard ether extraction method recognized by the Association of Official Agricultural Chemists. A comparison of test findings with the two procedures is shown in the table

Creamed cottage cheese (3 samples), yogurt (1 sample) and buttermilk (6 samples) gave results within 0.1% of official (ether extraction AOAC) method results.

Charring and column separations were frequently encountered in determinations on ice creams. More work is needed to attempt to establish optimum conditions for this product. Difficulties were found in determinations on ground beef (3 samples) as there is not enough mixing space in the Gerber test bottles to effect rapid sample solution. Up to 10 minutes of shaking was required.

COMPARISON OF ETHER EXTRACTION (AOAC) AND

GERBER METHODS FOR FAT DETERMINATION OF DAIRY PRODUCTS

Product & No. of Samples	Average Difference Fat %	Maximum Difference Fat %		Overall Average
		Individual Determinations	Duplicate Averages	
Milk, whole (37)	0.034%	0.10%	0.08%	Gerber averaged 0.020% higher fat
Half & Half (22)	0.184%	0.50%	0.50%	Gerber averaged 0.025% lower fat
Table Cream (9)	0.300%	0.90%	0.90%	Gerber averaged 0.24% higher fat
Whipping Cream (11)	0.490%	1.3%	1.0%	Gerber averaged 0.36% higher fat
Sour Cream (8)	0.540%	1.2%	1.2%	Gerber averaged 0.15% higher fat
Chocolate Milk (17)	0.056%	0.15%	0.12%	Gerber averaged 0.01% lower fat

4. A recent innovation in rapid moisture testing apparatus is one that will use varying size samples, thus not requiring adjustment of sample to a precise weight, usually 10.00 g. Such an instrument was obtained and its use is being evaluated. The balance mechanism incorporated into this instrument operated erratically. However, correction of this by the manufacturer is expected.

Trials in moisture determinations on various products show possibilities for its use in testing solid or semi-solid foods and animal feeds by inspectors or contractors. It is unsatisfactory for routine laboratory use since only one sample can be analyzed at a time. Each test determination generally took 30 to 45 minutes. Thus, the number of samples analyzed per day is limited and the man hours involved are excessive for routine laboratory purposes. The instrument does not work properly with liquids.

Summary and Conclusions:

1. It was found that with proper methods of sample handling and preparation for analysis, 2% merthiolate + 1.4% sodium borate would adequately preserve ice cream, and samples could be prepared in such a manner that satisfactory analyses for fat and solids could be made. The proper procedure for this was published in Technical Data Letter No. 4 and recommended for use by Army Medical Laboratories.

2. Potassium acid pthalate, NBS primary standard for acidimetry, was used in trials with the recording cryoscope to determine its possible use as a reference standard for milk cryoscopy. Solutions giving readings in the -0.424°C and -0.522°C ranges were readily prepared and have remained stable for a period of three months. Additional work will be done in other freezing point ranges.

3. Fat analyses of homogenized milk and creams, chocolate milks, butter milk, and cottage cheese, by use of Gerber methods and equipment have given comparable (generally within 0.1%) results to present AOAC methods, in less time. Results with heavy creams (as whipping cream), sour cream, ice creams, and meats have not been as satisfactory. Further work is needed before recommendation for use of this equipment and method can be made.

4. Evaluation studies of a rapid moisture testing apparatus were made. Difficulties were experienced because of a faulty balance mechanism. However, it was determined that the apparatus could have use as a rapid screening method for semi-solid or solid foods and animal feeds by field inspectors or manufacturers.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 803, Military Internal Medicine

Task 02, Metabolism and Nutrition (Clinical Use of Radioisotopes)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Biophysics
Division of Nuclear Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Maj Richard C. Reba, MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 02 Title: Metabolism and Nutrition (Clinical Use of Radioisotopes)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Author: Maj Richard C. Reba, MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

In an attempt to evaluate renal glomerular substances labelled with a gamma-emitting radioisotope, Cobalt-57 vitamin B_{12} was studied. Plasma and tissue binding sites were saturated prior to the study with stable vitamin B_{12} and the renal clearance of free vitamin B_{12} was compared to that of inulin. In addition, the renal extraction ratio was compared in dogs. These studies agree with the concept that free vitamin B_{12} is handled as a glomerular substance by both dog and man.

BODY OF REPORT

Project No. 3A 0 12501 A 803 Title: Military Internal Medicine

Task No. 02 Title: Metabolism and Nutrition (Clinical Use of Radioisotopes)

Description: A glomerular substance, i.e., one that is excreted solely by glomerular filtration, and labelled with a gamma-emitting radioisotope would result in a simple and rapid measurement of GFR. Based on the observation that at high plasma levels (greater than 12 mugm/ml of stable B_{12}) the renal clearance of free vitamin B_{12} (non-protein bound) was very near that of inulin, a correlation study was performed comparing these two substances. Binding sites were presaturated with stable B_{12}; however, determination of the total and free portions was not performed.

Progress: Initially 42 correlative measurements of glomerular filtration rate and 49 correlative measurements of the renal extraction ratios were performed in five mongrel dogs. The regression line (least squares) for the clearances was $C_{in} = 1.045\ (C_{B12}) + 0.77$. Correlation coefficient was .95. B_{12}/inulin clearance ratio was .88 ± .16 (1 S.D.) with a standard error of .02. The regression line for the extraction ratios is best described by $E_{in} = .98\ (E_{B12}) + .02$ with a correlation coefficient of .97.

This study was then extended to human patients and comparisons were made for 47 clearance periods in five patients. The least square regression line for this data is best described by $C_{in} = 1.02\ (C_{B12}) + .1$ with a correlation coefficient of .98. $\frac{C_{B12}}{C_{in}}$ ratios for these 47 comparisons was found to be .99 ± .07 (1 S.D.) with a standard error of the mean .01.

Comparison was also made between Creatinine/inulin clearance ratios and B_{12}/inulin clearance ratios. In a total of 68 comparisons in both man and dog, including some extraction ratios as well as clearance in which creatinine determinations were performed on aliquots of the same sample, comparisons were made. By statistical analysis of these selected comparisons, there is a significant difference between these two groups, $P<0.1>0.05$.

Summary and Conclusions: Using the present method it was concluded that the renal clearance of free vitamin B_{12} is an accurate and precise measurement of glomerular filtration rate.

List of Publications: None

ANNUAL PROGRESS REPORT

Project: 3A 0 12501 A 804 MILITARY PSYCHIATRY
(Analysis of Therapeutic Methods)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Psychiatry
Division of Neuropsychiatry

Period Covered by Report: 1 July 1962 - 30 June 1963

Principal Investigators: Lt Col Kenneth L. Artiss, MC
Lt Col Joseph V. Brady, MSC
Lt Col Harold S. Kolmer, Jr, MC

Assistants: Major Edward F. Krise, MSC
Major Elsie Krchnavi, ANC
Major Mary L. Bradley, ANC
Charles B. Ferster, Ph.D.*
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Pfc Gary Durst (B.A.)
Pfc Peter Stanley (B.A.)
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

*Institute For Behavioral Research, Silver Spring, Md.

ABSTRACT

Project No. 3A 0 12501 804 — MILITARY PSYCHIATRY (Analysis of Therapeutic Methods)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 - 30 June 1963

Authors: K. L. Artiss, Lt Col MC
J. V. Brady, Lt Col MC
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

The use of a therapeutic milieu as a treatment regime for chronic alcoholic soldiers has furnished a central method for the operation of a laboratory investigating a series of related studies including the job-oriented unit, patient government, group psychotherapy behavior, selected operant conditioning techniques, specific use of symptoms to achieve social ends and their disappearance when no immediate symptom-based interaction with the fate-determining staff is possible.

Following the successful completion of the operant conditioning project dealing with the quantitative study of human operant performance, an expanded program was initiated in the milieu therapy setting for the primary purposes of (a) controlling patient behavior by bringing under experimental control the maximum number of environmental variables in the alcoholic ward culture, and of (b) improving ward communication by integrating into the ward culture behavioral referents and concepts to supplement the existing terminology employed in patient-staff communication.

Further implementation of follow-up studies concerning schizophrenic soldiers treated with Milieu Therapy has included the location of control groups and preparations for the first publication next year.

BODY OF REPORT

Project No. 3A 0 12501 A 804

MILITARY PSYCHIATRY
(Analysis of Therapeutic Methods)

Description:

a. Human Clinical Experimental Studies of Chronic Alcoholic Soldiers 15 August 1961 to 30 June 1963, continued to explore 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.

b. The aversive and reinforcing contingencies of the ward environment were functionally analyzed and arranged, wherever possible, into appropriately structured contingencies for the shaping of desired patient behavior. Emphasis was placed upon the utilization of positive means of controlling patient behavior, and aversive control was discarded wherever positively reinforcing techniques proved sufficient. The initial arrangement of the environmental contingencies made it possible for the patients to obtain generalized reinforcers for successful completion of segments of programmed instruction dealing with the functional analysis of behavior. The ward staff was also provided with the same programmed instruction; however, explicit reinforcing contingencies were not available to the staff. Generalized reinforcers earned by the patients could be exchanged for passes and other privileges.

c. All patients treated in the Walter Reed experimental Milieu Therapy ward, 1956-1961, have been followed up regarding progress and status at intervals of not over six months since 1957, records and charts being maintained in an office at Forest Glen.

Progress:

a. The Chronic Alcoholic Soldier and Milieu Therapy:
A modified treatment open psychiatric ward became operational on 15 August 1961 and remained so until 15 June 1963, studying the biosocial development and the total milieu of the chronic alcoholic soldier. Individual, as well as group psychotherapy was conducted and analyzed in concert with those aspects of group process which aid in the formation of therapeutically beneficial sub-groups. Field social anthropological studies have been 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 more informational value, after which the studies were adjusted and re-evaluated. During the year the techniques for obtaining a ten-year follow-up on the results of treatment in the experimental ward have continued to develop.

In addition, certain selected techniques were established to critically test the value of operant conditioning as a therapeutic adjunct. These studies were conducted with the assistance of Dr. Charles B. Ferster of the Institute of Human Behavior, University of Maryland. Regular consultation has been obtained with Dr. David McK. Rioch and Lt Col Kenneth L. Artiss, of this Institute, Dr. Dexter Bullard of Chestnut Lodge, Dr. Alfred C. Wood, Temple University School of Medicine, Philadelphia, Pa., and Dr. Stanley Eldred, McLean Hospital, Harvard University, Boston, Massachusetts.

b. Patient activity at the beginning of the program was marked by a low rate of performance, while the staff members with no explicit reinforcer progressed rapidly through the material. Within a brief period, however, the patients' mean rate of progress showed marked positive acceleration that soon exceeded and remained well above the steadily decreasing mean rate of performance of the ward staff. The patients' performance on the programmed material and participation in socially instructional activities continued to be successfully maintained on a ratio schedule.

The programmed instruction in the analysis of behavior was supplemented for patients and staff by formal and informal tutorial sessions during which particular emphasis was placed upon the detailed functional analysis of interpersonal interactions, examples of which were selected from behavior observed within the ward culture. Many of the referents and behavioral concepts presented by the several instructional methods became thoroughly integrated into the working vocabulary of the ward culture providing valuable tools for patient-staff communication.

c. Follow-up investigators discovered some four years ago that previously treated schizophrenic soldiers would respond actively and informatively when queried by personal letter and not subjected to a "form-letter" approach. This method brought the response rate up to 100% at times, previously unheard of in this area and encouraging to the workers. The last point is of no small importance for follow-up work is largely thankless and the amount of effort often unsung and unappreciated.

This study has become integrated and accepted as essential in the department. Now five years along, its members are assembling matched control cases and preparing for the first assemblage of data for publication next year.

Summary and Conclusions:

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

The successful maintenance of patient behavior on programmed instruction through the use of intermittent reinforcement suggests optimistic possibilities for the reading research project recently begun. The primary experimental tool is a basic reading course in programmed form. The basic aims of the project are: (a) to study the effects of intermittent reinforcement employed with programmed instruction of basic reading; (b) to establish where the process of learning to read breaks down in alexic and dislexic subjects; (c) to create a program that can serve as a tool in diagnosing brain damage isolating lesions by establishing which faculties of learning and recall are inoperative in the brain damaged subject.

Follow-up studies on soldiers previously treated via Milieu Therapy techniques are progressing satisfactorily and first publication is being planned.

List of Publications:

Artiss, K. L. Communicating with Schizophrenic Persons, 1962 Annual Sigma Phi Gamma Lecture, Spring Grove State Hospital, Baltimore, Md., November 1962 (to be published in Psychiatry).

Artiss, K. L. Human Behavior Under Stress - From Combat to Social Psychiatry. Mil. Surgeon (in press).

Artiss, K. L. Psychoendocrine Aspects of Acute Schizophrenic Reactions, (with Sacher, E. J., Mason, J. W., and Kolmer, H.S.), Psychosom. Med. (in press).

Artiss, K. L. Modes of Communication Used by Military Patients Diagnosed as Schizophrenic (Acute) as Alcoholic (Chronic) and by Military Offenders (AWOL), (with Kolmer, H. S., and Kurke, L.) Proc. Assn. for Research in Nervous & Mental Diseases, (in press).

Brady, J. V. and Schuster, C. R. The Discriminative Control of a Food Reinforced Operant by Interoceptive Stimulation. Pavlovian Journal of Higher Nervous Activity, Podsosensky Per. 21 Moscow 5-64, USSR, 1963 (in press).

Brady, J. V. Operant Methods in the Production of Altered Physiological States, in Honig W. (ed.) Operant Behavior and Psychology, Appleton-Century (in press).

Shellhase, L. J. A Study of the Self-governing Activities of a Schizophrenic Group. Int. J. Soc. Psych, August 1962, pp 211-219.

Shellhase, L. J. Social Service and the Psychiatric Patient, Howard Allen Inc., Cleveland, O., fall 1963 (in press)

ANNUAL PROGRESS REPORT

Project: 3A 0 12501 A 804

MILITARY PSYCHIATRY
(Preventive Psychiatry)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Psychiatry
Division of Neuropsychiatry

Period Covered by Report: 1 July 1962 - 30 June 1963

Principal Investigators: Major Lewis Kurke, MC
Major Leslie J. Shellhase, MSC
David H. Marlowe, Ph.D.

Assistants: SFC (E-7) Gilbert Brenner
Dorothy A. Baker, MSW*
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 804

MILITARY PSYCHIATRY
(Preventive Psychiatry)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 - 30 June 1963

Authors: L. Kurke, Major, MC
L. J. Shellhase, Major, MSC
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

The application of techniques of personal interview, study of unit social interaction and analysis of the family group developed in the therapeutic milieu subtask has been attempted in a study of the Army AWOL Offender. Since the feasibility of these techniques were demonstrated in an exploratory phase of this field study, and a field team established at Ft Meade, intensive data acquisition has proceeded for the past year.

During the past year, field materials relating to inter-group conflict gathered in Somalia East Africa, were organized and analyzed. Analyses have been made of the functions of both inter-group conflict and intra-group conflict in respect of the processes of certain types of social systems and the organization and patterning of concommitant social behavior.

BODY OF REPORT

Project No. 3A 0 12501 A 804

MILITARY PSYCHIATRY
(Preventive Psychiatry)

Description:

a. During the year the AWOL Offender Study proceeded to intensive data gathering by means of group and individual depth interviews with stockade prisoners, unit contacts and interview with company commanders, NCO's and enlisted men, and visits to the homes of stockade prisoners in order to interview their family members and to evaluate the setting of the family in its community. Emerging regularities of socio-economic level, educational background, work history and geographical origin have made possible the development of increasingly parsimonious criteria for the selection of subjects to conform with self-imposed restrictions on travel, now essentially limited to the Washington, Baltimore and Philadelphia areas, and their adjoining rural environs.

b. Materials have been published on the relationship between the incidence of availability of conflict to groups as a problem solving mechanism in response to changed expectations within their political systems. Further materials have been published on the relationships between concepts of group membership and social bounding processes as these control the choice of aggressive behavior in both potential and actual conflict situations. Particular attention has been paid, in this work, to the relationship of modes of extension of group membership; kinship, political alliance, geographical alliance, etc., to potential choices of aggression in the solution of disputes and the ordering of affiliation and alliance when such problems arise. Materials have also been published on the behavioral circumscriptions for and against participation in conflict situations that allocated roles of individual participants. A recent field trip to Kenya, Somali, East Africa, was used by the investigator for clarification in such areas.

Progress:

a. Data gathering has been completed for a demographic study of the Ft Meade stockade population during the period 1 January - 31 March 1962. The information available from the OPMG 498 has been coded, punched on IBM cards, and sorted for and tabulated for a large number of factors and for a series of paired variables distinguishing the AWOL offenders from the remainder of the stockade population. This data has provided the basis for two Master's thesis offered in partial fulfillment of degree requirements by students at the National Catholic School of Social Science, Catholic University of America. Among the suggestive findings of this demographic study are the preponderance of first enlistment RA volunteers in the AWOL sample, who are also school dropouts, and unskilled recurrently unemployed members of the lowest socio-economic group in the country. There has emerged little support

for the assumption that this population is characterized by a history of civil delinquency arrests, although there is some suggestion that these individuals are deviant even within their own social class.

Interview with family members in their homes have met with a marked degree of cooperation from both prisoners and families. While it is clear that AWOL behavior does not occur in isolation from family membership, there is some evidence of the existence of a mutual disorder in the use and signification of symbols within the family, well adapted to the language behavior within the family but at the same time crippling, to a degree, beyond its limits. Evidence has accumulated that the AWOL Offender and his family share a sense of absolute deprivation, a limitation on the range of possible work and social options, and a limited repetoire of behaviors necessary for adaptation to our complex commerical-industrial society.

In order to accumulate data and test the validity of certain sociometric insights, a self-administered questionnaire was developed and given to about 250 stockade prisoners and to about 700 members of functioning operational units at Ft Meade. In addition to the opportunity for developing sociometric charts for entire battery and company sized units, the questionnaire also endeavors to shed some light on the validity of emergent generalizations from the interview data. This data, which is not yet coded, is an attempt to evaluate the behavior of the prisoner population against a background of adequately functioning enlisted men affiliated in groups. It is expected that this data will provide an estimate of the meaningfulness of the interview data in comparison with a group of enlisted men comparable by background, but differing in perceptions of themselves and of their social roles.

b. At present work is in process, which it is expected will be completed and published during the coming year, that explores some of the general and theoretical problems and possibilities generated by the work completed on the Somali. This work, for the most part, relates the area of conflict to the social system as a structure of differential concensuses and contradictory models for its participants. Further materials are also in preparation on the role of bargaining and negotiatory processes in conflict situations, the problems of social deviance as they relate to the ordering of conflict, and further explorations of the relationship between group membership and the use of differential evaluative processes in the preception of the conflict producing event.

Summary and Conclusions:

During the past year, the AWOL Offender Study has continued data gathering through the study of confined prisoners, their families, and their units. In addition, a demographic analysis of prisoners at the Ft Meade stockade has been coded, and a sociometric questionnaire completed by a sample of prisoners and of members of operational military units. Data has emerged that suggests regularities in the behavior of their social peers. It is expected that the next year will see completion of the data gathering phase of the study and further concentration on analysis.

Analyses have been made of the role played by aggressive conflict in the life of a specific social group. Special relationships between the ordering of group membership and the availability of conflict as a problem-solving mechanism have been enunciated for this group, the Somali. Future work contemplates a more general theoretical exploration of a number of factors and assumptions derived from the field materials and their use in the analysis of conflict in wider social orders and in relationship to other areas of social behavior.

List of Publications:

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Marlowe, D. H. "Commitment, Contract, Group Boundaries, and Conflict", Science and Psychoanalysis, Grune & Stratton (in press).

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Brand, C. G. The Uneducated Soldier - A Study of School Drop-Out Among AWOL Offenders. Unpublished MSW Thesis, National Catholic School of Social Service, The Catholic University of America, Washington, D. C. 1963.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 804, Military Psychiatry (Psychological, Physiological, Metabolic and Endocrinological Homeostatic Mechanisms in Health and Disease.)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Departments of Neuroendocrinology and
Experimental Psychology
Division of Neuropsychiatry

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

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ABSTRACT

Project 3A 0 12501 A 804, Military Psychiatry (Psychological, Physiological, Metabolic and Endocrinological Homeostatic Mechanisms in Health and Disease.)

Reporting Installation: Walter Reed Army Institute of Research
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Authors: John W. Mason, MD; Joseph V. Brady, Lt Col, MSC; Edward H. Mougey, MS; John W. Boren, PhD; Bernard Migler, Capt, MSC; William C. Black, Capt, MSC; JacSue Kehoe, PhD; Bernard Beer, MS; William Hodos, Capt, MSC; Gerard Smith, Capt, MC; and David Werdegar, Capt, MC

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

Neuroendocrine studies of stress continue to be focused primarily on the systematic investigation of hormone response patterns in monkeys and human subjects to both acute and chronic psychological stress. Stereotyped response patterns, involving marked changes in adrenal, gonadal and thyroid hormones, have been found in various types of psychological stress. Our approach to stress physiology has been broadened in two ways during the past year. First, with the development of new methods, we have been able to make observations of selected cardiovascular and gastrointestinal functions so that the integration of endocrine and autonomic regulatory changes can be studied. Secondly, we have initiated the study of patterns of hormonal change in a variety of physical stress situations such as muscular exertion, cold, trauma, and hemorrhage so that comparisons can be made with the regulatory changes in psychological stress. Clinical studies of psychiatric and medical patients continue to indicate that the psychoendocrine approach may have far reaching implications in both the study of mental and physical illness as well as in elucidating normal mechanisms of bodily integration during stress.

The experimental analysis of behavioral stress has continued to focus upon temporal and environmental factors related to adaptive mechanisms in laboratory animals. The research effort has been aimed at three major problem areas: the development of methods for inducing environmental stress, the analysis of stress-induced changes in behavioral performance, and the exploration of basic behavioral phenomena which can provide measures of the effects of environmental stress. The maintenance of avoidance behavior for prolonged periods of time has been most heavily emphasized as a stress-inducing procedure, although fear stress and the stress generated by demanding performance requirements have also been studied. The behavioral

phenomena under investigation include temporal spacing of avoidance behavior, learning of complex behavioral chains, variability of response, preference among behavioral chains, stimulus generalization gradients, and precision timing behavior.

BODY OF REPORT

Project 3A 0 12501 A 804, Military Psychiatry (Psychological, Physiological, Metabolic and Endocrinological Homeostatic Mechanisms in Health and Disease.)

Description:

Patterns of hormonal change in psychological stress have been studied in the rhesus monkey during 72-hour avoidance sessions, during adaptation to the restraining chair, and during various types of chronic conditioned emotional stress. Clinical psychoendocrine studies have been concerned with parents of children with leukemia, severely depressed patients, and pilot studies have been initiated with selected medical, surgical, and neurological patients. The study of patterns of hormonal change in physical stress have involved experiments on hemorrhage, muscular exertion, and cold in the rhesus monkey and studies on the effect of surgical trauma in patients with back injury. Basic work on the development of new hormone assay methods and on basic neuroendocrine physiology necessary for the interpretation of stress experiments has continued.

The following report describes the progress made toward an experimental analysis of behavioral stress. The research has been carried out in the laboratory with animal subjects. Investigation has been concerned primarily with the production of behavioral stress, the analysis of the stress-induced decrements in behavioral performance, and the study of basic behavioral phenomena which can serve as dependent variables in the analysis of stress.

Progress:

I. Neuroendocrine Studies of Stress

A. Hormonal Patterns in Psychological Stress

Studies of hormonal patterns (17-hydroxycorticosteroids, epinephrine, norepinephrine, estrogens, aldosterone, androgens, and thyroid hormones) before, during and after 72-hour conditioned avoidance sessions in the rhesus monkey have been continued, with the addition this year of concurrent measurements of heart rate, blood pressure, gastric secretion of pepsin and acid and gastric motility. These experiments indicate that every endocrine and autonomic system studied undergoes marked and characteristic changes in association with this type of stress and emphasizes the importance of studying stress in its full temporal aspects, including relatively prolonged recovery period. Some modification of hormone response pattern has been observed with repetition of 72-hour avoidance sessions at monthly intervals. More profound changes in hormonal response patterns however have been observed recently in an animal on a

chronic stress program involving only four days of rest between each of a long series of repeated 72-hour conditioned avoidance sessions. Basal 17-hydroxycorticosteroid levels are extremely low, approximately 25% of normal, and frequently it is observed that 17-hydroxycorticosteroid levels are suppressed even lower during the avoidance session. When building alterations are permitted, cubicles will be constructed to permit additional chronic stress experiments of this type, in which relationships between experimentally induced chronic hormonal imbalances and somatic illnesses will be studied. Continuing investigation of endocrine responses to chair restraint indicates that some hormones, for example the androgens, may require as long as three or four weeks before stable levels are reached after the animal is installed in the chair.

Within the past year a clinical study has been completed in which the chronic mean urinary 17-hydroxycorticosteroid levels of individual parents of children with leukemia have been successfully predicted by two psychiatrists on the basis of evaluation of emotionality and defensive structure. These studies have drawn attention to the major importance and the need for investigation of normal mechanisms of psychological defense which serve to prevent, minimize or counteract emotional disturbance during stressful life experiences. It appears that the psychoendocrine approach may be a useful adjunct in the systematic classification of various styles of defense. Studies of depressed patients indicate that depression syndromes constitute a heterogeneous clinical category from a psychoendocrine viewpoint. While many depressed patients show strikingly elevated urinary 17-OH-CS levels, especially during crises, others may show very low corticosteroid levels. These findings have stimulated the close clinical reevaluation of the two subgroups which indicates considerable likelihood that they may be subsequently differentiated on behavioral grounds as well. Preliminary studies of manic patients have shown very low urinary 17-OH-CS levels, suggesting that emotional distress is minimal in manic phases of psychiatric illness in contrast to the higher level of distress during the depressed phases in cycling subjects.

Studies of hormonal patterns in selected medical patients in diagnostic categories such as anorexia nervosa, obesity, and rheumatoid arthritis indicate pronounced disorders of both basal hormonal balance and hormonal response to psychological stress. These studies are among the most intriguing of the recent year and indicate an urgent need for extending the psychoendocrine approach to the study of various medical illnesses.

B. Hormonal Patterns in Physical Stress

Several methods have been devised for the study of muscular exertion in the rhesus monkey. One of the most successful has been to require monkeys to lift varying amounts of weight in order to obtain food. With such a technique, it has been possible to increase muscular

workload from a basal level of about 300-foot pounds per day to over 10,000-foot pounds per day. Such increased exertion is rather consistenty accompanied by moderate urinary 17-hydroxycorticosteroid elevations, generally not exceeding about a twofold increase over basal levels. There is a close correlation between the amount of exertion on a particular day and the urinary corticosteroid level, suggesting that the physical rather than the psychological component in these experiments may be critical. In order to evaluate this problem, further, monkeys are also being studied during a natural climbing form of exercise in a floor to ceiling cage. Hormonal patterns are being studied in both situations.

A series of about ten monkeys has now been completed in which the effects of prolonged hemorrhagic shock have been studied upon both autonomic and endocrine systems. These experiments have been conducted with normal, unanesthetized, chair-adapted monkeys with chronic indwelling aortic catheters for blood pressure and heart rate measurement, and have eliminated the influences of anesthesia, acute surgery and environmental stimuli present in most work on hemorrhagic shock. Marked plasma 17-OH-CS and aldosterone changes have been observed and measurements of gonadal and thyroid hormones are currently being made.

A controlled temperature environmental chamber has been modified to permit the study of cold on hormonal balance in the monkey. Preliminary results indicate a slow but substantial urinary 17-OH-CS elevation during a three-day exposure to 50°F. temperature. Other hormonal measurements will follow.

Plasma and urine samples have been collected from five patients undergoing surgery for ruptured intervertebral disks. Urinary 17-OH-CS elevations of two to threefold magnitude have been observed. Measurements of hormonal patterns are underway and indicate that androgen and estrogen responses to trauma may differ from those to psychological stress.

C. Other Studies

There is continued emphasis on basic biochemical methodology since it appears that a major limitation preventing psychoendocrine research from widespread application in psychiatry and medicine is the costly and time-consuming nature of most hormone assay procedures. Two general analytical principles offer realistic hope of breakthrough in this area, gas chromatography for small molecular hormones and radio-immunochemical methods for the peptide and protein hormones. During the past year, Dr. Tolson has developed a gas chromatographic method for urinary androgen measurement which will at least triple our former output. In work under way only four months, excellent standard curves for plasma insulin assay have been obtained, using the Berson-Yalow procedure, indicating that amounts of insulin as low as .001 microgram can be measured. In a physiological evaluation of a new method for measuring tri-iodothyronine and thyroxine separately in plasma, distinctly different response patterns to TSH injection have been demonstrated in the monkey. In other basic

physiological studies it has been shown that virtually every hormone measured follows a regular diurnal variation in level including estrogens, androgens and aldosterone.

II. Experimental Analysis of Behavioral Stress

A study has been completed which describes the stimulus control produced by multiple warning stimuli in the adjusting avoidance procedure. The basic procedure arranged that a certain amount of shock-free time was stored for each avoidance response. When multiple stimuli indicated the amount of shock-free time, the animal subjects spent most of the time relatively close to the shock and started to respond only when the shock was near. The more adequate the stimuli, the more closely the animals approached the shock. When all stimuli were removed, the animals usually kept the shock as far away as the procedure permitted.

The effects of prolonged fatigue and avoidance stress have been studied with the adjusting avoidance technique. Deterioration of the behavioral performance, with and without multiple warning stimuli, was compared during a continuous three-day session. While the shock frequency rose sharply over the three days, the increase was approximately the same for the two stimulus conditions. However, the temporal distribution of responding without stimuli was markedly shifted toward the shock, whereas with stimuli the distribution changed only slightly.

A technique for studying the learning of behavioral chains has been developed which retains the advantages of the individual subject approach and of steady state phenomena. Monkeys were trained in a chamber containing four groups of three levers. For each daily session the monkey's task was to learn a new four-response chain by pressing the correct lever in each group. After a number of sessions, a stable pattern of learning was established, and the number of errors declined to a steady state.

The technique was then used to determine how "instruction" stimuli (lights indicating the correct levers) and punishment of errors by time outs affected the acquisition of new chains. When the instruction stimuli were removed it was found that the performance had not been improved. The application of time outs after incorrect responses, however, was quite effective in reducing errors (more than a tenfold effect). The basic effect of the time outs was to prevent the addition of "superstitious" members to the behavioral chain.

A variation of the paced avoidance procedure has been developed as a stress-inducing situation. The pacing procedure requires that the monkey must respond within a narrow time interval or receive a shock. The new procedure automatically adjusts the time interval on the basis of shocks per hour so that the difficulty is regulated to a level commensurate with the animal's ability to meet the requirement. The adjusting procedure allows the experimenter to set the average difficulty of the task (thus,

the amount of stress), and it also permits an accurate assessment of the animal's timing behavior. Collaborative work on stress-induced gastric secretion is being planned, where the stress will be produced by task difficulty and by fatigue.

An experiment now in progress is studying how reinforcement schedules influence behavioral variability in a multi-choice situation. Rats are pretrained with a single lever on continuous, fixed ratio, and variable ratio reinforcement schedules and are then given access to four levers simultaneously. The early results indicate that continuous and fixed ratio pretraining generates a preponderance of responses on one or two levers, while variable ratio pretraining generates an even distribution over the four levers. In addition some evidence suggests that this effect is more pronounced in young animals and drcreases with age.

A study is in progress concerning the preference between behavioral chains. Animal subjects are given a choice between a DRL chain, where the food reinforcement is given only if the subject waits 15 seconds between two responses, and a fixed ratio chain, where the subject must make a fixed number of responses to be reinforced. Five fixed ratio chains are used, requiring either 10, 40, 70, 100, or 130 responses. Auditory cues indicate to the animal which fixed ratio chain is in effect. While the extensive training required for this study is not yet complete, the early results show that it is possible to obtain a stable curve for each subject describing his DRL-fixed ratio preference as a function of the fixed ratio chain available. Once such a curve is obtained under baseline conditions, the food deprivation level will be manipulated to see how the subject's preference curve is altered.

Studies have been carried out to determine the effects of stimulus generalization testing procedures on chains of operant responses. Rats were trained to make two consecutive responses precisely spaced in time to obtain food rewards, when a bright light was present. When the light was dim the precision timing behavior was never rewarded. In test sessions when lights of intermediate brightness were presented, the frequency of execution of the two-response chain was reduced. The timing behavior, however, was relatively unaffected across most of the stimulus intensity continuum.

Research has been carried out on the nature of stimulus generalization gradients. Rats were trained to make two consecutive responses precisely spaced in time to obtain food rewards in the presence of a low frequency click rate. In the presence of a high click rate the animals could execute the two responses at maximum speed. When tested at intermediate click rates the performance was characterized by intermediate behavior between the spaced responses and the high speed response sequences. A curve of the average performance across the stimulus continuum yielded a conventional stimulus generalization gradient. Closer inspection of the individual components of the behavioral performance

rather than the average data at each stimulus point revealed that the performance was in fact bimodal and the smooth stimulus generalization gradients were artifacts of averaging techniques.

Research has been completed which describes the effects of fear stress on precision timing behavior. Rats were trained to make two consecutive responses precisely spaced in time to obtain food rewards. Occasionally a five-minute warning signal was presented followed by an unavoidable foot shock. Behavior during the fear-producing warning signal was characterized by infrequent execution of the two-response sequence. The precision of the timing behavior, however, was relatively unaffected.

Summary and Conclusions:

Neuroendocrine studies of stress continue to be focused primarily on the systematic investigation of hormone response patterns in monkeys and human subjects to both acute and chronic psychological stress. Stereotyped response patterns, involving marked changes in adrenal, gonadal and thyroid hormones, have been found in various types of psychological stress. Our approach to stress physiology has been broadened in two ways during the past year. First, with the development of new methods, we have been able to make observations of selected cardiovascular and gastrointestinal functions so that the integration of endocrine and autonomic regulatory changes can be studied. Secondly, we have initiated the study of patterns of hormonal change in a variety of physical stress situations such as muscular exertion, cold, trauma, and hemorrhage so that comparisons can be made with the regulatory changes in psychological stress. Clinical studies of psychiatric and medical patients continue to indicate that the psychoendocrine approach may have far reaching implications in both the study of mental and physical illness as well as in elucidating normal mechanisms of bodily integration during stress.

The experimental analysis of behavioral stress has continued to focus upon temporal and environmental factors related to adaptive mechanisms in laboratory animals. The research effort has been aimed at three major problem areas: the development of methods for inducing environmental stress, the analysis of stress-induced changes in behavioral performance, and the exploration of basic behavioral phenomena which can provide measures of the effects of environmental stress. The maintenance of avoidance behavior for prolonged periods of time has been most heavily emphasized as a stress-inducing procedure, although fear stress and the stress generated by demanding performance requirements have also been studied. The behavioral phenomena under investigation include temporal spacing of avoidance behavior, learning of complex behavioral chains, variability of response, preference among behavioral chains, stimulus generalization gradients, and precision timing behavior.

List of Publications:

1. Boren, J. J. and Feild, G. E. An adjusting avoidance procedure with multiple auditory and visual stimuli. Paper read at the Eastern Psychological Association, New York, April 1963.

2. Boren, J. J. The effects of the tertiary and the quaternary forms of scopolamine upon fixed ratio and fixed interval behavior. Paper read at the American Psychological Association, St. Louis, September 1962.

3. Boren, J. J. Behavioral changes during extended avoidance sessions. Eastern Psychological Association, Atlantic City, 1962.

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8. Bunney, W. E. Jr., Mason, J. W. and Hamburg, D. A. Correlations Between Behavioral Variables and Urinary 17-Hydroxycorticosteroids in Depressed Patients. (To be published in Psychosom. Med.) (Abstract).

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ANNUAL PROGRESS REPORT

Project: 3A 0 12501 A 804 **Military Psychiatry**

(Blood Brain Barrier and the Responses of Cerebral Tissue to Injury (NP))

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Neurophysiology
Division of Neuropsychiatry

Period covered by report: 1 July 1962 through 30 June 1963

Principal Investigator: CAPTAIN LAWRENCE C. McHENRY, JR., MC

Assistants: Mrs. Ulla Slonicki
Miss Ronda Motter

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project: 3A 0 12501 A 804 — **Military Psychiatry**

(Blood Brain Barrier and the Responses of Cerebral Tissue to Injury (NP))

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Neurophysiology
Division of Neuropsychiatry

Period covered by report: 1 July 1962 through 30 June 1963

Author: CAPTAIN LAWRENCE C. McHENRY, JR., MC

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

An improved method for the quantitative determination of cerebral blood flow in man utilizing the Fick principle has been developed. The method is based on measuring a krypton desaturation curve following saturation of the brain. Readily available equipment is used to administer krypton and to analyze for krypton concentration. The results obtained in a study of 25 normal individuals are similar to accepted values. A study of the effects of passive hyperventilation on the cerebral circulation and metabolism of conscious man was performed using the method. The effects of anesthesia on cerebral circulation and metabolism are being evaluated. Studies of the cerebral circulation and intracranial pressure are being performed on patients with brain edema.

BODY OF REPORT

Project No. 3A 0 12501 A 804

Military Psychiatry

(Blood Brain Barrier and the Response of Cerebral Tissue to Injury (NP))

Sub-title: Changes in cerebral circulation associated with alterations in cerebral volume.

Description:

Alterations in cerebral volume, such as occur with brain edema, or brain "shrinkage" after hyperventilation, have been observed during craniotomy, but can not be directly estimated or evaluated in the intact patient except by the clinical manifestations of brain swelling. Since direct measurement of brain volume changes is not feasible, information must be obtained by using indirect methods.

Changes in the volume of intracranial contents will be reflected by alterations in intracranial pressure. Quantitative fluid shifts between intracranial vascular, CSF and tissue fluid compartments can not be evaluated by pressure change, but quantitative studies of cerebral blood flow will give some indication of changes in cerebral hemodynamics that occur with brain fluid shifts. Changes in cerebral volume or circulation are also reflected by alterations in brain electrical activity occurring on the electroencephalogram and by changes in electrical impedance measured by the rheoencephalogram.

The first step in carrying out this project was the development of a technically applicable method to quantitatively determine cerebral blood flow. In a previous report (WRAIR Annual Progress Report, 1 July 1961 through 30 June 1962) a method described by Albert to determine cerebral blood flow was evaluated and was considered to most likely be inaccurate. As a consequence of this experience a technique to determine cerebral blood flow was developed which is considered to be accurate.

During the past year, 5 separate phases or parts of the main project have been either completed or developed further:

1. The quantitative determination of cerebral blood flow in man by a desaturation technique.
2. The effects of passive hyperventilation on the cerebral circulation and metabolism of normal man.
3. An evaluation of the Schuhfried rheoencephalogram (REG).
4. The effects of halothane anesthesia and passive hyperventilation on cerebral circulation and metabolism.
5. Studies of cerebral circulation and intracranial pressure in patients with brain edema.

Progress:

1. The quantitative determination of cerebral blood flow in man by a krypton-85 desaturation method.

A desaturation method based on the Fick principle to determine cerebral blood flow originally was considered feasible by Kety, but was not utilized until Lewis' studies in 1960. Coronary blood flow had been studied previously using nitrous oxide desaturation by Bing and Goodale and renal blood flow studies were reported by Brun. Lewis studied cerebral blood flow using both nitrous oxide and krypton-79

with saturation and desaturation methods. In 1961 Kimpensky reported using a nitrous oxide desaturation method.

In spite of the existence of these various techniques, it was considered worthwhile to develop a krypton-85 desaturation method to simplify two technical problems in the determination of cerebral blood flow using the Fick principle.

First, when the saturation methods of Kety or Lassen are used, smooth curves are obtained only by maintaining a constant concentration of inert gas during inhalation. This is easily done when using nitrous oxide, but when using krypton-85 or krypton-79, special equipment is needed to maintain a precise concentration of the tracer. In the desaturation methods the maintenance of a precise concentration of inert gas during saturation is unnecessary. When a desaturation study is carried out, mask leaks are unimportant, and no special equipment is needed during the saturation phase of the process. Since there is no mask on the patient's face during the desaturation study, any influence of anxiety upon cerebral hemodynamics is reduced.

Secondly, in the method described here, a glass-jacketed Geiger tube is used to determine krypton-85 concentration. Manometric analysis for nitrous oxide is avoided by using a radioactive inert gas. In Lassen's method specially constructed cuvettes are used to determine krypton-85 concentration. The use of krypton-79 as described by Lewis, is not feasible because special preparation of the isotope is required and the half-life is only 34 hours. In the method described here, standard commercially available equipment can be used both for the administration of krypton-85 and for the analysis of its concentration.

Methods:

In order to carry out the desaturation procedure the brain is first saturated with approximately 8 millicuries of krypton-85 by rebreathing it for 7 minutes in the closed circuit of a BMR machine or a portable anesthesia machine. A seven minute period for saturation was chosen on the basis of Kety's studies of brain tissue concentration of gas in relation to venous blood concentration.

During the last minute of saturation, internal jugular venous and arterial blood samples are drawn continuously to determine the quantity of krypton at the end of saturation. At the end of saturation after the removal of the face mask, two 3cc continuous arterial and venous samples are obtained. Samples are drawn into 10cc syringes via '5 stop-cock' manifolds. Individual 3cc blood samples are then obtained during the remaining desaturation period at the end of the 2nd, 3rd and 4th minutes and thereafter every two minutes to the 12th minute. A 12 minute desaturation period was chosen to provide a slightly longer period of study than the 10 minutes of the nitrous oxide method. This may be of particular value in patients with a decreased cerebral blood flow.

After the removal of the syringes from the manifolds, 7cc of air are drawn in to a total of 10cc (3cc blood - 7cc air). Since the krypton is more soluble in air than in blood, 98% of the krypton is released into the air-phase. The air-phase is then injected into a glass-jacketed Geiger tube by the displacement of mercury and counted on a standard scaler.

The concentration or counts per minute of each sample is then plotted. The cerebral blood flow is calculated in a manner similar to the original method of Kety and Schmidt by using the Fick equation:*

$$CBF = \frac{(Ci/W) \times S \times 100}{\int_0^{12} (C_v - C_a)dt}$$

The (C_i/W) is assumed to be equal to the concentration of krypton in the venous blood at the end of saturation (C_{v_s}) minus the concentration at the end of desaturation (C_{vd}):

$$C_i/W = C_{v_s} - C_{vd}$$

The blood/brain partition coefficient, S, is only slightly above unity, i.e. 1.06 and is constant from patient to patient at normal hemoglobin levels. A value of S of 1.00 has been used throughout the study.

$$CBF = \frac{(C_{v_s} - C_{vd}) \times 100}{\int_0^{12} (C_v - C_a)dt}$$

The denominator of the equation is obtained from the trapezoid rule for the 2nd to 12th minute. The samples obtained during the first two minutes are already "integrated".

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* C_i = concentration of Kr^{85} given off by the whole brain during 12 minutes desaturation measured from the end of 7 minutes saturation.

C_v = concentration of Kr^{85} in the cerebral venous blood during 12 minutes desaturation.

C_a = concentration of Kr^{85} in the arterial blood during 12 minutes desaturation.

C_{vs} = concentration of Kr^{85} in the cerebral venous blood at the end of saturation.

C_{vd} = concentration of Kr^{85} in the cerebral venous blood at the end of desaturation.

W = weight of brain.

S = partition coefficient of Krypton.

Results:

Using the krypton desaturation method, studies of the cerebral circulation were performed on 25 normal males (mean age 27 years) with the following results:

	Mean	S.D.
Cerebral blood flow (CBF) cc blood/100 gm brain/minute	56.5	7.7
Cerebral vascular resistance (CVR) mm Hg/cc blood/100 gm brain/minute	1.52	0.89
Cerebral metabolic rate ($CMRO_2$) cc O_2/100 gm brain/minute	3.37	0.5

The values are similar to those obtained using the nitrous oxide method of Kety and Schmidt and the krypton saturation method of Lassen and Munck.

In order to test the reproducibility of this method two studies (1) (2) were performed on five normal individuals at an interval of 1? minutes. Both studies were done after 7 minutes saturation. The results were as follows:

	(1)		(2)	
	Mean	S.D.	Mean	S.D.
CBF	55.3	10.4	56.2	7.9
CVR	1.50	0.145	1.39	0.30
$CMRO_2$	3.17	0.54	3.22	0.60
ACO_2	46.62	1.37	45.86	3.03

There is a high degree of correlation between the first (1) and second (2) studies with no significant changes in the values obtained. This indicates that the method is most likely highly reproducible in normal individuals.

In order to evaluate the influence of the period of saturation prior to the desaturation CBF study, two separate studies were performed in each of 6 patients with an interval of 10 minutes. The first study (1) was performed after 7 minutes saturation and the second (2) after 15 minutes saturation. Although the mean CBF values obtained, i.e. (1) 45.4cc and (2) 44.2cc are quite close, in the case with a very low CBF there was a decrease of 15% between the first (1) value, 17.3cc and the second (2) 14.7cc obtained after longer saturation. This most likely indicates that only in normal individuals is 7 minutes saturation adequate. In patients with diminished cerebral blood flow a longer period of saturation is probably indicated.

Discussion:

As mentioned the use of a 7 minute saturation period was based on Kety's experimental findings of almost the same concentration of inert gas in the brain and cerebral venous blood from 7 minutes on, i.e. $C_v \times S = C$ brain. Actually complete equilibrium is not achieved before infinitely long saturation has been carried out. Lassen has estimated that in normals there is about 10% lower brain tension than cerebral venous tension of inert gas after 10 minutes saturation, i.e. $C_v \times S > C$ brain.

Let it be assumed then, that at 7 minutes the error is also about 10%. Owing to this effect, the present desaturation method overestimates the CBF in normal males by about 10% when compared with saturation techniques. Lassen's mean CBF values in normal individuals was 52cc and in this desaturation method the mean value was 57cc or 10% above the value obtained by the saturation technique. Lassen has mentioned however, that this desaturation method will give much more accurate results, using 20 blood-krypton samples of high accuracy when compared to the 10 samples obtained in nitrous oxide method of Kety and to the 3 samples in the Scheinberg and Stead modification of the Kety method.

This project was carried out through the cooperation of Col. Arthur J. Levens, MC, and Major Darrell Buchanan, and the Neurology Service Department of Psychiatry and Neurology, WRGH. It is described in an initial report, Research Project No. 63-4-5- MEDEC -- GNN, WRGH.

2. The effects of passive hyperventilation on the cerebral circulation and metabolism of normal man.

It has long been known that variations in carbon dioxide concentration are more potent than any chemical agent or physiological mechanism in the regulation of the cerebral circulation. The effects of alterations in CO_2 tension have been studied by a variety of methods. In 1946, Kety, using the nitrous oxide saturation technique, reported the effects of active and passive hyperventilation on cerebral blood flow and oxygen consumption. In order to re-evaluate the effects of passive hyperventilation, cerebral blood flow studies were performed using the krypton desaturation technique. The results from Kety and Schmidt studies of 5 individuals are compared with values obtained in this study of 8 normal men:

	CBF		CVR		$A\text{-}V_{O_2}$		$CMRO_2$		pH		pCO_2	
	C	HV	C	HV	C	HV	C	HV	C	HV	C	HV
Nitrous oxide saturation	66	41	1.4	2.4	7.2	11.4	4.7	4.7	7.39	7.56	43	24
Krypton desaturation	53	38	1.7	2.3	6.3	8.8	3.3	3.3	7.39	7.53	44	27

C = control; HV = hyperventilation.

The degree of hyperventilation was greater in Kety's study: a 36% fall in CBF was found in Kety's study and a 28% fall in the present study. As shown by Kety, there is no change in cerebral oxygen consumption following passive hyperventilation. The pH and pCO_2 changes in both studies were approximately the same. The present study, using a different technique, but based on the same principle, substantiates the findings obtained using the original nitrous oxide technique for the determination of cerebral blood flow.

This project was carried out with the cooperation of Col. Harvey C. Slocum,MC, formerly Chief of Anesthesia and Operating Service, WRGH and Capt. Hollis Bivens,MC.

3. An evaluation of the Schuhfried rheoencephalogram (REG)

Rheoencephalographic studies have been performed in over 75 normal individuals and patients with organic brain disease. Many problems have been encountered not only in the use, but particularly in the interpretation of the REG.

Standardization and calibration of the REG in order to have reproducible data has been difficult, particularly in the determination of standard values for normal individuals. Evaluation of the REG tracing consists of (1) measuring the EKG R wave to the REG peak interval in seconds. (2) determining angle of inclination of the first part of the REG wave, (3) measuring height of REG wave in milliohms, (4) determining the area under the REG wave.

At the present time REG data are being evaluated in order to establish a range of normal values. Further data are being obtained, using a standard calibration. The findings of Jenkner and others will be compared with our data. At the present time no statement can be made as to the clinical or research value of the REG.

4. The effects of halothane anesthesia and passive hyperventilation on cerebral circulation and metabolism.

Previous studies in the awake individual have shown a reduction in cerebral blood flow without a reduction in oxygen consumption following passive hyperventilation. It is generally known that anesthetic agents themselves reduce cerebral oxygen consumption. Oxygen electrode studies have shown a reduction of local cortical pO_2 with hyperventilation. On the other hand, Pierce has shown that in patients under thiopental anesthesia there is no further reduction in cerebral oxygen consumption after hyperventilation. This study will be undertaken to demonstrate the effects of halothane anesthesia as well as hyperventilation on cerebral circulation and metabolism.

These investigations are being carried out by performing cerebral blood flow studies on anesthetized volunteers prior to elective general surgery. CBF studies are performed before and after 45 minutes of standard hyperventilation. Continuous internal jugular venous pressure, mean arterial pressure, EKG, and EEG are recorded. Intermittant blood samples for pH and pCO_2 are drawn before and during the studies.

These studies are being carried out in conjunction with Col. George J. Hayes, MC; Lt. Col. John Jenicek, MC; and the Neurosurgical and Anesthesia Services, WRGH. This is the same project as WRGH Research Project No. 63-4-1-MEDEC-GSN.

5. Studies of cerebral circulation and intracranial pressures in patients with brain edema.

The objectives of this study are the detection, measurement, and possible control of brain swelling that occurs in neurosurgical patients operated on for brain tumors.

Post-operative patients will be monitored by the continuous recording of intraventricular fluid pressure (IVFP), internal jugular venous pressure (IJVP), and electroencephalogram (EEG). Intermittant quantitative cerebral blood flow studies will be performed to evaluate cerebral hemodynamics and metabolism. The value of the REG as the detector of brain swelling will be determined.

Patients with malignant brain tumors will be chosen for study by Col. Hayes. This study will be carried out in conjunction with Col. George J. Hayes, MC, Lt. Col. John Jenicek, MC, and the Neurosurgical and Anesthesia Service WRGH. This is the same project as WRGH Research Project No. 63-4-1-MEDEC-GSN.

Summary and Conclusions:

An improved method for the quantitative determination of cerebral blood flow in man utilizing the Fick principle has been developed. The method is based on measuring a krypton desaturation curve following saturation of the brain. Readily available equipment is used to administer krypton and to analyze for krypton concentration. The results obtained in a study of 25 normal individuals are similar to accepted values. A study of the effects of passive hyperventilation on the cerebral circulation and metabolism of conscious man was performed using the method. The effects of anesthesia on cerebral circulation and metabolism are being evaluated. Studies of the cerebral circulation and intracranial pressure are being performed on patients with brain edema.

Publications:

A paper "The determination of c erebral blood flow in man by a krypton desaturation technique" was presented at the American Academy of Neurology meeting. An abstract of the paper was published in Neurology, Vol. 13, April 1963.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 806, Military Preventive Medicine

Task 01, Communicable Disease (Arthropod-Borne Infections)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D.C.

Departments of Virus Diseases & Entomology
Division of Communicable Disease & Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators:

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Captain Bruce F. Eldridge, MSC	E. Rosensweg, Ph.D.**
Lieutenant E. G. Thompson, MSC	Herbert C. Barnett, Ph.D.**
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project 3A 0 12501 A 806 Title: Military Preventive Medicine

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Reports Control Symbol: MEDDH-288

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A strain of Aedes aegypti highly resistant to infection with Plasmodium gallinaceum was derived by selection from susceptable lines. Viruses recovered from Pakistan sandflies in 1961 can be grouped into 5 classes immunologically. Naples and Sicilian strains, and 3 other unrelated agents are represented. Cache Valley virus has been recovered repeatedly from salt marsh mosquitoes collected on the Eastern shore of Virginia. This virus, from serological surveys of human and animal residents, of Maryland and Virginia, infects wild and domestic ungulates frequently; man resident in Tidewater areas is also infected; no disease has been associated with infection. Immunization of soldier volunteers with dengue I virus vaccine, further attenuated from the original Sabin vaccine, can be effected with minimal reaction, and no loss of duty time. Immunized persons are immune to challenge with virulent dengue I virus 60 days after immunization. Serial superinfection of burros with Group A arbo viruses results in patterns of infection and antibody response which appear to vary with the infection sequence. The value of this procedure

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for producing plurally reactive antisera is shown. Interference with entero viruses in cell culture systems has been used successfully to detect unmodified dengue viruses; the same technique applied to clinical specimens from patients with Far Eastern hemorrhagic fever has failed to yield a transmissible agent.

BODY OF REPORT

Project 3A 0 12501 A 806 Title: Military Preventive Medicine

Task 01 Title: Communicable Disease (Arthropod-Borne Infections)

Description: Purpose - to define the biologic and immunologic behavior of agents transmitted by biting arthropods to man, and to evaluate, by ecologic, entomologic and microbiologic investigation the significance of these agents in the production of human and animal disease of real or potential military importance. During the report period most investigations at this Institution have centered upon arthropod-borne viruses, the only exception being basic investigations of model systems employing plasmodia.

Progress:

1. Susceptibility and Resistance of Mosquitoes to Infectious Agents.

a. Studies on the genetic aspects of susceptibility to malarial infection in Aedes aegypti have continued. At the phenotypic level, mosquito susceptibility is a continuous or quantitative trait. Early observations on the variability of malarial oocyst number suggested that this was an example of quantitative inheritance due to multiple genes. Later studies on crosses between strains altered this concept. The fact that the F_2, F_3 and F_4 of crosses between susceptible and resistant strains were no more variable than the F_1 and the bimodality exhibited by backcrosses among the susceptible and resistant strains indicated that a single Mendelian factor or a group of closely linked genes was involved. The intermediate values of the F_1 generation of all crosses indicate that incomplete dominance is involved.

b. A gentic model (Table I) has been described to account for the major portion of the genetic variance for susceptibility. The genotype S_1S_1, denoting low or no susceptibility, has a skewed distribution with more than 70% of the individuals showing no or virtually no oocyst development. The genotypes for susceptibility, S_1S_2 and S_2S_2, are normally distributed and are virtually indistinguishable phenotypically. This model assumes that there is some overlapping of classes between genotypes.

Table I

Genetic Model for Susceptibility to Infection

Genotype	Class of Susceptibility	Phenotype Oocyst Counts	
		Median Class	Range
S_1S_1	Low or no susceptibility	0-1	0-32
S_1S_2	Susceptible	65-128	9-256
S_2S_2	Highly susceptible	129-256	33-257+

c. The significance of these findings to the epidemiology of malaria will require an integrated study of both laboratory and field populations of anopheline mosquitoes. Some of the differences in vectorability of mosquito populations may be attributed to variations in gene frequency in different regions. In areas where mosquito populations are fairly stable, in terms of population size and absence of migration and selection, the level of mosquito susceptibility should remain constant. Considerable variation in susceptibility might be expected in areas where there is extensive mosquito migration and marked shifts in anopheline population size.

2. Sandflies and sandfly fevers: Work on the verification and characterization of viral agents isolated from sandflies collected in Iran and Pakistan during 1959 was continuted. These isolates have been grouped into five classes, two consisting of the Naples and Sicilian strains, respectively, and three into new (not previously described) categories. In order to exclude the possibility that these isolates were not indigenous mouse agents, strains from normal suckling mice were blind passed according to the same techniques used with the isolates. Neutralization tests were also run with several of the isolates and randomly selected sera from older colony mice. Neither of these experiments indicated that the sandfly isolates originated from the test mice employed. Further evidence that these isolates are not mouse-borne agents was provided by the demonstration that they are inactivated by chloroform and sodiumdesoxycholate - a characteristic shared by most arboviruses.

a. Transmission attempts with Phlebotomus papatasi and the Naples and Sicilian strains of sandfly fever virus were undertaken using a mouse-sandfly-mouse system. Since the results of these attempts have been negative experiments with a mouse-sandfly-chick system are currently underway.

b. The preparation of hyperimmune sera against the prototype viruses in rabbits and roosters and in ascitic fluid of adult mice is underway. The characterization of isolates from Sergentomyia females and from male sandfly lines are projected upon the completion of adequate hyperimmune sera.

3. Ecology of Arboviruses on Chincoteague-Assateague Islands.

a. Study of arbovirus ecology on the Chincoteague-Assateague Island complex was continued during Spring-Fall, 1962, with emphasis being placed upon identifying EEE and Cache Valley viruses in naturally occurring arthropods, and establishing the natural vertebrate host range of Cache Valley virus in the area, and in the states of Maryland and Virginia. As in 1961, there was again little or no evidence for dissemination of EEE virus; none was recovered from over 90,000 mosquitoes collected between 27 April and 29 October (Table III), and there was no overt disease in man or equines during this interval. However, serological evidence of infection of a single catbird (Dumetella carolinensis) with EEE virus was obtained; this adult bird, first netted on the south end of Assateague

Island, was found without antibody on 7 May 1962. When recaptured 8 miles north on 11 July, it was found to have developed neutralizing antibody (LNI 2.0). Of 1327 avian plasmas examined for neutralizing antibody to EEE virus, only 19 were found positive (LNI 1.7). These included Catbird (14), Blue Jay (2), Brown Thrasher (1), Black-polled Warbler (1), and Yellow-breasted Chat (1). Plasmas from 14 additional birds (8 species) gave equivocal tests. Serological study of mammalian and reptilian plasmas collected concurrently is in progress.

b. While no EEE virus was recovered from collected mosquitoes 9 viruses, pathogenic for suckling mice, were recovered from Ae. sollicitans, C. salinarius and An. bradleyi-crucians complex (Tables III, IV). It should be noted that Ae. sollicitans and An. bradleyi-crucians complex yielded Cache Valley-like viruses in 1961 (ABVIE #6 October 1962). Further, continued study suggests that our identifications of An. crucians in 1961 were not accurate. The adult females of 3 species An. crucians, bradleyi, and georgianus cannot be distinguished. Larvae reared from eggs obtained from adult anophelines collected on Assateague in late September, 1962 were identified as An. bradleyi. An. crucians has been reported from the area in the past, and for these reasons the term "Anopheles bradleyi-crucians complex" will be used to identify these potentially mixed collections of adult females.

c. While the 1962 viruses are as yet incompletely identified, at least 6 appear to have the propagation characteristics of Cache Valley-virus and at least one other is different from the 1961 mosquito viruses from Assateague.

Table III *

Virus isolations from mosquitoes collected on Assateague and Chincoteague Islands, Virginia, during 1962

	Total specimens	No. pools tested	No. pools positive
Aedes sollicitans	79,851	856	7
Culex salinarius	8,187	118	1
Anopheles bradleyi-crucians complex	2,626	48	1
Aedes taeniorhynchus	2,024	42	0
Aedes cantator	1,167	23	0
Psorophora ciliata	9	3	0
Aedes vexans	9	1	0
Culiseta inornata	1	1	0
Total	93,874	1,092	9

* Table II omitted

TABLE IV

Collection Histories, Arthropod Pools Yielding Assateague Island Viruses, 1962

Mosquito Pool	Species	Pool Size	Date	Collection Type	Area
M784/62	Ae. sollicitans	100	7/11	Aspirated from man	Assateague Is.
M875/62	Ae. sollicitans	100	7/23	" "	" "
M911/62	Ae. sollicitans	100	7/30	Chicken house	Chincoteague Is.
M1014/62	Ae. sollicitans	100	8/13	Aspirated from man	Assateague Is.
M1035/62	Ae. sollicitans	100	8/15	" "	" "
M1410/62	Ae. sollicitans	100	9/25	" "	" "
M1439/62	Ae. sollicitans	47	10/1	Opossum-baited trap	" "
M1441/62	C. salinarius	108	10/1	" "	" "
M1533/62	An. bradleyi crucians complex	100	10/12	" "	" "

d. The use of the precipitin reaction to indicate the source of mosquito blood meals was initiated in 1962. Blood smears from engorged mosquitoes collected on Assateague and Chincoteague were tested with six antisera prepared in rabbits against human, horse, deer, racoon, rodent (rat) and avian (chicken) sera, respectively. Of 246 engorged mosquitoes collected by sweeping and from light traps, 243 were Aedes sollicitans, 2 were Aedes taeniorhynchus and 1 was Culex salinarius. Sixty-one percent of the sollicitans smears yielded equine positive reactions, 17 percent produced human positives, 11 percent reacted with anti-deer serum, two percent were avian positives, one percent reacted with anti-racoon sera, and seven percent showed no reaction with any of the antisera. The smears from A. taeniorhynchus and C. salinarius yielded equine positive reactions. No positive reactions with rodent antisera were observed.

4. Evidence for dissemination of Cache Valley Virus in Maryland and Virginia.

a. The occurrence of Cache Valley virus on Assateague Island prompted serological surveys of vertebrate populations of the area, and later of Maryland and Virginia for antibody to the Assateague Island strains of this virus. It was originally shown that the incidence of neutralizing antibodies was high in the wild ponies of Assateague. This observation prompted investigation of equines and other ungulates resident in other parts of tidewater Maryland and Virginia, and eventually to other portions of the state, and other wild animals. The results of such surveys are summarized in Tables V and VI. Horses and cattle resident in tidewater areas apparently are regularly infected with this virus, and infection frequency appears to decrease in animals resident in higher country. Thus fewest reactors are found in cattle reared in Garrett County, western Maryland. Goats and sheep of Montgomery County, Maryland, near Washington, D.C. also show evidence of infection with Cache Valley virus; pigs in Queen Anne's County are less frequently involved. From Table IV, it will be noted that wild carnivores of either tidewater or Piedmont, Virginia are infrequently found with antibody; however, 4 of 10 White-tailed Deer from mountainous Virginia possessed antibody.

b. Man in tidewater areas also possesses antibodies to this virus (Table VII). The highest frequency of positive reactors has been found thus far in the residents of Chincoteague, Virginia. Like the data with domestic animals, the frequency of positive reactors appears to decrease in other than tidewater counties, the exceptions being Frederick and Montgomery Counties, Maryland.

c. Thus while disease in neither man or animals has as yet been associated with Cache Valley virus, it would appear from preliminary tests that this agent has been rather widely disseminated among ungulates and man resident in tidewater Maryland and Virginia for several years. Evidence for natural infection of wild animals and birds tested thus far is minimal; this suggests but does not establish, that the important natural transmission cycle involves saltmarsh mosquitoes and large vertebrates and man. Further investigation of the ecology of this virus is in progress.

TABLE V

Occurrence of Antibody to Cache Valley-like viruses in Domestic Animals of Maryland and Virginia

Species	Area	Date	Frequency of Positive Reactors
Horses	Pungoteague-Keller, Va.(T)	June 1957	16/16
"	Onancock, Va.(T)	June 1957	6/10
"	Chincoteague, Va.(T)	June 1957	14/14
"	Assateague Is.,Va.(T)	June 1957	5/5
"	" " " (T)	May 1961	22/23
"	Chincoteague, Va.(T)	Oct 1962	20/20
Cattle	St.Mary's County, Md.(T)	Oct 1962	18/29
"	Talbot County, Md.(T)	Oct 1962	11/20
"	Carroll County, Md.(TP)	Nov 1962	6/20
"	Prince Georges Co.,Md.(TP)	Nov 1962	12/15
"	Howard County, Md.(P)	Nov 1962	9/15
"	Montgomery Co., Md.(P)	Nov 1962	3/4
"	Garrett Co., Md.(M)	Nov 1962	2/15
Goats	Montgomery Co., Md.(P)	Nov 1962	4/13
Sheep	" " " (P)	Nov 1962	6/22
Pigs	Queen Anne's Co., Md.(T)	Dec 1962	2/10

Counties arranged for each species in ecological order from Tidewater (T) through Piedmont (P) to Mountainous (M) country.

TABLE VI

Occurrence of Antibody to Cache Valley-like Viruses in Wild Mammals, Virginia

Species	Place	Date of Bleeding	#+/TOTAL
Wild Rodents	Assateague-Chincoteague Area	Aug-Nov 1962	3/211
Raccoon-Opossum	" " "	Aug-Nov 1962	0/8
Red fox	Central Piedmont, Va.	1960	0/10
Gray fox	" " "	1960	1/10
Opossum	" " "	"	0/4
Raccoon	" " "	"	3/10
Woodchuck	" " "	"	1/4
Squirrel	" " "	"	0/3
Cotton Rat	" " "	"	0/3
Cottontail Rabbit	" " "	"	0/3
White-tailed Deer	Eastern Appalachian Area, Va.	"	4/10
Avian Sera	Chincoteague-Assateague Area	May-Oct 1961	0/61

Many of these sera furnished by Dr. Ben Elisberg of this Institute.

2 Brown rats (Rattus norvegicus), 1 Meadow vole (Mircotus pennsylvanicus) positive.

61 Avian sera pools species specific, representing 30 species, 239 individuals.

TABLE VII

Occurrence of Neutralizing Antibody to Cache Valley-like Viruses in Man: Maryland and Virginia

Area Source of Human Sera	Age Range (Years)	Date of Bleeding	#+/TOTAL
Talbot-Dorchester Counties	20-39	Dec 62-Jan 63	1/13
Wicomico County	20-36	" "	4/24
Anne Arundel County	20-40	" "	0/25
Prince Georges County	20-40	" "	0/25
Cecil County	20-40	" "	0/24
Montgomery Country	20-38	" "	1/25
Frederick County	20-40	" "	3/22
Garrett County	21-39	" "	0/22
Chincoteague, Va.	8-85	May 1961	33/176

Sera of Maryland residents provided by Dr. C. A. Perry, Chief, Maryland Bureau of Laboratories; sera of Chincoteague, Virginia residents provided by Dr. Martin B. Marx, State Department of Health, Richmond, Virginia.

Age range of positives 13-67 years.

5. Evaluation of An Experimental Dengue Type I Virus Vaccine In Man. In February 1963, a collaborative study with Dr. C. L. Wisseman and Hq. XVIII Airborne Corps, Fort Bragg, North Carolina was begun to evaluate the effectiveness, and reaction rates of an experimental, further attenuated vaccine to dengue virus, Type I. These studies were motivated by requirements of Strategic Army Command, and were designed to fit them.

a. On 26 February, 107 soldier volunteers were recruited from the 55th Medical Group, Fort Bragg. These men were inoculated with the Md.-1 strain of dengue I virus subcutaneously. This strain was derived by further mouse passage, and limiting dilution passages from the original dengue I vaccine strain (Sabin) by Dr. C. L. Wisseman. The following dose schedule was used: 10,000 mouse $1CLD_{50}$ was given to 35 men; 8,9,7,6, and 6 were given 1000, 100, 10, 1, and 0.1 LD_{50} respectively; and 36 men were

administred a placebo inoculation. Reactions to vaccination were sought daily for 18 days in all inoculated men. On 2-3 April, this procedure was repeated using 114 men from the 55th Medical Group, The Advanced Medical Technician's School, Special Warfare Center, and the 82nd Airborne division. The dose schedule in this experiment ranged from 4000 to 0.04 LD_{50}. All volunteers were bled prior to and 30 days following inoculation for antibody determinations. Six men, carefully selected from the initially immunized group were challenged intradermally with 100 human infectious doses of virulent dengue I virus on 29 April, approximately 60 days following immunization.

b. No reactions resulting in loss of duty were observed in any of the immunized personnel in the 18 days following inoculation. Oral temperaturesover 100^0F for at least one day were observed in 1of 140 vaccinees, and in 3/81 control subjects. Headache, respiratory and gastrointestional symptoms were observed with roughly comparable frequency in both groups. However, a morbilliform eruption in the 3rd week following inoculation was observed in 14 of the 140 vaccinees; none was seen in the control group. The occurrence of rash was not anticipated from earlier, more limited studies on prisoner volunteers done by Dr. Wisseman. The exanthem observed in vaccinees was similar qualitatively to that seen in classical dengue, although less extensive, and of slightly shorter duration. Petechiae were observed in 2 of the vaccinees on the 3rd and 4th day of rash. Itching was not uncommon among those vaccinees developing exanthems. However, the rash generally was unaccompanied by any other systemic manifestations. Of interest was the fact that the occurrence of rash did not appear to be dose dependent since it was observed in persons inoculated with 10, 100, 400 and 10,000 LD_{50} of virus. Further the distribution of rash was not uniform in these service units to which volunteers were assigned, the incidence ranging from 0% in the 82nd Airborne Division troops to approximately 20% in the Special Warfare Center students. No explanation can be given for the observation at this time. The nature, duration, and manifestations of the rash were not considered by unit Medical Officers as a significant complication of the immunization, since it did not result in loss of duty time.

c. Antibody determinations upon vaccinated men are as yet incomplete. However, all men in the initial experiment (26 Feb 63) thus far tested who received 10 or more mouse LD_{50} developed neutralizing antibody to dengue I virus; many of those receiving 1 LD_{50} also developed antibody. Thus from preliminary analysis of data it would appear that the human infective dose for vaccine virus closely approximates the mouse LD_{50}.

d. On 28 April 5 vaccinees (3 receiving 10,000 LD_{50}, 1 each receiving 10 and 100 LD50) and one receiving a placebo were challenged with 100 LD_{50} of virulent dengue I virus after hospitalization at Womack Army Hospital. Each of the 5 vaccinees possessed significant amounts of neutralizing antibody 30 days prior to challenge; none

developed clinical illness in the 20 days following challenge. The single control subject developed mild but typical dengue 7 days following challenge, was febrile for 4 days, and made an uncomplicated and uneventful recovery. Thus immunization with the further attenuated Md-1 strain of dengue 1 virus confess immunity to homologous infection for at least 60 days. Further studies are in progress and will be reported at a later date.

6. Serial infection of equines with Group A Arthropod-borne Viruses.

a. Serial group A arboviruses infections in burros, made in collaboration with the Department of Veterinary Medicine, University of Maryland were continued. In March 1962 experiments to test effect of EEE virus inoculation in burros with previous Group A virus infection were initiated. Eight experienced animals were involved, four previously exposed to WEE virus and four exposed to TC 80 VEE Virus. Three control animals (seronegative to EEE) were, with the above animals, inoculated with 10^4 $WMICLD_{50}$ of EEE Virus (Cambridge) of low passage in avian tissue. The 4 animals with previous WEE experience and the 3 control animals converted with log neutralization indices (LNI) of 2.2 to 3.8 secondary to inoculation with EEE virus. Amongst the 3 VEE virus immune animals, none developed fever or viremia and a single animal developed a significant LNI (3.4) though all had negative LNI vs EEE virus in pre-inoculation sera. Inoculation of the above series of animals produced no change in neutralizing antibodies to WEE virus. It is of interest that all of the animals with WEE experience, one of the 3 VEE immunes and one of 3 control burros developed significant LNI (2.2 to 4.8 or greater) to Semliki Forest Virus. This broadening of specificity of neutralizing antibodies is also reflected in the CG antibody response.

b. In addition, further EEE virus challenges of burros and ponies with prior infection with other Group A viruses were made. Two burros which had received VEE virus initially followed by EEE virus in low dosage (approximately 100 $WMICLD_{50}$) to which they did not respond either clinically or serologically received in turn 10 4.5 $WMLD_{50}$ with prompt production of neutralizing antibodies (average LNI=4.5). Two other burros, similarly exposed to a small inoculum (approximately 100 $WMICLD_{50}$ of EEE virus) had responded initially with development of neutralizing antibody. Inoculation of such burros with the larger inoculum of EEE virus produced only a modest elevation from the pre-inoculation LNI. Two ponies, previously exposed to VEE virus, developed, upon inoculation with EEE virus, high LNI to EEE virus but without evident disease. Of three ponies with no previous exposure to Group A arboviruses, two seroconverted (LNI 3.2 and 3.9 respectively) but the third remained negative throughout. Neutralizing antibodies to Semliki Forest virus were again demonstrated in 3 of the 4 burros utilized though all were seronegative to this agent prior to inoculation. Similarly one pony, seronegative initially, demonstrated neutralizing antibodies to Semliki Forest Virus 21 days post-inoculation.

c. Further experiments to determine clinical and serological responses were instituted. Four burros with previous exposure to WEE virus (approximately 18 months before) plus four seronegative to Sindbis virus by TC testing, were inoculated with 10^4 $TCID_{50}$ of Sindbis virus of hamster kidney cell culture origin. No evidence of clinical disease was obtained. Using hamster kidney cell culture no viremia through the 4th day post inoculation was detected. Further only one animal, a Sindbis seronegative control, developed significant level of neutralizing antibodies. Tests for Sindbis HI antibody were similarly negative on serial post-inoculation bleedings with the single exception of a WEE seropositive burro that demonstrated minimal antibody levels (1:20 and 1:10 on days 10 and 14 respectively post-inoculation). CF values on this particular experiment are incomplete at present.

d. In a subsequent experiment (March 1963), the effect of Chikungunya virus in burros with multiple, serial Group A arthropod viruses experience was studied. Three burros had been previously exposed to EEE, WEE and VEE viruses, while one burro had had experience only with EEE and VEE viruses but by neutralization test had significant neutralizing antibodies to Semliki Forest Virus. Four control burros with no previous experience were also included in this particular experiment. In this later group 2 control burros were given $10^{4.4}$ $SMICLD_{50}$ subcutaneously. 2 other controls as well as those previously infected with other viruses were given $10^{7.3}$ $SMICLD_{50}$ of Chikungunya virus. Serology is incomplete in these animals to date but neutralization tests of pre and 21 day post inoculation sera established significant rises in the LNI to Chikungunya virus in the previously experienced burros and one control burro. Three Mexican burros without prior inoculations appear to have naturally occurring neutralizing antibodies to Chikungunya virus. Whether this represents natural infection with this virus in Mexico requires further investigation.

e. Earlier experience with EEE immune burros suggested that the CF antibody response was considerably broadened following superinfection with VEE Virus. In order to determine how plurally reactive such antisera could be, sera from 7 burros with different schedules of inoculation with EEE, VEE and/or WEE virus were tested against 10 different Group A arbovirus by Dr. Jordi Casals. Antisera of high homologous titer (1:64 1:128 or greater) were found to react to significant titer to Getah, Bebaru, Sindbis, Semliki, and Chikungunya viruses. Reactions to Mayaro, and Middleberg viruses were detectable but of low titer (1:16-1:32). Broad patterns of reactions were observed in each of the following combinations of virus infection: EEE followed by VEE and VEE followed by EEE and WEE, but not regularly when WEE was followed by EEE. To obtain large volumes of potentially broadly reactive antisera, two other burros previously immune to EEE virus and 2 susceptible controls were inoculated with VEE virus, and bled copiously on the 20th post inoculation day. These antisera reacted with Group A arthropod-borne viruses as anticipated. Those with dual infection responded with high titers to all of the above

viruses except Middelburg and Sindbis, those with single infection responded only to VEE virus. The plurally reactive antisera seem adequate to serve as reagent for possible grouping of unknown arboviruses.

7. Detection of Dengue Viruses In Cell Culture Systems.

a. The detection of dengue viruses in fluid cultures by their interference with the cytopathogenic effect (CPE) of polio type 2 virus was investigated to confirm previous observations by Major S. Halstead, SEATO Laboratory, Bangkok.

b. Investigations showed that both mouse adapted and impassaged dengue viruses types 1 and 2 could be detected in African green monkey (Cercopithicus aethiops) kidney (GMK) cultures after 3, 5, 7 and 10 days of incubation at 37°C when challenged with 10 and 100 TCD_{50} of polio type 2 virus. The human sources comprised one acute phase serum containing dengue type 1 virus and another containing dengue type 2 virus. Mouse adapted sources of dengue types 3 and 4 viruses were similarly detected in GMK cultures.

c. An attempt to titrate a low passage source of dengue type 1 virus in H-p2 fluid cultures was undertaken. After a 7-day incubation period at 37°C, the tubes were challenged with 100 TCD_{50} of polio type 2 virus. Since no interference occurred, it is probable that the cell line fails to support dengue type 1 virus.

d. A comparative titration of a low passage source of dengue type 1 virus in GMK and human embryonic kidney cultures showed no significant difference in sensitivity.

e. Studies are in progress to determine viremias in various laboratory animals and to demonstrate the transmission of the agents in mosquitoes using the interference phenomenon as an indicator system.

8. Attempts to isolate the virus of Far Eastern Hemorrhagic Fever from acute phase blood samples and autopsy material.

a. Studies to isolate the virus of Far Eastern Hemorrhagic Fever from acute phase whole blood and autopsy specimens collected during the Korean conflict have been begun. The principle of interference with the cytopathogenic effects (CPE) of a number of viruses is being applied to detect the agent.

b. One blood sample to date has been screened in African green monkey (Cercopithicus aethiops) kidney (GMK) cultures and separately challenged with 10 to 100 TCD_{50} of Coxsackie B1, ECHO 11 and polio type 2 viruses after 14 days of incubation at 37°C; GMK cultures are separately challenged with 100 TCD_{50} of Eastern equine encephalomyelitis (EEE) and polio type 2 viruses after 5 days of incubation; human embryonic kidney

cultures and separately challenged with 10 to 100 TCD_{50} of ECHO II, polio type 2, vaccinia and herpes simplex viruses after 14 days of incubation; and Hep 2 cultures and separately challenged with 10 to 100 TCD_{50} of Coxackie B1, polio type 2 and EEE viruses after 14 days of incubation. The specimen was inoculated at 1:10 and 1:100 dilutions. No interference was observed. The remaining specimens will be similarly treated. If the results are negative, other tissue systems and challenge agents will be considered.

Summary and Conclusions:

1. A strain of Aedes aegypti highly resistant to infection by Plasmodium gallinaceum was derived from a susceptible strain by 26 generations of genetic selection. An analysis of the genetics of susceptibility to malaria was made and a genetic model of susceptibility is described. The possible significance of these findings to the epidemiology of malaria is mentioned.

2. The characterization of the isolates from sandflies collected in Iran and Pakistan in 1959 has continued. These isolates have been grouped into five classes, two representing the Naples and Sicilian strains, and three representing new categories. Transmission experiments with Phlebotomus papatasi and the Naples and Sicilian strains are in progress. The preparation of hyperimmune ascitic fluid in mice and hyperimmune sera in rabbits and roosters against the prototype sandfly isolates is underway.

3. No evidence was obtained for dissemination of EEE virus in the Assateague Island area, spring summer 1962. However, Cache Valley virus, and at least one other as yet unidentified virus were recovered from Aedes sollicitans, Culex salinarius and Anopheles bradleyi-crucians complex. Precipition tests on blood found in engorged mosquitoes showed that collected mosquitoes were feeding primarily upon equines, to lesser extent upon man and deer. Rodent blood was not identified in the collections tested.

4. Serological surveys of human and animal residents of the states of Maryland and Virginia show that Cache Valley virus commonly infects the domestic and wild ungulates of tide water districts. The evidence of serological reaction decreases in ungulates in the Piedmont and Mountainous areas. The frequency of prior human infection is less than in ungulates, but is also greatest in tide water areas. No disease has thus far been associated with Cache Valley virus infection in man or in animals, and the economic importance of the infection remains undetermined.

5. Preliminary experiments in soldier volunteers has shown that the Md-1 strain of dengue I virus, further attenuated from the original Sabin vaccine virus by Dr. C. L. Wisseman Jr., can be administered to troops with minimal reaction rates, and no loss of duty time. Vaccinees develop antibody by 30 days post-inoculation, and are apparently resistant to

challenge with fully virulent dengue I virus 60 days after immunization.

6. Serial exposure of both experienced and virginal burros to Group A arthropod-borne viruses, WEE, VEE, EEE, Sindbis, and Chikungunya were undertaken. Confirmation of the protective character of previous VEE inoculation against subsequent EEE inoculation was obtained; however, seroconversion to EEE may or may not occur in such protected animals. Seroconversion, viremia and/or fever are to be anticipated, however, in WEE immunes challenged with EEE and to a lesser extent vice versa. Infection of burros with Sindbis virus was attempted, particularly in previously WEE immune animals; it is apparently difficult to infect both such WEE immune animals and seronegative controls with the dosage used. Inoculation of Chikungunya virus into serially infected and therefore WEE, EEE and/or VEE immune burros results in seroconversion, as it does with seronegative virginal animals. The effects of WEE virus inoculation into Sindbis and EEE virus inoculated animals is currently being determined. Broadly CF reactive sera can be obtained from burros serially infected with Group A arthropod-borne viruses. The extent of cross reactivity and titer of response appears to be broadest with the particular sequence of EEE virus inoculation followed later by VEE virus inoculation. Additional inoculations with Middelburg and/or Mayaro virus may be indicated to completely cover all members of the Group A arthropod-borne viruses.

7. The detection of both mouse adapted and human sources of dengue types 1 and 2 viruses, and mouse adapted sources of dengue 3 and 4 viruses in cell cultures of the African green monkey (Cercopithicus aethiops) kidney cells are discussed. Their interference with the cytopathogenic effect of polio type 2 virus was used as a detector system. The results confirm previous observations. Dengue type I virus can be demonstrated in human embryonic kidney cultures but not in the HEp 2 cell line. Studies are in progress to determine dengue viremias in various laboratory animals and to demonstrate transmission of the agents in mosquitoes using the interference phenomenon as an indicator system.

8. Studies are in progress to isolate the agent responsible for Far Eastern hemorrhagic fever. The principle of interference with other viruses in fluid culture systems are being employed. To date, only one blood specimen has been screened with negative results.

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2. Gould, D. J., Barnett, H. C. and Suyemoto, W.: The Transmission of Japanese Encephalitis Virus by the Mosquito Culex gelidus Theobald. Trans. Roy. Soc. Trop. Med. Hyg., 56 429-435, 1962.

3. Ward, R. A.: Preservation of Mosquitoes for Malarial Oocyst and Sporozoite Dissections. Mosquito News 22: 306-307, 1962.

4. Byrne, R. J., Collins, M. J., Russel, P. K. and Gould, D. J.: Eastern Equine Encephalomyelitis in the Chesapeake Bay Area in 1960. Am. Jour. Trop. Med. Hyg., (In press).

5. Ward, R. A.: Genetic Aspects of the Susceptibility of Mosquitoes to Malarial Infection. Experimental Parasitology (In press).

6. Byrne, R. J., French, G. R., Yancey, F. S., Gockenour, W. S., Russell, P. K., Ramsburg, H. H., Grand, G. A., Scheider, F. G. and Buescher, E. L.: Experimental Infections With Group A Arboviruses in Animals, I Clinical and Laboratory Findings in Burros. Am. J. Vet. Rsch. (In press).

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 806 MILITARY PREVENTIVE MEDICINE

Task 01, Communicable Diseases (Rickettsial diseases)

Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
	Department of Rickettsial Diseases Division of Communicable Diseases and Immunology
Period Covered by Report:	1 July 1962 through 30 June 1963
Principal Investigators:	Earl L. Atwood, B.S. F. Marilyn Bozeman, M.S. Janis M. Campbell, B.S. Bennett L. Elisberg, M.D. Henry S. Fuller, M.D. Garrison Rapmund, Capt, MC
Assistants:	PFC Ronald J. Helmke, B.A. James W. Humphries PFC George J. Munro, B.S. Akira Shirai, B.A.*
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

* Department of Health, Commonwealth of Virginia

ABSTRACT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Rickettsial diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. The indirect immunofluorescent test provides a means of specifically diagnosing and studying antibody responses in Rickettsia tsutsugamushi infections and is a tool for screening sera for detection of past infection.

2. Investigations to localize and recover a group-specific antigenic fraction of Rickettsia tsutsugamushi are in progress. Purified suspensions of Gilliam strain have been prepared which are highly infectious and relatively free of contaminating yolk sac material. These suspensions are potent complement-fixing antigens. Methods of obtaining various fractions of the whole rickettsiae, including cell walls and intracellular substances, are currently under study.

3. Material antigenically related to Proteus OX19 lipo-polysaccharide has been demonstrated in urine of guinea pigs infected with Siberian tick typhus and murine typhus rickettsiae. A search is continuing for substances in guinea pig urine related to erythrocyte sensitizing substance of various rickettsiae. Studies of urine from four cases of spotted fever failed to identify either category of substance.

4. a. In order to study the factors affecting the maintenance, distribution and dispersion of Rocky Mountain spotted fever in nature, an experimental plan was designed and initiated which should provide

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quantitative data on the dynamics of the interaction among the tick vectors, small mammalian hosts and Rickettsia rickettsii in an endemic focus.

b. Of 2,819 cases of spotted fever in civilians, 253 cases (9 per cent) were contributed by 117 families. Disease occurred in sibling children in more than 50 per cent of these families. A negative history of tick-bite does not rule out spotted fever in the face of an otherwise strongly presumptive diagnosis of the disease.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Rickettsial diseases)

Description: The work of this task is designed (1) to evaluate further the use of immunofluorescence for diagnosing and studying serological responses in scrub typhus; (2) to determine the localization of a common or group-specific antigen of Rickettsia tsutsugamushi which can be used as a diagnostic antigen and ultimately might be used as a vaccine; (3) to determine the presence of rickettsial antigen in urine during the acute phase of disease in the hope of obtaining a useful method for the early diagnosis of the rickettsioses; (4a) to devise and institute an experimental plan designed to quantitatively study the dynamic interaction among the tick vectors, vertebrate hosts and R. rickettsii in nature, and (4b) to perform epidemiologic analyses of the occurrence of multiple cases of Rocky Mountain spotted fever in families and military groups.

Progress:

1. Further Evaluation of Immunofluorescence for Studying the Serological Responses in Scrub Typhus.

a. The use of indirect immunofluorescence for the specific serodiagnosis of scrub typhus was presented in the Annual Progress Report, WRAIR, 1 July 1961-30 June 1962 (p. 197, par. 1). The procedure of the test was to overlay Rickettsia tsutsugamushi-infected yolk sac smears (Karp and Gilliam strains) with dilutions of the patient's serum. An antigen-antibody reaction, if present, was detected by staining the complex with a fluorescein-labelled anti-human globulin and noting the intensity of fluorescence of the rickettsiae. The intensity of fluorescence was recorded 4+ (maximal fluorescence) to ± (organisms barely visible). The highest dilution of serum which gave a 1+ degree of fluorescence was considered the immunofluorescent, or I.F. titer. All of 15 patients tested in the original study showed marked increases in I.F. antibody, some ranging in titer from <1:10 in the early specimen to 1:10,240 in the convalescent serum.

b. The use of immunofluorescence was explored more extensively to further evaluate the test (1) for the laboratory diagnosis of scrub typhus, (2) to study the pattern of antibody response and persistence, and (3) to determine its usefulness as a test procedure for screening sera for the detection of past R. tsutsugamushi infections.

c. The sera included in the study were collected from (1) patients in two outbreaks of acute febrile disease which occurred in jungle operational troops in Malaya in 1958 and 1959, (2) volunteers in chloramphenicol field trials conducted in Malaya between 1948 and 1951, and from laboratory personnel who became infected while working with the strains used in the Malayan field studies, and (3) Border police and civilians residing in northern Thailand in November and December, 1962. The latter sera were collected during the course

of studies conducted to evaluate the existing and potential military importance of rickettsial diseases in that country.

d. Table I presents the serological results obtained from the jungle operational troops in Malaya. Both outbreaks occurred during the relative dry season of the year, when the risk of contracting scrub typhus is usually reduced, and were in jungle areas where the disease previously had not been recognised. In the first outbreak, attempts to isolate an etiological agent were made from 9 of the 12 cases. Four strains of R. tsutsugamushi, which were not lethal for mice, were recovered. Only two of the patients exhibited signs and symptoms usually associated with scrub typhus. In the immunofluorescent tests on the paired sera, 9 of the 12 patients had a 4- to 16-fold increase in I.F. titer, the convalescent serum titers ranging from 1:160 to 1:2560. In the second outbreak which occurred a year later in an area about 20 miles from the first epidemic, none of the 11 patients had clinical features suggestive of scrub typhus. The results of fluorescent tests showed 10 of the cases to have significant rises in scrub typhus fluorescent antibody. None of the patients in these two outbreaks had rises in Proteus OXK, OX19 or OX2 agglutinins, and complement fixation tests for typhus, Q fever, spotted fever and psittacosis were negative, as were hemaglutination-inhibitions tests for dengue and hemolytic tests for leptospirosis.

e. Figures 1 through 4 are concerned with the volunteer studies done in Malaya in 1948 to 1951. The graphs show the immunofluorescent titers obtained on the designated days after onset of illness, the antibody levels at the time of challenge or reinfection, and subsequent antibody titers. The open bars represent the titer when the serum was tested on Karp strain rickettsiae, and the slashed bars the antibody to Gilliam strain rickettsiae.

f. Figure 1 shows the response of two individuals who were exposed to scrub typhus by sitting in the grass for six days in a hyperendemic area near Kuala Lumpur. Patient 114 had a mild illness of short duration and developed no Proteus OXK agglutinins, while case 134 presented a typical picture of scrub typhus, and his Weil-Felix titer was 1:1280. The fluorescent antibody responses following the field infection were similar for both volunteers, the antibody titers found were essentially the same with the two antigens. Seventeen months later each man was inoculated with Gilliam strain rickettsiae and both were immune. The fluorescent tests showed that their levels of antibody had dropped one 4-fold dilution by the time of challenge. Each responded with a boost in titer to both strains, but in the second case the Gilliam antibody response was much more dramatic. Fifteen months after the first challenge, they were again inoculated with Gilliam and developed no signs of disease. Case 114 had essentially no change in titer while 134 had a slight increase to Gilliam, but the heterologous Karp antibody remained the same.

g. Figure 2 shows the response of 2 of 4 patients who were inoculated with Gilliam rickettsiae, and 14 and 15 months later were challenged with the same strain. Each one had an early, rapid rise in Gilliam antibodies following the first exposure. In the case of the first patient, there was also a good Karp antibody rise. The level of antibody to both strains dropped significantly by 14 and 15 months when they were challenged, yet neither developed

overt disease. There was a 4-fold rise in Gilliam antibody on day 12 in the first volunteer, while the Karp antibody level did not change. In the second case, it is interesting that even though this man did not become sick after challenge, rickettsiae were isolated from his blood on the 20th day, when his Gilliam antibody had risen to 1:640. Karp antibody, which was undetectable at the time of reinoculation, rose to 1:10.

h. Figure 3 shows the results of 2 of 4 volunteers who were infected initially with Gilliam and after 16 and 15 months, respectively, were inoculated with the heterologous Karp strain rickettsiae. Both men developed scrub typhus. The first case had a Gilliam titer of 1:640 by the 6th day of disease in the initial infection and the titer remained the same throughout the entire period of study. The antibody response to Karp antigen after the first infection developed more slowly and subsequently dropped by the time of reinoculation. After the Karp challenge there was an increase in Karp antibody. The second case exhibited a similar response except that the low Karp antibody level evoked by the initial Gilliam infection was not detectable after 15 months. The Karp challenge caused a rise in Karp antibody, while, as in the other cases, the initial homologous antibodies remained the same.

i. Figure 4 depicts the persistence of antibodies in two individuals over a number of years. The top graph, Case CP-25, shows the antibody response following a natural field infection. The levels of antibodies to both the Karp and Gilliam antigens were about the same. Upon challenge with Gilliam 19 months later, there was a boost in antibody to both strains. The heterologous antibody, however, dropped sonner, and after 12½ years only the homologous was detectable. Case V-10 shown in the lower graph, resulted from a laboratory infection. The high titers obtained with the Karp antigen and the sustained titer of 1:640 after three years suggest that the original disease was probably due to a Karp strain infection. The individual was resistant to homologous challenge in 1950. In 1951, while working extensively with the Gilliam strain, he experienced another episode of scrub typhus. At that time, his I.F. titer to Gilliam was only 1:10. There was a sharp rise to Gilliam, as well as a slight increase in Karp antibody. Antibodies to both Karp and Gilliam persisted at relatively high levels for at least 6½ years after the last known infection.

j. Table II presents the results of the fluorescent antibody screen tests done on 194 human sera collected in northern Thailand in November and December, 1962. Immunofluorescent titers of 1:40 or greater were found in 25 of the individuals, while a titer of 1:10 was observed in 43 other persons. The significance of the 1:10 titers cannot be determined at this time, since in previous studies the sera from a single case of leptospirosis in Puerto Rico exhibited minimal fluorescence at this dilution (Bozeman, F.M. and Elisberg, B.L., Proc. Soc. Exp. Biol. & Med. 112:568, 1963). A titer of 1:40, however, is considered significant and represents a present or past R. tsutsugamushi infection.

2. An Antigenic Study of the Gilliam Strain of Rickettsia tsutsugamushi.

a. Among the numerous immunologic techniques used for the study of the antigenic characteristics of strains of Rickettsia tsutsugamushi are included the serum protection and neutralization tests, complement fixation tests utilizing soluble-type antigens, vaccination-challenge and toxin neutralization tests, all of which have revealed the heterogeneity which exists within the species.

Only mouse cross-immunity tests (in which mice infected with one strain resist challenge with known lethal strains) demonstrate close intra-group relationships among the different strains. The last mentioned observation suggests the existence of a common antigen which is associated with the production of a state of immunity.

b. The purpose of the present work was to localize and recover the common or group-specific antigen in the Gilliam strain of R. tsutsugamushi. This antigenic component would have great potential as a diagnostic reagent, and, depending upon its immunogenic properties, might be suitable for use as a vaccine.

c. The general approach was to prepare purified rickettsial suspensions from infected yolk sacs, attempt separation of cell walls, intracellular components and other fractions, after disrupting the rickettsiae by various physical and chemical means. The antigenic properties of the various fractions will be tested with respect to their ability to elicit antibodies in laboratory animals and their antigenic reactivity in complement fixation tests.

d. The Gilliam strain was selected as the test organism because (1) it grows profusely in the yolk sac of embryonated eggs and (2) higher antibody titers in convalescent sera of a series of scrub typhus patients were found in indirect immunofluorescent tests with this strain as compared with the Karp strain. Other biological properties are unique to this strain. Rich suspensions of the Gilliam strain are lethal only in low dilutions (10^{2} - 10^{3}) but are capable of infecting mice at dilutions of 10^{8} - 10^{9} and protecting them against subsequent challenge with the lethal Karp strain. In addition, a toxic effect can be demonstrated with heavy suspensions of the Gilliam strain.

e. Two methods of purification were employed. The first was patterned after the method of Wisseman et al. for obtaining highly purified suspensions of R. mooseri (J. Immunol. 67:123, 1951). It involved absorption of 20% yolk sac suspensions with Hyflo Supercel (celite), a treatment with 6% bovine serum albumin and digestion with 0.1% crystalline trypsin, along with a series of differential centrifugations. Suspensions prepared by this method and modifications of it were anticomplementary in a range too close to the titer of the specific antigen. The lethal and infectious titers obtained indicated that many of the rickettsiae were lost or destroyed in the process. The second method used was first described by Kitaoka et al. in Japan (Annual Rept., Army Contract No. DA-92-557-FEC-33903, 1960). In a batch process, a weak cation exchange resin of the Amberlite series was used for adsorbing out contaminating yolk sac material. The suspension was further purified with bovine serum albumin, in a sequence of low and high speed centrifugations. The final preparation was a 4:1 concentration of the initial 50% yolk sac suspension. The latter method has been adopted because it provides higher titered suspensions containing less contaminating yolk sac material.

f. The purified preparations, as well as the original 50% crude suspensions, were inoculated into mice to obtain lethal and infectious titers and into rabbits and guinea pigs to study antibody responses. Smears were made at each step of the purification process and stained with Giemsa and Wright's stains and by immunofluorescence. The final suspensions, as well as

aliquots from each step, were titrated as antigens in complement fixation tests against a Gilliam rabbit antiserum. To check the purification procedures, i.e., removal of contaminating yolk sac material, each fraction was tested against serum from a rabbit hyperimmunized with uninfected yolk sac suspensions. Complement fixation tests were performed using a 50% end-point and standardized 5 unit complement, by the methods currently being employed in the Dept. of Serology, WRAIR. Results of preparations of the Gilliam, as well as of the Kato and Karp, strains purified by the latter method are presented in Table III. The Kato and Karp strains were selected for comparative purposes. Furthermore, the Japanese workers indicated that it was possible to detect present and past infections caused by all strains of scrub typhus found in Japan with complement fixation tests using purified rickettsial suspensions of these three strains (Gilliam, Kato, Karp).

g. Figures 5 and 6 show the antibody responses of rabbits and guinea pigs which received 50% yolk sac suspensions and final purified preparations of the Gilliam strain. The sera were tested against four antigens: (1) the purified rickettsial suspension, (2) an uninfected normal yolk sac suspension purified by the same process, (3) an ether-extracted soluble-type Gilliam antigen, and (4) a normal yolk sac soluble-type antigen. The rabbit sera titered 1:5120 or greater with the purified rickettsial antigen and were 32- to 64-fold less with the ether-extracted soluble-type antigen. In the guinea pig sera the difference in antibody titers demonstrated by the two types of rickettsial antigens was more pronounced. It should be noted that although the guinea pig is the animal of choice for other groups of rickettsiae, it has been used infrequently in studies on scrub typhus. Although these animals get sick when inoculated with certain strains of R. tsutsugamushi, their antibody level has been so low as to be undetectable with the soluble-type antigens. The inability to prepare high titer hyperimmune serum in guinea pigs undoubtedly is a reflection of the poor antigenic potency of the soluble-type antigen. In both species of animals, regardless of the type of normal yolk sac antigen employed, there were lower antibody titers to contaminating yolk sac material in the animals which received the purified suspension

h. Rabbits, guinea pigs and mice have been immunized with each of the three strains (Gilliam, Kato and Karp). These immune sera will be used for the detection of group-reactive complement fixing activity in the following antigen preparations: (1) living intact organisms, (2) inactivated intact organisms, (3) sonicated whole organisms, (4) cell walls (intact), (5) cell walls (sonicated), and (6) intracellular substances.

i. Work is currently in progress to obtain intact rickettsial cell walls. The usefulness of sodium desoxycholate, which has been employed to obtain cell walls from a number of microorganisms, including R. mooseri, is being evaluated. Separation of the cell walls and other fractions will be done with density gradients. Preparations will be examined by electron microscopy. The results of these investigations will be the subject of future reports.

3. Urinary Excretion of Rickettsial Antigens.

a. Ten years ago, in the Department of Rickettsial Diseases, urine from scrub typhus patients was examined for OXK-like antigen of Rickettsia

tsutsugamushi, without success. More recently, two different antigens of R. mooseri were reported to be detectable in urine during the first week of disease of patients and animals infected with murine typhus (Fleck et al., Am. J. Hyg. 72:351, 1960). Therefore, a re-evaluation of this phenomenon has been undertaken to determine its applicability to the diagnosis of rickettsial disease during the acute phase of illness. Excretion of antigen was studied in guinea pigs infected with either scrub typhus, Siberian tick typhus or murine typhus rickettsiae.

b. The hemagglutination-inhibition technique was used for the detection of antigen. In this procedure, urine is incubated with serum of known antibody titer. Antigen, if present in the urine, combines with antibody. When the serum/urine mixture is titered for hemagglutinating (HA) antibody by the addition of sensitized red blood cells, a reduction in the expected HA titer of antibody is observed. This reduction represents a measure of inhibiting antigen present in the urine.

c. Control antigens capable of sensitizing red blood cells were prepared from OX19, and OXK strains of Proteus bacilli, and from R. sibericus and R. mooseri. Serum from patients convalescent from rickettsial diseases was used as a source of antibody. The considerable non-specific hemagglutination inhibitory activity of urine was removed by adjusting the urine to pH10, heating to 100°C for 30 minutes, followed by dialysis for 16 hours in normal saline. All assays to date have been performed on unconcentrated specimens.

d. Animal urine studies: Following preliminary studies of the methods involved, 24 hour urine specimens were collected from guinea pigs for three days prior to, and 28 days following inoculation with 1.0 ml of 10% yolk sac suspension of various rickettsiae. All urine specimens have been processed from (1) 8 of 13 animals infected with R. sibericus, (2) 8 animals infected with R. mooseri, and (3) 8 of 12 animals infected with R. tsutsugamushi. Analysis of these specimens for rickettsial antigens has not been completed, but preliminary results can be reported.

(1) Siberian tick typhus (first 12 post-inoculation specimens tested): 6 of 8 animals excreted daily an OX19-like material, 2 of these beginning on the first day, 2 on the 6th day, and 2 on the 8th day after inoculation. Similar material was detected irregularly in urine from the remaining 2 animals. Examination for material related to rickettsial erythrocyte sensitizing substance (ESS) has not yet been done.

(2) Murine typhus (first 12 post-inoculation specimens tested): 4 of 8 animals excreted an OX19-like material. In 3 of the 4 animals it was detected in only one specimen each, on the 3rd, 8th and 10th days, respectively, and in the fourth animal on the 5th and 7th days. In only one animal ESS-like material was found in the urine excreted on the 5th, 7th, 9th and 11th days.

(3) Scrub typhus (first 9 post-inoculation specimens tested): OXK-like material was not detected in urine from 8 animals.

e. It is thought that all animals were successfully infected. All animals inoculated with R. sibericus developed fever, 8 of 13 developed scrotal

reaction and one died. All animals developed CF antibody. All animals inoculated with R. mooseri developed fever and scrotal reaction, and one animal died. All surviving animals developed CF antibody. Ten of 12 animals inoculated with R. tsutsugamushi developed fever, and of these five died. One of two animals which did not develop fever, also died. The CF antibody response in this group of animals has not yet been determined.

f. The urinary excretion of antibody was not a factor in the negative results which were obtained. None of the animals developed Weil-Felix or ESS agglutinating antibodies, a finding characteristic of rickettsial infection in guinea pigs.

g. Human urine studies: Efforts are being made during the present tick season to collect blood and urine specimens from locally occurring cases of Rocky Mountain spotted fever. At least two specimens from each of 4 cases have been studied to date; the initial specimens were collected on days 4, 4, 7 and 12, respectively, after onset of illness. Neither OX19-, nor ESS-like materials have been detected in these urines. In the two cases with specimens obtained early in the course of disease, antibody was not found in the serum at the time of the urine collection. In one of these R. rickettsii was isolated from the blood. In the two later cases (DD 7 and 12), however, Proteus OX19 agglutinating antibody in high titer was present in the serum when urine specimens were first collected.

4. Ecology of Rocky Mountain Spotted Fever.

a. Factors affecting the maintenance, distribution and dispersion of Rickettsia rickettsii in nature.

(1) One of the principal efforts of the collaborative investigations undertaken by the Department of Rickettsial Diseases with the Virginia State Health Department during the two year period 1960-1962, has been concerned with an evaluation of the role of native, wild animals as vertebrate hosts for Rickettsia rickettsii in the Commonwealth of Virginia. The field aspects of this phase of the project were centered around the occurrence of human disease throughout the whole of the state. For the most part, animals sent to the Department of Rickettsial Diseases for the detection of current or past Rocky Mountain spotted fever infection were trapped in areas suspected of being the source of infected ticks. These collections were supplemented with larger animals principally obtained during the course of other activities of State and Federal agencies concerned with wild life management and disease control. The laboratory attempted to recover rickettsiae from the tissues of wild mammals and to demonstrate complement-fixing antibodies in the sera of wild mammals and birds. Data collected in this manner implicated 14 different species of mammals included among five different Orders; viz., Rodentia, Lagomorpha, Carnivora, Marsupialia, and Artiodactylia. Similarly, 18 species of birds belonging to three different Orders, the majority of which were Passeriforme were also incriminated. The postulated ecological significance of these results was reported last year (Annual Progress Report, 1 July 1961-30 June 1962, p. 199, par. 2).

(2) The above-mentioned approach has had wide application in studies of a variety of other arthropod-borne diseases. In this instance,

the findings impressively described the complexity of the ecosystem in which Rickettsia rickettsii is maintained. It also provided information to a certain extent, about the various communities within the ecosystem which are involved in this tick-borne rickettsiosis. Another type of experimental design is required to gather information on (a) the interaction among the various component communities; i.e., vector ticks, vertebrate hosts and the rickettsiae, (b) the influence of environmental circumstances on these portions of the biocenose, and (c) the relative importance of the different factors associated with the perpetuation of an endemic focus and the extension of disease from nature to man.

(3) During this current year, the major effort has been devoted toward formulating an experimental design and testing critical procedures which should provide an understanding of certain aspects of the kinetics of Rickettsia rickettsii in nature. Through the collaborative efforts of rickettsiologists, an entomologist, a biostatistician, mammalogists and field biologists, a feasible operational plan was devised. A brief description of the three phases of the projected program will be presented at this time, to serve as background for future reports. Studies have already been initiated within the scope of each of the parts of the program, but the work has not progressed far enough to permit reporting of the results.

(4) Phase 1 is concerned with a field investigation of a known endemic focus of Rocky Mountain spotted fever. The highest number of human cases of Rocky Mountain spotted fever in Virginia have been reported from the Piedmont Province. On a 400 acre farm located in this physiographic region a 40 acre plot has been selected. Included in the study plot are the various land utilization types found in the area. The proportion of field and forest are representative of the distribution throughout this region. Here, throughout the calendar year, the population dynamics of the resident small mammals and vector ticks are being simultaneously quantitated. Regular evaluations of the incidence of infected ticks and the immunologic status of the animals are being made also. These observations will be correlated with each other and also with climatic conditions and plant succession.

(5) In phase 2, attempts will be made to collect information on specific aspects of the ecological relationships encountered in the field. These are to be undertaken in the laboratory under controlled conditions approximating those found in nature. Two examples of these types of investigations already in progress are (a) the determination of the factors influencing the behavioural characteristics of vector ticks, and (b) the evaluation of the efficiency of selected wild mammals as reservoirs or hosts for the rickettsiae. With respect to the latter study, experiments are being undertaken with the cotton rat (Sigmodon hispidus) to determine the pathogenesis of infection, and the degree and duration of rickettsemia and antibody response.

(6) To implement phase 3, an outdoor vivarium has been constructed utilizing natural vegetative cover. Peromyscus and Dermacentor variabilis already have been introduced into this closed ecosystem. Eventually Rickettsia rickettsii also will be included and the reactions and interactions of these three components of the natural cycle of Rocky Mountain spotted fever followed.

(7) It is realized that a more complete understanding of the ecology of Rocky Mountain spotted fever could be obtained if other wild and domestic animals, principally dogs, as well as birds, were included in the study program. Careful epidemiological investigations of the occurrence of human disease should also be undertaken. However, limited availability of personnel and funds have precluded the extension of the project to encompass these other considerations at this time.

b. Multiple cases of Rocky Mountain spotted fever in families.

(1) The present report deals with an outgrowth of studies of the ecology of spotted fever in Virginia, where apparently greater numbers of cases and higher county rates have occurred in the Piedmont Province and Fall-line zone. This observation led to the following sequence of questions: Is this apparent distribution in Virginia a real one? Is it also found in the eastern piedmont area in states north and south of Virginia? Is it likewise observed in the western piedmont area of the Appalachian chain? Within the range of the eastern vector species of ticks, Dermacentor variabilis and Amblyomma americanum, what are the precise localities in which the reported cases of spotted fever are known to have been acquired? Answers to these questions are clearly essential to an understanding of the ecology of spotted fever in the eastern United States.

(2) The source materials in the present study were the records maintained at the Rocky Mountain Laboratory by Dr. R.R. Parker, who provided laboratory diagnostic services whenever possible, and who made a thorough painstaking investigation of each case by means of correspondence with health officers, attending physicians, the patients, and their families. For the present study, epidemiologic analysis was made of records of cases occurring east of longitude 100° during the period 1931-1950. The western boundary was selected because (a) it is roughly the western limit of the range of the usual vector, D. variabilis, in this region, and (b) it has been recognized since the 1890's as a zone of biogeographic change between the flora and fauna of the eastern United States, and the biota of the Great Plains region and areas westward thereof. By 1931 it was generally recognized that spotted fever occurred in several of the eastern states. By 1950, the widespread use of broad spectrum antibiotics had undoubtedly ameliorated many cases to the extent that subsequent reporting could not give a meaningful picture of the true amount and distribution of human infection. Furthermore, owing to the death of Dr. R.R. Parker, meticulous records of each case were not maintained after 1950. Although the analysis of more than 5,000 records has not progressed sufficiently to permit tentative answers to the main questions posed above, it has served to emphasize the importance of multiple cases of spotted fever in family groups. The pertinent facts are the substance of this report.

(3) Instances of multiple infections in one household during the same tick season are not uncommon. In fact, examples were presented by Anderson (Hygienic Lab. Bull. No. 14, 1903), and their occurrence was discussed briefly by Parker and Oliphant (In: Pullen, (Editor): Communicable Diseases, pp. 733-4, 1950). However, since the facts and their significance appear to be neither widely disseminated nor fully appreciated, an analysis of the Rocky Mountain Laboratory records is timely. Of 5,031 case records, 2,819 contained information suited to the present purposes.

(4) Familial cases: Instances of multiple cases in the same household are summarized in Table IV. It is noteworthy that 117 families contributed 253 cases, or 9 per cent of the total number. From the biostatistical standpoint, the numbers of families with specified numbers of cases appear to follow a "contagious" frequency distribution. This finding is not unexpected in view of the multiplicity and heterogeneity of the biological, medical and administrative factors which generated these observations.

(5) Family relationships among patients: The intra-familial relationships of patients in 116 families are summarized in Table V, where it is noted that disease occurred in siblings in more than 50 per cent of the total families affected. In the family which produced 7 cases, the patients were: a grandmother, the husband and wife, their three sibling children, and the attending physician who treated them all at home.

(6) Month of onset: Of the 253 case records in this series, the month of onset was stated in 233. The dates of onset ranged between 7 April and 15 September. During this period, the numbers of cases per month were: April - 6; May - 55; June - 63; July - 78; August - 25; and September - 6. These observations merely reflect the seasonal activity of the adult stages of the two vector species of ticks in this region: D. variabilis, and Amblyomma americanum.

(7) Intervals between onset of successive cases: Of particular significance to the clinical diagnostician is the period which has elapsed between the onset of two or more cases of a febrile disease in the same group of subjects. The pertinent data are summarized in Table VI, where one notes that, in 21 instances, two patients became ill on the same day, the median interval between onsets was 2.5 days, and in 94 instances the interval between onsets was one week or less. In such situations the clinician is first inclined to think he is dealing with an infection transmitted by interpersonal contact (e.g., the common exanthemata; via the respiratory or intestinal routes), or acquired from a common source. Hence, these observations underscore the absolute necessity for an adequate history and an acute awareness of the possibility of an arthropod-borne infection.

(8) Nature of exposure: Among the 106 families of which each gave rise to two cases, the records for 86 were satisfactory for analysis. Of the remaining 20 families, the nature of exposure was not recorded for either member of 17 pairs of patients; in 3 families, one member of each pair had a history of tick-bite, but the nature of exposure of the other patients was not stated. Data on these 86 families are summarized in Table VII. A positive history of tick-bite requires no further comment here. In the absence of a history of other types of exposure, such as removal of ticks from an animal or another person, or crushing ticks, the reliability of a negative history of tick-bite is highly questionable. Many fastidious persons regard ticks as vermin, reminiscent of lice, and, hence, as evidence of uncleanliness, to be denied at all costs. Secondly, a tick may feed and drop off unnoticed. Finally, ticks may attach in such areas of skin as the umbilicus or the scalp and escape detection, even by the physician. In instances where two persons occupy the same bed, a single tick may feed partially on one, detach, and then attach to the other occupant. The point which emerges is that a negative history of tick-bite need not rule out spotted fever in the face of an other-

wise strongly presumptive or obvious diagnosis of the disease.

Summary and Conclusions:

1. The indirect immunofluorescent test provides a means of specifically diagnosing and studying antibody responses in Rickettsia tsutsugamushi infections, and is a tool for screening sera for detection of past infection. Repeated infections can be diagnosed by this method. The Weil-Felix test is of little value in these instances since there is rarely an increase in OXK agglutinins following reinfection. The heterogeneity which exists among strains of R. tsutsugamushi also was demonstrated. In human volunteer challenge experiments, it was shown that homologous antibody rises more rapidly, attains a higher level, and persists for a much longer period of time than heterologous antibody. Upon challenge there was usually a boost in titer to the infecting strain, while residual antibody remained essentially the same. In two individuals where specimens were available, fluorescent antibodies were still detectable at significant levels after 6 and 12 years.

2. Investigations to localize and recover a group-specific antigenic fraction of Rickettsia tsutsugamushi are in progress. Purified suspensions of Gilliam strain have been prepared which are highly infectious and relatively free of contaminating yolk sac material. These suspensions are potent complement-fixing antigens. Methods of obtaining various fractions of the whole rickettsiae, including cell walls and intracellular substances, are currently under study.

3. Results to date suggest that study of unconcentrated urine specimens, even by the very sensitive method employed (detection of 0.001 microgram/ml inhibiting antigen), will not reveal antigen consistently enough for the method to be of diagnostic value. Assay of concentrated urine specimens will be undertaken and studies of presently available material from guinea pigs will be completed. Effort is being made to collect urine from as many spotted fever cases as possible which occur in the Washington area this year, in order to extend present minimal experience with human urine examinations.

4. a. In order to study the factors affecting the maintenance, distribution and dispersion of Rocky Mountain spotted fever in nature, an experimental plan was designed and initiated which should provide quantitative data on the dynamics of the interaction among the tick vectors, small mammalian hosts and Rickettsia rickettsii in an endemic focus.

b. Of 2,819 cases of spotted fever in civilians, 253 cases (9 per cent) were contributed by 117 families. Disease occurred in sibling children in more than 50 per cent of these families. A negative history of tick-bite does not rule out spotted fever in the face of an otherwise strongly presumptive diagnosis of the disease.

List of Publications:

1. R. Traub and B.L. Elisberg, "Field Test on Diethyltoluamide (Deet); A Highly Effective Repellent Against Mosquitoes in the Nipah Palm-Mangrove Swamp in Malaya." Pacific Insects, 4:303-313, 1962.

2. R. Traub and B.L. Elisberg, "Comparative Efficacy of Diethyltoluamide Skin-Application Repellent (Deet) and M-1960 Clothing Impregnant Against Mosquitoes in the Nipah Palm-Mangrove Swamps of Malaya." Pacific Insects, 4:314-318, 1962.

3. H.S. Fuller, "Ecology of Rocky Mountain Spotted Fever." First International Wildlife Disease Conference, High View, New York, 28 June 1962.

4. _____. Rocky Mountain Laboratory, Hamilton, Montana, 24 September 1962.

5. J.A. Morris, B.L. Elisberg, W.L. Pond and P.A. Webb, "Hepatitis Associated with Coxsackie Virus A 4." New Eng. J. Med., 267:1230-1233, 1962.

6. H.S. Fuller, "Ecology of Rocky Mountain Spotted Fever." William Bowman Gray School of Medicine, Winston-Salem, N.C., 15 December 1962.

7. F. Marilyn Bozeman and Bennett L. Elisberg, "Serological Diagnosis of Scrub Typhus by Indirect Immunofluorescence." Proc. Soc. Exp. Biol. & Med., 112:568, 1963.

8. G. Rapmund, "Urinary Excretion of Rickettsial Antigens." Commission on Rickettsial Diseases, AFEB, 1 March 1963.

9. F.M. Bozeman, "Further Evaluation of Immunofluorescence for Serological Diagnosis of Scrub Typhus." Commission on Rickettsial Diseases, AFEB, 2 March 1963.

10. B.L. Elisberg, "Rickettsial Disease Study in Thailand." Commission on Rickettsial Diseases, AFEB, 2 March 1963.

11. B.L. Elisberg, "Report of Arbovirus Studies by USAMRU (Malaya)." Commission on Viral Infections, AFEB, 11 March 1963.

12. J.A. Morris, F.M. Bozeman, C.G. Aulisio and A. Shirai, "New Murine Hemadsorbing Virus." Fed. Proc., 22:324, 1963.

13. J.A. Morris, F.M. Bozeman, C.G. Aulisio and A. Shirai, "New Murine Hemadsorbing Virus." Proc. Soc. Exp. Biol. & Med., 1963 (in press).

14. H.S. Fuller, "Ecology of Rocky Mountain Spotted Fever." Societe de Pathologie Exotique Institut Pasteur, Paris, 7 May 1963.

Table I

OCCURRENCE OF SCRUB TYPHUS IN JUNGLE OPERATIONAL TROOPS IN MALAYA

Outbreak Unit	Operational Area	Ecological Habitat	Cases	Serological Tests Weil-Felix OXK Test pos.	neg.	I.F. Test pos.	neg.
22 SAS	Kledang Saiong Forest Reserve	Primary upland dipterocarp forest	12	0	12	9	3
1st Bn., N.Z. Rgt.	Bukit Payong	Secondary hill dipterocarp forest	11	0	11	10	1

Figure 1

SCRUB TYPHUS FLUORESCENT ANTIBODY RESPONSE: INITIAL INFECTION DUE TO FIELD EXPOSURE AND SUBSEQUENT CHALLENGE WITH GILLIAM STRAIN

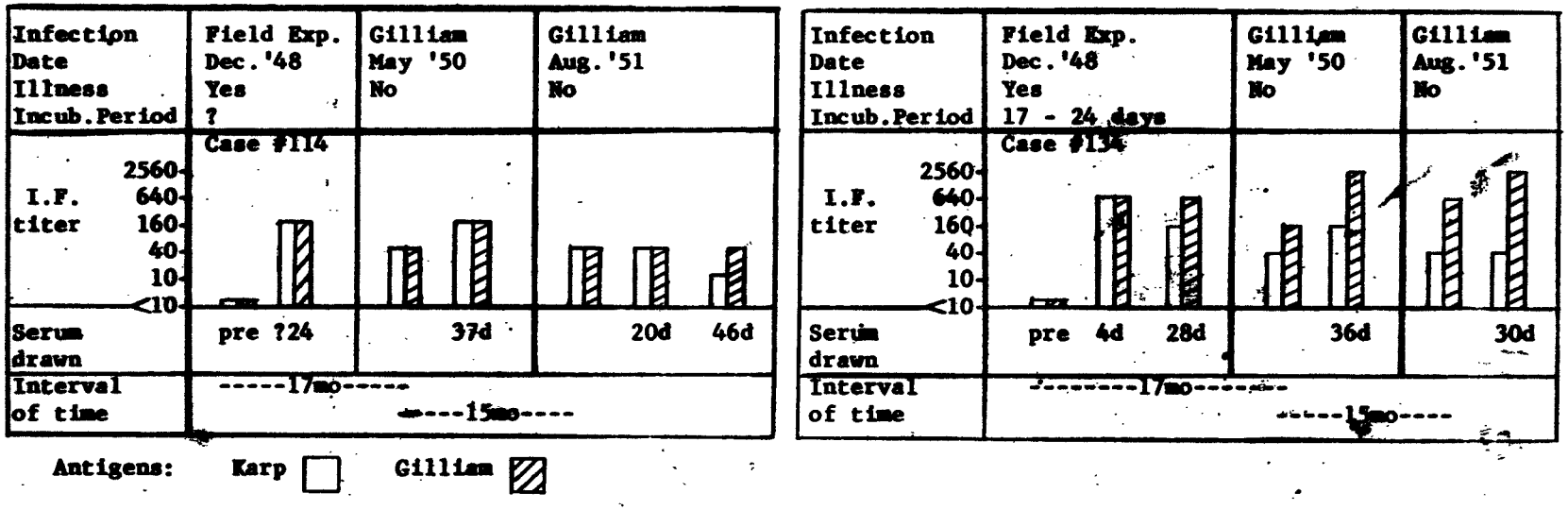


Figure 2

SCRUB TYPHUS FLUORESCENT ANTIBODY RESPONSE: INITIAL INFECTION WITH GILLIAM STRAIN AND SUBSEQUENT CHALLENGE WITH GILLIAM STRAIN

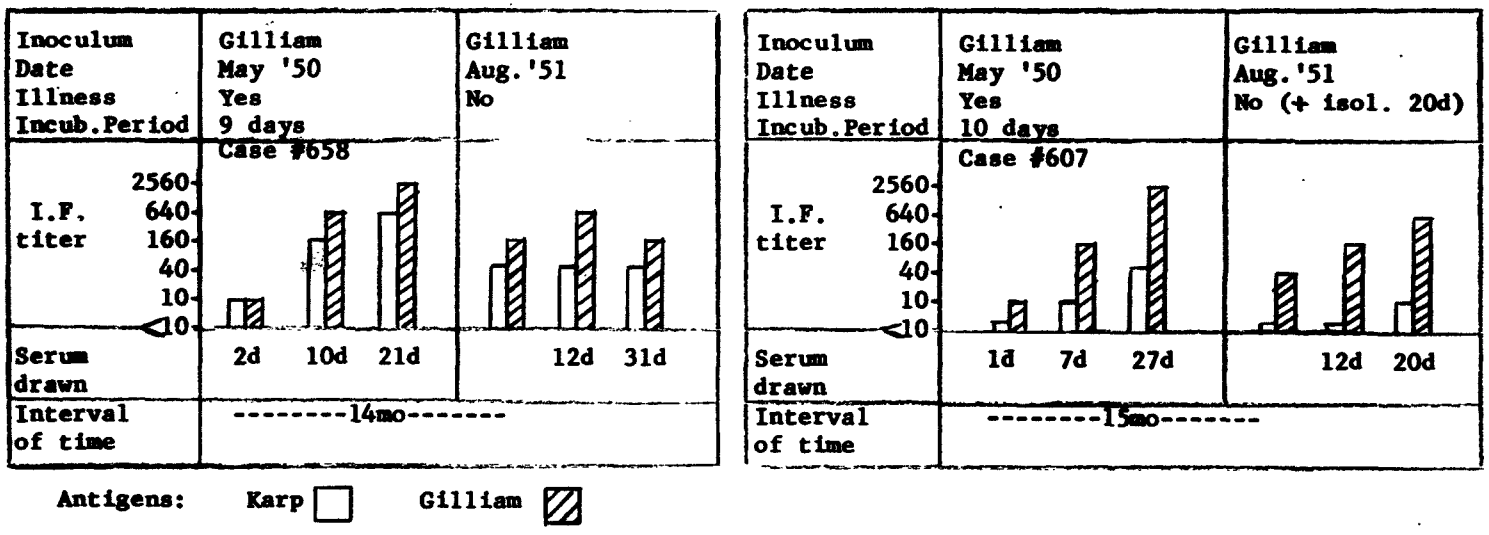


Figure 3

SCRUB TYPHUS FLUORESCENT ANTIBODY RESPONSE: INITIAL INFECTION WITH GILLIAM STRAIN AND SUBSEQUENT CHALLENGE WITH KARP STRAIN

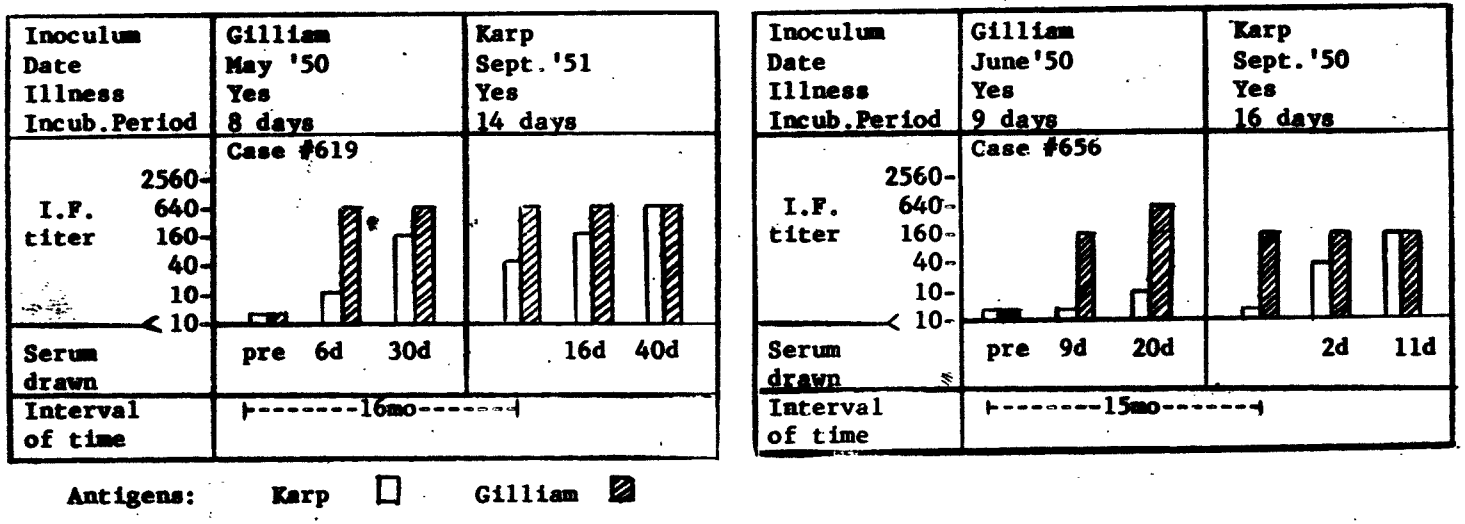


Figure 4

PATTERN OF SCRUB TYPHUS FLUORESCENT ANTIBODY RESPONSE FOLLOWING RE-INFECTIONS WITH RICKETTSIA TSUTSUGAMUSHI

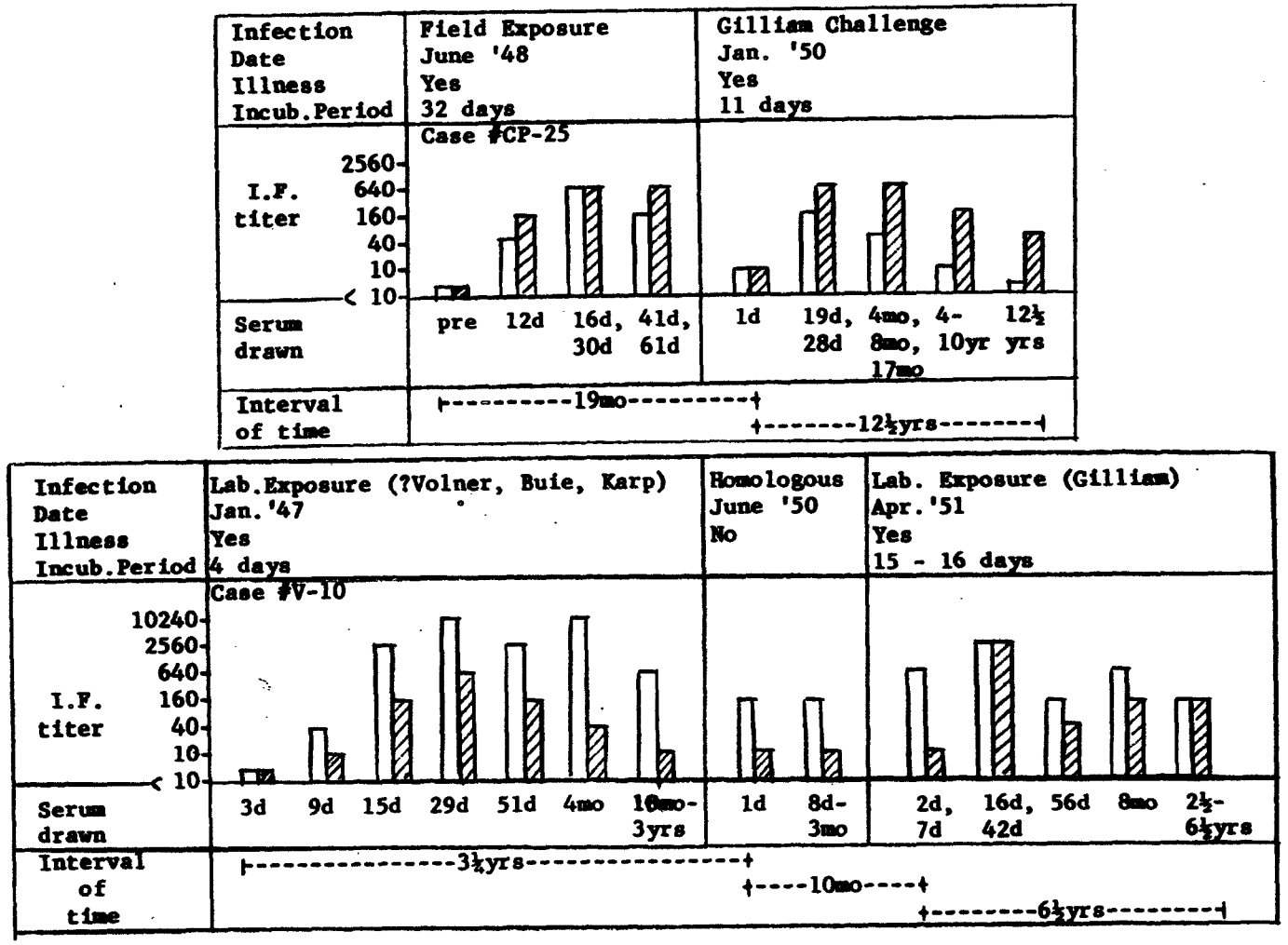


Table II

RESULTS OF IMMUNOFLUORESCENT TESTS ON THAILAND HUMAN SERA FOR DETECTION OF PAST *R. TSUTSUGAMUSHI* INFECTION

	R. tsutsugamushi I.F. Test		
Location	Number tested	Number positive 1:10	1:40
Huai Mae Sanam	93	15	8
Border Police N.W. Thailand	49	15	6
Khon Kaen Forest Reserve	44	13	9
Border Police Chong Mek	8		2
Totals	194	43	25

Table III

MOUSE INFECTIVITY AND COMPLEMENT-FIXING ACTIVITY OF THREE STRAINS OF *R. TSUTSUGAMUSHI* PURIFIED BY TWO METHODS

METHOD OF PURIFICATION	STRAIN	MOUSE TITERS LD_{50} PRE	LD_{50} POST	ID_{50} PRE	ID_{50} POST	COMPLEMENT-FIXING TITER OF FINAL PREPARATION
1	Gilliam	$10^{-2.0}$	$10^{-1.0}$	$10^{-7.0}$	$10^{-4.9}$	1:80
2	Gilliam	ND	ND	$10^{-7.3}$	$10^{-7.0}$	1:80
	Gilliam	$10^{-3.0}$	$10^{-1.8}$	$10^{-8.3}$	$10^{-6.9}$	1:160
	Gilliam	$10^{-2.0}$	$10^{-2.3}$	$10^{-9.0}$	$10^{-8.0}$	1:160
	Kato	$10^{-7.3}$	$10^{-6.3}$	$10^{-7.8}$	$10^{-6.8}$	1:160
	Karp	$10^{-8.4}$	$10^{-7.1}$	$10^{-9.4}$	$10^{-6.9}$	1:160

Figure 5

COMPARISON OF ANTIBODY TITERS IN RABBITS INFECTED WITH R. TSUTSUGAMUSHI WHEN TESTED WITH PURIFIED AND SOLUBLE ANTIGENS

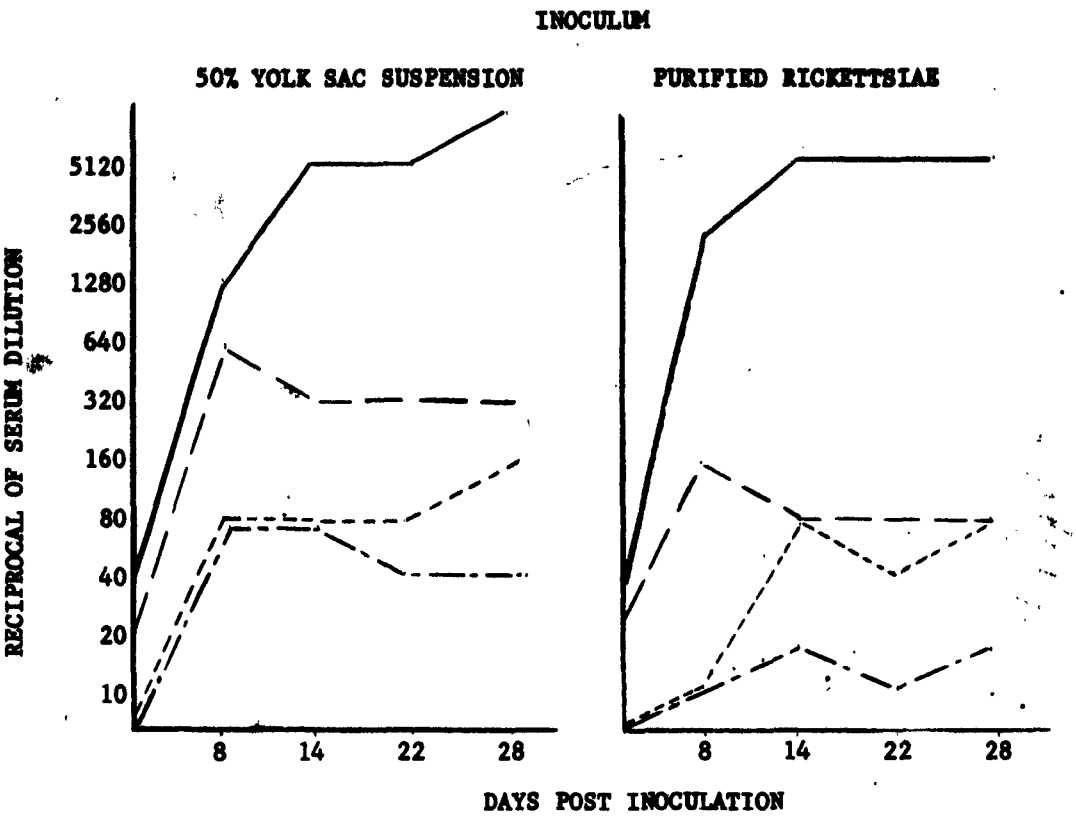
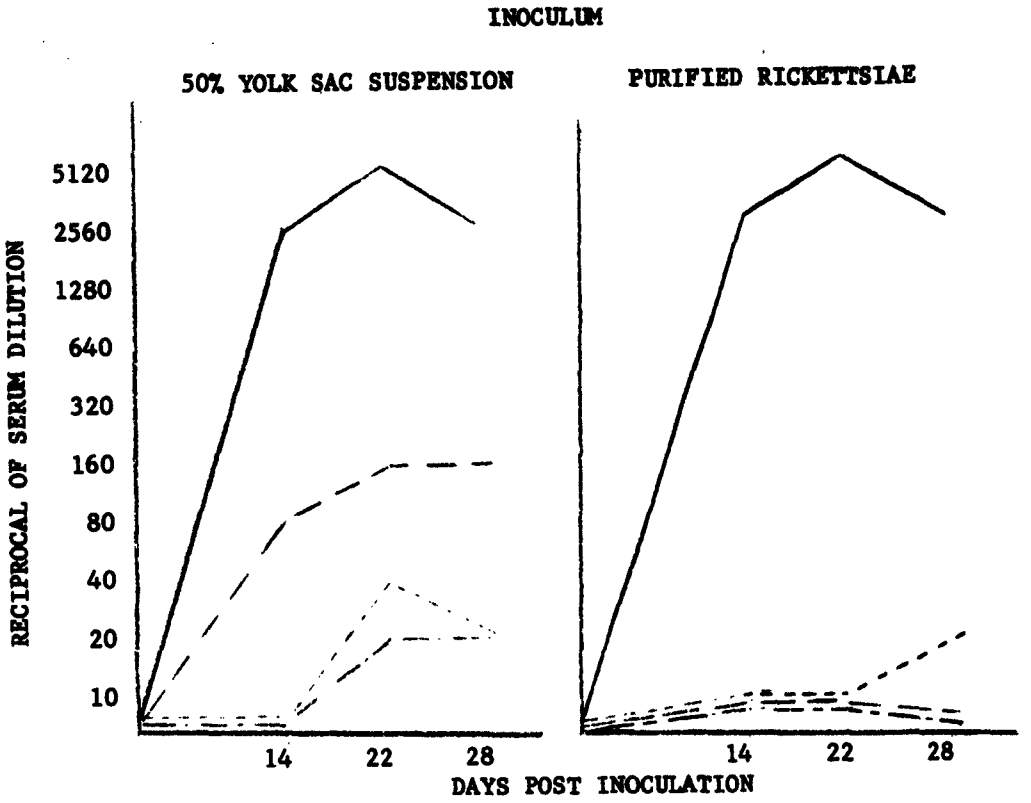


Figure 6

COMPARISON OF ANTIBODY TITERS IN GUINEA PIGS INFECTED WITH R. TSUTSUGAMUSHI WHEN TESTED WITH PURIFIED AND SOLUBLE ANTIGENS



Antigens:
- Purified Gilliam ————
- Soluble Gilliam - - - - - -
- Purified Normal Yolk Sac — — —
- Soluble Normal Yolk Sac —·—·—

Table IV

ROCKY MOUNTAIN SPOTTED FEVER IN FAMILIES

Number of Cases per Family	Number of such Families	Total Number of Cases
2	106	212
3	6	18
4	4	16
7	1	7
TOTALS:	117	253
TOTAL CASES NOT IN FAMILIES:		2,566
TOTAL CASES:		2,819

Table V

MULTIPLE FAMILIAL CASES OF SPOTTED FEVER:

RELATIONSHIPS AMONG PATIENTS *

Relationship	Number of Families with: 2 cases	3 cases	4 cases	Total Cases
Sibling children	58	1	2	127
Parent and child	26			52
Husband and wife	11			22
Cousins	1			2
Mother and 2 children		3		9
Husband, wife, daughter		1		3
Husband, wife, 2 grandchildren			1	4
Mother, 2 daughters, cousin			1	4
"Relatives"	10	1		23
Totals	106	6	4	246

* A single family group with 7 cases is not included.

Table VI

ROCKY MOUNTAIN SPOTTED FEVER IN FAMILIES

Interval, days, between onset of successive cases	Number of such occurrences	Grouped Data
0	21	
1	19	
2	17	
3	10	
4	8	
5	8	
6	5	
7	6	First week 94
8	4	
9	2	
10	6	
11	2	
12	1	
13	2	
14	2	Second week 19
>14	10	
TOTAL:	123	
INFORMATION LACKING:	13	

Table VI

ROCKY MOUNTAIN SPOTTED FEVER IN FAMILIES

Interval, days, between onset of successive cases	Number of such occurrences	Grouped Data
0	21	
1	19	
2	17	
3	10	
4	8	
5	8	
6	5	
7	6	First week 94
8	4	
9	2	
10	6	
11	2	
12	1	
13	2	
14	2	Second week 19
>14	10	
TOTAL:	123	
INFORMATION LACKING:	13	

Table VII

ROCKY MOUNTAIN SPOTTED FEVER

Nature of exposure in 86 families in each of which two cases occurred:

Tick bite:	Number of families	Number of patients	Number of bitten patients
Yes, both members of pair	49	98	98
One "yes," other "no"	14	28	14
No, both	18	36	0
Exposure other than tick bite:	5	10	1*
TOTALS:	86	172	113

* One member of this pair was bitten, while the other "had ticks crawling on him."

113 of 172 patients, or 66 per cent, gave positive history of tick bite.

ANNUAL PROGRESS REPORT

Project: 3A 0 12501 A 806, Military Preventive Medicine

Task 01, Communicable Diseases (Mycotic Diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Bacteriology
Division of Communicable Diseases and Immmology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Robert L. Taylor, Major, MSC, Ph.D.
Charlotte C. Campbell, B.S.

Assistants: Grace B. Hill, B.S.*
Barney T. Falgout, B.S.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

*Veterans Administration Central Laboratory for Clinical Pathology and Research

ABSTRACT

Project No.: 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Mycotic Diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Robert L. Taylor, Major, MSC, Ph.D.
Charlotte C. Campbell, B.S.
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. The development and comparative evaluation of a complement fixation test based on micro-titer techniques versus the standard 50% end point tube test was undertaken. The approximately 12,000 CF tests conducted annually in this laboratory prompted the determination of the feasibility of utilizing this time saving technique. Preliminary results are encouraging and further evaluation is planned.

2. Metabolic studies of both the yeast phase and mycelial phase of Histoplasma capsulatum and Sporotrichum schenckii were initiated in an effort to obtain definitive information on the metabolic capabilities of these pathogenic fungi. Preliminary investigations have produced data on the oxidative dissimilation of various carbohydrates. More complete investigations, including the use of cell-free extracts, are planned.

3. One hundred ninety-nine small rodents collected in the State of New Mexico were examined for evidence of naturally acquired coccidioidomycosis. Disease could not be proved in any of the animals, however, these animals were collected for another study and were not suitable for cultural recovery of mycotic agents. Preliminary serologic data on a limited number of these animals suggests naturally acquired infection in these animals and a follow-up study is planned.

4. The reference and diagnostic facilities furnished by this laboratory have recently provided verification of one of the few cases of histoplasmosis recognized in India. Histoplasma capsulatum was recovered from an oral lesion and serial complement-fixation titers on the patients serum are being obtained.

5. The histoplasmin and coccidioidin skin test survey initiated in Honduras in 1962 is continuing and preliminary data show a moderate incidence of exposure to H. capsulatum among the indigenous population.

BODY OF REPORT

Project No.: 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Mycotic Diseases)

Description: Investigation of techniques to improve serologic methods for the rapid diagnosis of mycotic diseases were initiated. Studies relating to the ecology, epidemiology and geographic distribution of H. capsulatum and C. immitis are continuing. Fundamental explorations of the metabolic capabilities of the pathogenic fungi are also in progress.

Progress: During the current reporting period the research program conducted by the Mycology Section was of necessity interrupted several times due to personnel changes, the prolonged illness of a key employee and the movement of the laboratory with the accompanying problems encountered in the re-establishment in new quarters. Miss Campbell resigned as Chief of the section in July 1962 and was on leave from mid August until 14 September 1962 at which time Major Robert L. Taylor assumed the duties of Chief of the section. During September the section moved into new laboratories in the Siler Pavillion of the Institute of Research. The services of Mrs. Grace B. Hill were lost in November 1962 due to an illness which has not permitted her return to work. Since this time the reduced staff has initiated several new studies and maintained the diagnostic and reference services of the section.

1. Investigations of techniques to improve methods for rapid diagnosis of mycotic infections.

The demand for complement-fixation tests as an aid in the diagnosis of mycotic diseases is increasing annually. Since this laboratory supplies this service for the Armed Forces medical installations, many of the Veterans Administration hospitals, plus local civilian hospitals; attempts were initiated to more effectively meet this increasing workload.

A complement-fixation test utilizing micro-titer techniques was devised which would effect a 50% saving in man-hours. A comparative evaluation of the micro-titer complement-fixation test with the standard 50% endpoint complement-fixation tube test is currently in progress. Comparative results have been obtained with approximately 100 sera having titers with at least one of the four antigens employed (whole-yeast-phase Histoplasma capsulatum, histoplasmin, ground-yeast-phase extract of Blastomyces dermatitides and coccidioidin).

These preliminary results show a comparative correlation between the standard tube test and the micro-titer test of ninety per cent (plus or minus one tube). The development of a standard for reading the micro-titer tests plus additional modification of incubation time and simultaneous testing of sera in both tests should result in a correlation equal to that obtained by replication of the tube test.

Development of the complement-fixation micro-titer technique will have advantages other than the obvious labor-saving it will effect in the performance of the diagnostic tests. The capability of performing complement-fixation tests with as little as .025 ml of serum will make it technically possible to use the laboratory mouse for serologic investigations. If the mouse proves to be a reliable model for such experiments the scientific and economic advantages of replacing each experimental rabbit with 10-20 mice become obvious.

Development and evaluation of the complement-fixation micro-titer technique will continue.

2. Metabolic activities of the pathogenic fungi.

Attempts have been initiated to demonstrate any metabolic differences existent in the yeast phase and mycelial phase of the diphasic pathogenic fungi. This study is conducted in collaboration with Dr. James H. Rust, Jr., Department of Bacteriology, WRAIR.

Until complete methodology can be devised and tested the organism chosen for the preliminary investigations has been the yeast and mycelial phases of Sporotrichum schenckii.

Data have been obtained on the ability of intact cells of both phases of S. schenckii to utilize various carbohydrates. Standardized aliquots of the cells were studied in the Warburg Respirometer and O_2 uptake measured over the six hour observation periods.

The mycelial phase of S. schenckii was grown in a simple NH_4NO_3 dextrose medium on a rotary shaker at 37°C for a period of three days. The yeast phase of S. schenckii was grown in a basic casamino acid dextrose medium on a rotary shaker at 37°C. Attempts were made to grow the yeast phase on the same NH_4NO_3 medium used for the mycelial phase by increasing the CO_2 tension. CO_2 has been reported as stimulatory for the growth of the yeast phase, however, CO_2 tensions up to 10% failed to maintain the yeast phase in the simple NH_4NO_3 medium.

Growth curves were determined for both phases of S. schenckii using the growth conditions previously outlined. From these data it was determined that three days was the optimal time to harvest the cells since by this time good growth had been attained and the organism was still in the logarithmic growth phase.

Microscopic examination of the cultures at three days showed a 95% predominance of the yeast phase in the casamino acid medium and a similar predominance of short mycelial fragments in the NH_4NO_3 medium.

The cells were centrifuged from the growth medium, washed three times in KH_2PO_4-K_2HPO_4 buffered saline. The suspensions used in the Warburg Respirometer were standardized by adjusting to an optical density of 4.0 (1:160 dilution) at a wave length of 610 millimicrons on a model 14 Coleman spectrophotometer.

Preliminary data indicate the intact yeast-phase cells of S. schenckii can oxidatively utilize dextrose, mannose, galactose, fructose, sorbose, xylose and maltose. Rhamnose, lactose, ribose, trehalose, cellobiose, arabinose, sucrose, lytose and raffinose were not or only slightly utilized. A similar pattern of carbohydrate utilization was found when the mycelial phase was studied.

Further studies are planned including the use of cell-free extracts to determine the enzymatic composition of these organisms. Such knowledge may be of value in the determination of a specific chemotherapeutic agent.

Similar studies were begun with Histoplasma capsulatum and preliminary data indicate the carbon source used for propagation of the test organisms may play an important role in their enzymatic capabilities. Further investigations of the influence of various carbon sources on the production of adaptive enzymes are planned.

3. Ecology of Coccidioides immitis in New Mexico.

A limited study of the ecology of Coccidioides immitis was possible this year when small animals collected during a survey for animal plague were also made available for examination by this section.

The rodents were snap-trapped throughout the State of New Mexico, bled if possible, fleas removed, frozen with CO_2 and shipped to WRAIR. Upon arrival the frozen animals were stored in a mechanical freezer until they could be examined. A total of 791 animals were trapped in this study, however, only those animals collected in the Lower Sonoran Life Zone of New Mexico were examined for naturally acquired coccidioidomycosis. One hundred ninety-nine rodents met this criterion and were distributed among the following genera: Dipodomys, Neotoma, Onchomys, Citellus, Peromyscus, Reithrodontomys and Perognathus.

Since the animals were not trapped specifically for this study (snap-trapped) they were not ideally suitable for definitive culture studies for fungi, therefore, a cursory microscopic examination was made of the lungs of each animal and when possible homogenized portions of lung tissue were inoculated intraperitoneally into two mice. The inoculated mice were held for a period of four weeks, sacrificed and microscopic examination of lungs made. Naturally acquired coccidioidomycosis was not demonstrated by these techniques, however, little importance can be given the negative results since cultural studies were not conducted.

Complement-fixation tests conducted on fifty-one animal sera, using the micro-titer technique, indicate there may indeed be naturally acquired mycotic infections among the rodents of Southern New Mexico.

Current plans are in progress to alter the protocol in a proposed Plague survey in New Mexico to permit a definitive culture study for mycotic infections. The changes in protocol would include live trapping of the animals with immediate autopsy and culture.

4. Culturally Verified Case of Histoplasmosis in India.

In January 1963 a request for diagnostic assistance was forwarded by the U. S. Army Attache in New Dehli, India which led to verification of one of the few cases of histoplasmosis known to have occurred in India. The request was originated by the Indian Army Medical Department and the patient is a colonel in the Indian Army. Correspondence with the Indian clinician has resulted in the submission of serial serum specimens for the determination of complement-fixing titers in an effort to follow the progress of the disease. The fungus isolated from an oral lesion was also sent to WRAIR for confirmation and was identified as Histoplasma capsulatum. The epidemiologic importance of histoplasmosis in India assumes greater significance as more cases are recognized in this area of the world.

5. Geographic Distribution of Mycotic Agents.

Eight thousand-seven hundred and thirty-nine residents of Honduras, C.A. have been skin tested with histoplasmin in conjunction with a national tuberculin testing program. Histoplasmin testing was begun in 1962 through a collaborative program with Miss Virginia Perkins, Laboratory Advisor, Servicio Interamericano de Salud Publica.

Three different areas in Honduras have been surveyed and the 8,739 persons tested represent almost the entire population in these areas. The greatest percentage of reactors were found in the 40 to 44 year age groups where the range of hypersensitivity among the three areas tested was 39 to 50 per cent, indicating these areas are endemic for histoplasmosis. One case of clinical histoplasmosis originating in one of these areas has been confirmed at Gorgas Hospital, Canal Zone.

A cross section sampling of the country was obtained when 2,145 histoplasmin skin tests were completed on personnel in the Central Penitentiary. The maximum number of reactors among this population was also in the 40 to 44 year age group where hypersensitivity exceeded 60 per cent, indicating there are probably highly endemic areas in the country. Determination of the residence of inmates hypersensitive to histoplasmin may reveal the location of these areas which can be confirmed by additional skin tests.

Additional skin testing with histoplasmin and coccidioidin are planned.

Summary and Conclusions:

1. Encouraging results have been obtained in the developmental stages of a complement-fixation test for mycotic agents using micro-titer techniques.

2. Metabolic studies of the yeast phase and mycelial phases of diphasic pathogenic fungi have been initiated by investigating the oxidative dissimilation of various carbohydrates by S. schenckii and H. capsulatum.

3. Microscopic examination of one hundred ninety-nine rodents trapped in New Mexico and representing seven genera failed to show evidence of naturally-acquired mycotic infections. Serologic evidence indicated the presence of mycotic infections and future plans include a cultural survey of animals collected in Southern New Mexico.

4. A clinical case of histoplasmosis in a colonel of the Indian Army was culturally verified thereby establishing India as an endemic area for histoplasmosis.

5. Data obtained from 10,884 histoplasmin skin tests applied in Honduras indicate certain areas in this Central American country are also highly endemic for histoplasmosis.

Publications:

1. Robert L. Taylor. Georgraphic Variation in the Prevalence of Histoplasmin Sensitivity in the Panama Canal Zone. Am. J. Trop. Med. & Hyg., 11: 670-675, 1962.

2. Robert L. Taylor, M. H. Shacklette, and H. B. Kelley. Isolation of Histoplasma capsulatum and Microsporum gypseum from Soil and Bat Guano in Panama and the Canal Zone. Am. J. Trop. Med. & Hyg., 11: 790-795, 1962.

3. C. F. Abildgaard and Robert L. Taylor. Histoplasmosis Survey of Preschool Children in Panama. Am. J. Trop. Med. & Hyg., 11: 666-669, 1962.

4. Robert L. Taylor and M. H. Shacklette. Naturally acquired Histoplasmosis in the Mammals of the Panama Canal Zone. Am. J. Trop. Med. & Hyg., 11: 796-799, 1962.

5. Grace B. Hill and Charlotte C. Campbell. Commercially Available Histoplasmin Sensitized Partivles in an Agglutination Test for Histoplasmosis. Mycopathologia et Mycologia applicata, XVIII: 169-176, 1962.

6. Robert L. Taylor, Leonardo Benedetti, H. B. Kelley, Martha H. Shacklette and Julio J. Fuentes. Histoplasmin Sensitivity among 823 Panamanian Children. Archivos Medicos Panamenos, December 1962. (English and Spanish translation).

ANNUAL PROGRESS REPORT

Project: 3A 0 12501 A 806, Military Preventive Medicine

Task 01, Communicable Diseases (Acute Gastroenteritis)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Bacteriology
Department of Applied Immunology
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: William C. Branche, Jr., M.S.

Assistants: Felicenne M. Houston, B.S.*
Lucille W. Koontz, B.S.*
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Acute Gastroenteritis)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: William C. Branche, Jr., M.S.
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

A new combination of antibiotics for use in viral studies has been devised and tested. Prostaphlin, colistin sulfate and amphotericin B have successfully eliminated troublesome bacterial contaminants in specimens for viral studies. The Escherichia coli typing service received 134 cultures during the year. Serologic typing is being limited to the enteropathogenic and suspect pathogenic groups. A viral agent, PR10, recovered from a Puerto Rican infant with diarrhea, has been accepted by the Panel on Picornaviruses as the prototype of ECHO virus, type 32. Another agent, PR17, was identified as ECHO 30 for which a prototype had been submitted earlier. Studies on the role of E. coli bacteriophage in the human intestine have been completed and the results are being tabulated. The growth of Entamoeba histolytica on tissue culture has been continued. Bacterial contamination has been reduced to $<10^2$ organisms/ml.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Acute Gastroenteritis)

Description: The objective of this task is to study the problem of acute gastroenteritis to delineate more clearly the character of the disease, host-parasite relationships and improve the laboratory diagnostic techniques.

Progress:

1. Antibiotic Combination. To eliminate contamination of monolayer cell culture lines by antibiotic resistant bacteria or fungi usually present in specimens for viral studies, a new combination of antibiotics was devised. Thirty mcg/ml each of prostaphlin, a synthetic penicillin, colistin sulfate and amphotericin B were added to the maintenance medium of Rhesus monkey kidney, human amnion (WISH) and Hela cell cultures. The antibiotics produced no cytopathology on the above cells, at the concentrations used, nor was there any inhibition of $TCID_{50}$ of polio virus, type 2, ECHO virus, type 9, Coxsackie virus A9 or adenovirus type 2 in the 3 cell lines.

Duplicates of 10 control specimens from which viruses had been previously recovered were initially processed by incorporating 500 mcg/ml of prostaphlin, amphotericin B and colymycin. The same viruses, including polio, ECHO, Coxsackie, influenza and herpes, were successfully isolated again.

Based on these observations, use of the combination of prostaphlin, amphotericin and colymycin is recommended in situations where bacterial contamination is a problem in the recovery of viruses from patient specimens. Additional comparative studies on the efficacy of these antibiotics on specimens for viral studies are in progress.

2. Enteropathogenic Escherichia coli. During the period of 1 July 1962 through 30 June 1963 the Escherichia coli typing service received a total of 134 cultures from 51 infants for serological identification of enteropathogenic E. coli. From 19 of these infants, enteropathogenic E. coli, representing 8 serotypes were identified. Included were five E. coli 0102, three each of E. coli 073 and 0112, two each of E. coli 025, 076, and 0111, and one each of E. coli 074, 075, and 0127. Nine cultures were biochemically identified as organisms belonging to other genera in the family Enterobacteriaceae. Seventy-seven per cent of these cultures were sent from Walter Reed General Hospital.

3. Enteroviruses.

a. Of the two viruses submitted to the Panel for Picornaviruses (formerly the Committee on Enteroviruses) one, the PR10 strain, has been accepted as the prototype of ECHO virus, type 32. The other virus, PR17, was identical to ECHO virus type 30 for which earlier candidates

Bastianni, Frater, Prince and Giles) had been submitted.

The three remaining Puerto Rican viruses, PR20, PR22, and PR28 will be ready for submission to the Panel of Picornaviruses after determination of particle sizes.

b. The study of the remaining low titer group of viruses from fecal samples collected from infants with acute gastroenteritis at Children's Hospital, Washington, D. C. (See Annual Report, 1961-62) nears completion. Identification studies have been hampered by poor quality commercial tissue cultures. These adeno-like viruses lose tissue culture infectivity if frozen, therefore, identifications will be completed when high titer material can be obtained from 4-5 continuous and consecutive rapid passages in human amnion (WISH) or Hela cultures.

4. Bacteriophage. Evaluation of results from experiments to determine the role of bacteriophage on the fluctuation of Escherichia coli in the normal human intestine is still in progress. Methods to detect lysogeny and to differentiate between bacteriophage plaques and colicine zones were the same as those described in 1961-1962 Annual Reports: (1) Forty-six lysates from 338 U.V. irradiated E. coli cultures produced lysis when tested against 234 E. coli indicator strains; (2) a few strains produced colicine and bacteriophage simultaneously; (3) some irradiated E. coli produce colicines against themselves; (4) colicines detected by this method do not necessarily correlate with those detected by the simultaneous or chloroform assay methods. (Annual Reports, 1959-1960).

5. Entamoeba histolytica: Experiments are in progress to devise a system for producing axenic cultures of Entamoeba histolytica. Studies have been hampered due to the unavailability of satisfactory tissue cultures. Recent trials have shown the following medium to be excellent for maintaining and transferring amoebae in monolayer monkey kidney cells; bovine amniotic fluid containing 1 per cent male mouse liver extract and 100-250 micrograms/ml. of neomycin. These concentrations of neomycin reduced the bacterial contamination from 10^9 to fewer than 10^2 organisms/ml. and have allowed five successful serial transfers within a 12-day period without apparent damage to the amoebae. Previously it was observed that amoebae could be transfered for at least 40 days in the same medium with 2,5 mcg/ml. neomycin but bacterial contamination was only reduced to 10^6 organisms/ml.

The amoebae were stained in monkey kidney Leighton tubes with Mallory's phosphotungstic acid hematoxylin, and microscopic examination revealed that the amoebae appeared to be normal.

Summary and Conclusions: A new antibiotic combination for viral studies has been devised and tested. The Enteropathogenic Escherichia coli typing service received 134 cultures for serologic identification. PR10 virus has been accepted as the prototype of ECHO virus, type 32. Experiments on the effect of bacteriophage on the E. coli flora of the normal human intestine have been completed. Studies on the axenic growth of Entamoeba histolytic are being continued.

Publications:

1. Relationship of Host Antibody to Fluctuations of Escherichia coli. Serotypes in Human Intestine. Harriette G. Robinet. Journal of Bact., 84: 896-901, 1962.

2. A New Combination of Antibiotics for the Treatment of Contaminated Specimens for Viral Studies. L. D. Bourgeois and W. C. Brueche, Jr. Bacteriological Proceedings, 1963, p. 143.

3. Studies of Infectious Agents in Infant Diarrhea. Part III. Bacterial, Viral, and Parasitic Agents in Feces of Puerto Rican Children. V. M. Young, R. B. Lindberg, A. Ortiz, D. Haniel, M. Sochard and J. Hemphill. American Journal of Tropical Medicine and Hygiene. Vol. 11: 380-388, 1962.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 806, Military Preventive Medicine

Task No. 01, Communicable Diseases (Schistosomiasis and Other Parasitic Diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Medical Zoology
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicin

Task 01 Title: Communicable Diseases, (Schistosomiasis and Other Parasitic Diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Medical Zoology
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Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Experiments in schistosmiasis immunization were carried out by exposure of experimental animals to (1) homologous and heterologous cercariae, (2) irradiated cercariae, and (3) inoculations of preparations from schistosomes. The results indicated that immunization in schistosomiasis is indeed possible in experimental animals. However, the degree of immunity acquired varies greatly with the species of parasite studied, with the host species, and with the technique used in the process of vaccination.

Experiments tracing the development of Dirofilaria uniformis in the mosquito, Anopheles quadrimaculatus, indicated that the microfilariae penetrated the gut wall and developed in the fat body. In a related species, Dirofilaria immitis, motility of the microfilariae was found to be useful in distinguishing D. immitis infections from other filarial infections occurring in dogs.

Studies are being conducted on the biology and immunochemistry of malaria. An inverted slide method for staining blood smears was developed. By this method, staining occurs without deposition of dye in the form of artifacts and without transferral of parasites. A direct method for enumeration of malaria parasites was developed utilizing toluidine blue and a hemacytometer. Esterase activity in Plasmodium berghei has been detected histochemically. This activity appears to be resistant to various inhibitors. Immunodiffusion studies on a soluble antigen preparation from Plasmodium berghei have been performed. These studies are being continued.

BODY OF REPORT

Project No. 3A 0 12501 A 806 **Title: Military Preventive Medicine**

Task 01 **Title: Communicable Diseases, (Schistosomiasis and Other 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 response to treatment in the host. Schistosomiasis, filariasis, trichinosis, trypanosomiasis, leishmaniasis, and malaria were used as models for these studies.

Progress:

1. Resistance induced in rats by vaccination with fresh homogenates on adult Schistosoma mansoni worms. Failure to demonstrate acquired resistance to S. mansoni by vaccination has been reported by several investigators. All these reported attempts have been conducted using mice or monkeys as experimental animals. A series of experiments was set up (1) to determine whether artificial immunization to S. mansoni can be demonstrated in selected experimental hosts, (2) to determine the number of immunizing doses needed, and (3) to determine the interval between the antigen inoculations and the challenging exposure.

a. One-day and 14-day old albino rats were selected as test animals. Some groups were inoculated with adult worm homogenates prepared fresh for each experiment, some received bovine albumin as a nonspecific antigenic protein control, and others received commercially prepared nonpyogenic sodium chloride. In all experiments some rats from each group were used for serological studies.

b. The results of the first experiment are summarized in table 1. At necropsy, the controls inoculated with saline harbored a higher number of worms than the rats receiving homogenates, bovine albumin, or a primary infection with 100 cercariae. A biometrical analysis of the data indicate that the difference in worm recovery between the control group receiving saline and each of the other groups is statistically significant at the 0.05 level. No significant difference was observed among rats which received worm homogenates, bovine albumin, or cercariae.

c. In the second experiment (table 2), a considerably smaller number of worms was recovered in all groups. Nevertheless, the controls inoculated with saline (Group V) harbored a significantly higher percentage of worms than those receiving worm homogenates, (Groups I and II) and those exposed to cercariae (Group IV). In this experiment seven of the rats which had been exposed to 100 cercariae were not challenged and were kept as controls of the primary infection. At necropsy, they harbored an average of 0.9 worms per animal equivalent to a percent recover of 0.1.

d. In a subsequent experiment (table 3) essentially similar results were observed. The nonimmunized controls (Group V) harbored a significantly greater number of worms than those which had received worm homogenates (Groups I and II), bovine albumin (Group III), and cercariae (Group IV). Furthermore, the rats exposed to cercariae (Group IV) had a significantly smaller number of worms than those which were artificially immunized. No significant difference was observed in any of the first three experiments between the animals receiving three and those receiving 21 inoculations of worm homogenates.

e. Another experiment was set up to attempt to study the effect of repeated injections of worm homogenates or bovine albumin in older rats. This experiment was conducted essentially in the same manner as the previous three except (1) the rats were 14 days old at the time of the first inoculation or exposure, (2) those of Group IV were exposed to 200 instead of 100 cercariae, and (3) all the rats were necropsied 26 days later instead of 28. As indicated in table 4, at necropsy the control animals receiving saline injections had a considerably greater number of worms than those injected with worm homogenates or bovine albumin. These differences are highly significant. Unlike the previous three experiments, however, the group which had been exposed to 200 cercariae (Group IV) had a higher number of worms than the animals of the other groups. Four rats which had been exposed to 200 cercariae were not challenged and used as controls of the primary infection. At necropsy only one worm was recovered from this group.

f. The results of serological tests performed in the rats of the various groups in different experiments are summarized in table 5. Among the animals which were one day old at the beginning of each experiment (Exp. I, II, and III), no antibodies were detected by the FA technic in those groups which had been repeatedly inoculated with saline (Group V) or bovine albumin (Group III). Fluorescent antibodies were detectable toward the end of the experiment in those rats which were inoculated

with worm homogenates (Groups I and II) or exposed to 100 cercariae (Group IV). Among the rats which were 14 days of age at the beginning of the experiment (Exp IV), antibodies were detectable in all groups much sooner following challenge except for those which received saline injections (Group V).

Table 1

Number of Schistosoma mansoni recovered from one-day old rats challenged with 500 cercariae 28 days after the first inoculation or exposure and necropsied 28 days later. (Experiment I)

Group number	Number of rats	Inoculation or exposure	Mg nitrogen per rat	Number of inoculations or exposures	Worms recovered	
					Mean number	Percent cercariae
I	8	Worm homog.	2.35	21	21.1	4.2
II	3	Worm homog.	0.33	3	4.0	0.8
III	9	Bovine albumin	4.56	21	24.5	4.9
IV	9	100 cercariae	-	1	9.0	1.8
V	6	Saline	0	21	80.0	16.0

Table 2

Number of Schistosoma mansoni recovered from one-day old rats challenged with 500 cercariae 28 days after the first inoculation or exposure and necropsied 28 days later. (Experiment II)

Group number	Number of rats	Inoculation or exposure	Mg nitrogen per rat	Number of inoculations or exposures	Worms recovered	
					Mean number	Percent cercariae
I	16	Worm homog.	1.69	21	5.8	1.2
II	16	Worm homog.	0.24	3	7.2	1.4
III	20	Bovine albumin	4.50	21	11.0	2.2
IV	31	100 cercariae	-	1	3.0	0.6
V	13	Saline	0	21	12.5	2.5

Table 3

Number of Schistosoma mansoni recovered from one-day old rats challenged with 500 cercariae 28 days after the first inoculation or exposure and necropsied 28 days later (Experiment III).

Group number	Number of rats	Inoculation or exposure	Mg nitrogen per rat	Number of inoculations or exposures	Worms recovered	
					Mean number	Percent cercariae
I	21	Worm homog.	3.47	21	36.4	7.2
II	22	Worm homog.	0.50	3	44.0	8.8
III	18	Bovine albumin	4.12	21	34.1	6.6
IV	13	100 cercariae	-	1	19.7	3.9
V	27	Saline	0	21	53.1	10.6

Table 4

Number of Schistosoma mansoni recovered from 14-day old rats challenged with 500 cercariae 28 days after the first inoculation or exposure and necropsied 26 days later (Experiment IV).

Group number	Number of rats	Inoculation or exposure	Mg nitrogen per rat	Number of inoculations or exposure	Worms recovered	
					Mean number	Percent cercariae
I	14	Worm homog.	4.17	21	1.2	0.2
II	14	Worm homog.	0.60	3	1.7	0.3
III	16	Bovine albumin	4.17	21	1.2	0.2
IV	12	200 cercariae	-	1	16.2	3.2
V	18	Saline	0	21	7.2	1.4

TABLE 5

The early appearance of fluorescent antibodies on rats of different ages artificially immunized or exposed to S. mansoni cercariae prior to challenge

Time from 1st inoculation or exposure (in days)	Time from challenge (in days)	Results obtained with the fluorescent antibody test									
		Group I(Homog.21)		Group II(Homog.3)		Group III(BSA)		Group IV(Cer)		Group V(Saline)	
		Exper I-III	Exper IV	Exper I-III	Exper IV	Exper I-III	Exper IV	Exper I-III	Exper IV	Exper I-III	Exper IV
27	-1	-	-	-	-	-	-	-	-	-	-
35	7	-	+	-	-	-	-	-	+	-	-
43	15	-	+	-	+	-	-	-	+	-	-
50	22	-	+	-	+	-	+	+	+	-	-
55-57	26-28	+	+	+	+	-	+	+	+	-	-

2. Studies with irradiated Schistosoma mansoni cercariae in monkeys (In collaboration with Harvard University, School of Medicine, Boston, Massachusetts). The encouraging results obtained in attempts to induce immunity in mice with irradiated cercariae stimulated the setting up of further extensive investigations in monkeys. The ability of immunized monkeys to survive otherwise lethal infections was used as the principal indicator of resistance.

a. Szumlewics and Olivier suggested that the acquired resistance developed following exposure to irradiated cercariae may be transitory, resulting only in a delay in the development of the worms of the challenging infection. This possibility was taken into account in setting up experiments with monkeys. Therefore, monkeys immunized with irradiated cercariae were sacrificed four months after challenge when worms, if present, could be recovered even if their development had been temporarily delayed.

b. The results of our experiments with monkeys are summarized in table 6. They indicate that exposure to irradiated cercariae induced a marked resistance to a subsequent challenge of nonattenuated cercariae of S. mansoni. This acquired immunity was manifested by the ability of monkeys to survive otherwise lethal infections, by a greatly decreased worm burden, and by a much smaller number of eggs in the stools. Furthermore, at necropsy 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 developed ascites. The protection induced by five exposures to cercariae irradiated at either 2,500 or 4,000 rep appeared to be greater than that induced by five exposures to cercariae irradiated at 10,000 rep or a single exposure to five times as many cercariae irradiated at 4,000 rep. A considerably reduced number of worms was observed in the immunized monkeys as late as four months after challenge thus indicating that the acquired resistance following exposure to irradiated cercariae is not a transitory phenomenon, and that the development of the worms was not merely delayed but was altogether prevented. The percentage recovery of worms in immunized monkeys was also compared with that of normal monkeys which had been sacrificed at the same time (Groups XI and XII). It must be pointed out that the animals of these groups were exposed to a much smaller dose of cercariae since otherwise they would have succumbed to the infection as did the non-immunized monkeys which were exposed to 4,000 cercariae (Groups II, IV, VI, VIII, and X).

Table 6

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		Challenge	Survivors		Average time of survival	Time between challenge and necropsy (days)	Mean no. of worms	Percent recovery	NEPGF**
		No. of cercariae	Rep	No. of cercariae	No.	%					
I	5	5 x 5,000	2,500	4,000	5	100	-	126	184.0	4.6	29.0
II	5	-	-	4,000	0	0	49	-	938.3	23.4	281.0
III	5	5 x 5,000	4,000	1,000	5	100	-	60	179.2	17.4	18.0
IV	5	-	-	1,000	4	80	44	-	352.4	35.4	64.0
V	5	5 x 5,000	4,000	4,000	3	60	75	90	836.0	21.0	118.0
VI	5	-	-	4,000	0	0	50	-	1327.4	33.1	179.0
VII	5	5 x 5,000	10,000	4,000	3	60	61	90	686.0	17.1	191.0
VIII	4	-		4,000	0	0	57	-	960.0	24.0	159.2
IX	5	1 x 25,000	4,000	4,000	3	60	61	83	1321.0	33.0	145.0
X	4	-		4,000	0	0	50	-	1101.3	27.5	159.2
XI	5	-	-	450	5	100	-	124	101.0	22.0	45.0
XII	4	-	-	350	4	100	-	63	87.0	24.5	33.0

** Number of eggs per gram of feces

3. Parasite migration and host reaction in mice exposed to irradiated cercariae of Schistosoma mansoni. (In collaboration with Harvard University, School of Medicine, Boston, Massachusetts). Detailed histopathological studies were conducted on mice which were sacrificed at regular intervals after having been exposed to approximately 200 cercariae irradiated at the following levels: 2,500 rep, 5,000 rep, 50,000 rep. From each of these groups samples of lung, intestine, and lymph nodes of the skin were obtained at various time intervals.

a. The histopathological observations confirmed previous parasitological data indicating that the life span, migration and maturation of irradiated cercariae are affected in proportion to the irradiation level. Stunted young adults derived from cercariae irradiated at 2,500 rep. These attained their greatest number 40 days after exposure, but by the 50th day most of the schistosomula had died leaving small granulomas and calcified foci as a residue. When cercariae were exposed at 5,000 rep, only a few stunted worms were found in the liver. The majority of schistosomula appeared to die in the lungs although a few of them were stopped in the skin. When cercariae were irradiated with 50,000 rep most of the schistosomula died in the skin and subcutaneous tissues. Attention should be called to the fact that variations of biological vigor are such that even at 50,000 rep some parasites were able to migrate from the skin to the lungs and reach the liver. The results are summarized in table 7.

Table 7

Relative numbers of schistosomula and eggs in different organs at successive intervals following exposure to 200 cercariae of S. mansoni irradiated at different levels.

Group	Tissue	Time									
		Hours	Days								
		1	1	2	3	5	7	14	28	40	50
0	Skin	++++	+++	++	++	+		-	-	-	
	Lymph node	-	-	-	+	-					
	Lung			-			+++	++	-	-	+ E
	Liver			-			-	+	+++	+++EE	+ EEEE
	Intestine										+ EEEE
1	Skin	++++	+++		++	+		-			
	Lymph node	-	-	-	-	-					
	Lung			+			+++	+		-	+
	Liver				.			+		++	-
	Intestine										
2	Skin	+++	+++	++	+	+		-			
	Lymph node	-	-	-	-	+					
	Lung			+			+++	+++	+	-	-
	Liver							+	+	-	-
3	Skin	++++	+++	+++	+++	+++			-		
	Lymph node	-	-	-	-	-					
	Lung			-			+	-	-	-	-
	Liver			-			-	-	-	-	-

Legends: 0 = Non-irradiated controls
1 = Cercariae given 2,500 rep
2 = Cercariae given 5,000 rep
3 = Cercariae given 50,000 rep

Blank space = No sample taken
(+) to (++++) = Relative number of schistosomula
(E) to (EEEE) = Relative number of eggs
- = Sampled and found negative

4. Histopathologic studies on the role of Schistosoma mansoni eggs in resistance. (In collaboration with Harvard University, School of Medicine, Boston, Massachusetts). The results of parasitological and serological studies on the role of S. mansoni eggs in producing immunity against the cercarial form of the schistosome cycle had been reported previously. Four groups of mice were used in these studies. The animals of group 1 received 18,000 S. mansoni eggs each, group 2 received saline, group 3 received 12,000 Divinyl-benzene-copolymer beads, and those of group 4 received 0.5 mg of bovine albumin. Forty days after inoculation all the animals were challenged with approximately 200 S. mansoni cercariae. Fifty days later all animals were necropsied and perfused for worms. Intact livers, lungs, and portions of the small and large intestine were fixed in buffered 10% formalin for sectioning, staining, and subsequent pathological descriptions.

a. Histopathologic findings were in harmony with the parasitological results. All four groups showed schistosome infections of comparable severity with numerous eggs and large granulomas in the liver, often with central necrosis and accumulations of eosinophils. Severe periportal inflammation, focal liver cell necrosis and blockage of portal radicles were common. Eggs and granulomas were numerous in the small and large intestine, and were sporadically found in the pancreas, spleen, and lungs.

b. Histological differences between the groups were noted principally in the lungs. Eggs were found in the lungs of all mice in group 1, but in only 23% of the mice in the other three groups. There was little difficulty in distinguishing granulomas due to previous egg injection from those due to the recent challenge infection. Large cellular granulomas were seen in the control groups, and only group 1 showed lesions in a healing stage, featuring markedly degenerated eggs. In group 4, plastic beads and eggs could sometimes be observed together in the same field, the former without much inflammatory reaction, the latter surrounded by recent granulomas. Immature adult worms were found in the lungs of three animals all belonging to group 4 (plastic bead pre-injection).

c. Arteriolar inflammation was focal and individually variable in all groups. However, it was most severe in group 1 where vessels exhibited marked thickening, intimal proliferation, occulsion, and in a few instances multiple channel formation. A comparable degree of arteritis was present only in two animals of group 4, and only adjacent to stunted ectopic young adult worms in lung arterioles. With this exception, arteritis was mild or minimal in all control groups, and featured mild thickening, endothelial swelling, and intravascular clumping of leukocytes.

d. Mantling of arteioles and particularly of venules with lymphoid cells and a few eosinophils was seen in all groups, but was distinctly more diffuse and intensive in group 1 (pre-injected with schistosome eggs). Focal "alveolitis" i.e., alveolar cell desquamation and leukocyte infiltration unrelated to sites of egg deposition also occurred in all groups.

e. In the spleen, foci of eosinophilic cells were found in the cords of the red pulp in occasional animals. They were most frequent and largest in group 1, and mitotic division of granulated eosinophils were observed in some of them. Such foci were rare in group 3.

5. The natural history of infection with Schistosoma mansoni in capuchin monkeys. Most of the chemotherapeutic and immunobiological studies of schistosomiasis in primates had been carried out in the rhesus and African green faced monkeys. However, the rhesus monkey usually undergoes a spontaneous cure within one year after exposure. Although the African green faced monkey carries the infection for a longer period of time, the known schistosomicidal compounds have given unsatisfactory results in this animal. Furthermore, this primate is incapable of developing an adequate immunological and serological response to infection. In view of these considerations the natural history of infection with S. mansoni was studied in Cebrus apella commonly called the "tufted capuchin monkey".

a. Six capuchin monkeys were divided into three groups and exposed in the following manner: Group I received 50 S. mansoni cercariae each. Those of Group II received 250 cercariae each, and those of Group III received 1,000 cercariae each. Stools from these animals were examined at regular intervals for the presence of eggs. Each monkey was bled every two weeks from the beginning of the experiment and sera were collected for a study of the development of schistosome antibodies.

6. Procedure for the mass production of Schistosoma mansoni cercariae. (In collaboration with the Instrumentation Division, WRAIR). Since numerous cercariae are required for immunologic and diagnostic investigations of schistosomiasis, a method of mass-producing infected snails with a minimum of effort and laboratory space is needed. To this end a snail colony facility for the routine production of adequate quantities of cercariae was devised and placed in operation.

a. The snail units are constructed of angle iron and measure 5 1/2 x 2 x 6 feet high. They are divided into two sections, the top half of which consists of two 1/2 inch plywood shelves 18 inches apart covered with thin aluminum sheeting. These two shelves hold 10 ten-gallon aquaria. A single row of air valves mounted between the two rows of aquaria regulates the air supply. Behind each aquarium is an 18-inch fluorescent light wired in series for individual operation. The bottom section contains covered refrigerator pans which are used to house infected snails. These pans are supported by their flanges in aluminum frames attached to suspension slides as found in filing cabinets. Five rows of frames with two frames per row produce space for 30 pans within a three-foot height. By extending the frames to their full 15 inches, easy access to the pans for feeding or removal is obtained. The air supply is regulated by outlets, mounted on the pan section and frames, connected by flexible tubing to the individual pans. Water conditioning is accomplished by attaching a mixer valve and thermometer to the hot and cold water inlets. The water is purified and dechlorinated by passage through an activated charcoal filter.

7. Urine antigen in schistosomiasis. Recently Okabe described the presence of precipitins in the urine of patients with Schistosoma japonicum. The presence of the antigen in urine is of interest both from immunological and serological view points. Investigations were undertaken to determine if Okabe's findings could be repeated in laboratory animals experimentally infected with Schistosoma mansoni.

a. Adult rabbits were utilized to obtain antisera either by intravenous injection of antigen or by exposure to S. mansoni cercariae. In addition mice infected with cercariae were utilized as an antiserum source. Antibody titers were determined by the complement fixation (CF) test with Chaffee type adult S. mansoni antigen or by the S. mansoni cercarial antigen slide flocculation test. Highly reactive sera were selected for the study of urine. Infected rabbits and mice were utilized for the collection of urine. Urine from normal animals was used as control material.

b. The rabbit and mouse urine was concentrated by pervaporation and dialyzed for 24 hours against running tap water according to the described procedure. This solution was used as antigen in the precipitin and CF tests.

Urine samples from infected and normal rabbits and mice treated as described above were used undiluted as antigen in the precipitin test. Antigen was overlayed on an equal volume of undiluted S. mansoni antiserum in capillary tubes. The CF 52 test was employed. The proteins in pooled mouse urine were fractionated using the technic of Cohn et al , method 10. These fractions were tested for antigen activity in the CF test with S. mansoni monkey antiserum.

c. Urine samples taken from rabbits 11 days after infection were tested with normal and immune rabbit serum in the precipitin test. After two hours at room temperature, no precipitate was observed; after 48 hours at 4-6° C a precipitate was observed in the tubes containing the immune serum and none was visible in the tubes containing normal serum. However, the results were obscured by the presence of a precipitate in the immune serum controls without urine. This difficulty was overcome by filtering the sera and the urine samples and a precipitin reaction then became visible after three days at 4-6° C with appropriately negative controls.

d. In all other cases, precipitin reactions were either negative or precipitate appeared in both experimental and control tubes. On the other hand urine samples from infected mice as well as urine samples from normal mice tested in the CF test reacted with both normal and immune sera. Likewise, the protein fractions prepared from infected mouse urine reacted both with the normal serum and the immune serum when titrated in the CF test.

8. The use of the antigen-antibody complex as a possible means of immunization in schistosomiasis. Investigations were continued to determine if animals can be experimentally immunized with the schistosome cercarial antigen-cholesterol-lecithin complex and to determine if the concurrent administration of antibody enhances the immunization process.

a. Initially, young mice were selected for the study. Five groups of mice were used and injected with (1) saline, (2) cholesterol-lecithin crystals, (3) antigen combined with cholesterol-lecithin crystals, (4) immune monkey serum after absorption with antigen, and (5) antigen-cholesterol-lecithin complex with antibody obtained by absorption of the immune monkey serum. Following two injections with standardized volumes of the materials the mice in all groups were exposed to 150 cercariae each from the same cercarial pool and necropsied six weeks later. The numbers of worms and size of worms in each mouse were tabulated. No significant differences were obtained among the groups.

b. In the next experiment young white rats were used since this animal is partially resistant to Schistosoma mansoni infection, and also since the rat might be a more sensitive test animal. Five groups of rats were treated in essentially the same manner as the mice, but the rats were exposed to 500 cercariae. At necropsy, there were no detectable significant differences among the groups.

9. Cross-absorption studies between Schistosoma mansoni and Trichinella spiralis. The in vitro cross-reaction between Schistosoma mansoni antigens and serum from patients with Trichinella spiralis infections has been shown by several authors. This cross-reaction is apparently not reciprocal because high titer S. mansoni antisera did not react frequently with antigen of T. spiralis larvae. A technic employing washed, packed, and essentially dried antigen absorbed onto cholesterol-lecithin crystals was developed in this laboratory to absorb the serologically reactive antibodies from a given serum sample with a single calculated dose of antigen. The presen: report deals with absorption studies employing this technic to determine the relationship between the trichinosis and schistosome antigens in the one-way cross-reaction in sera from individuals with trichinosis.

a. Sera obtained from humans with well documented cases of trichinosis were absorbed with T. spiralis antigen and with S. mansoni antigens according to the described technic. The combinations of absorptions performed are presented in table 8. Absorption and reabsorption with bovine serum albumen sufficed as a control to determined if the absorption technic itself significantly reduced the titer of the pool. Of the two remaining portions, each was first absorbed with either T. spiralis or S. mansoni, then divided into two aliquots and one aliquot was reabsorbed with the same antigen that was used initially, the other portion was reabsorbed with the heterologous antigen. Following absorptions and reabsorptions and all sera were tested quantitatively in both tests.

b. Initially, representative sera from a group of patients with proven schistosomiasis and another group with trichinosis were tested in both the slide flocculation test for trichinosis (TsSF) and the slide flocculation test for schistosomiasis (SmSF). The results are presented in table 9. While none of the sera from the schistosomiasis patients reacted with T. spiralis antigens, all the sera from the trichinosis patients reacted with both T. spiralis and S. mansoni antigens. The results obtained are consistent with previous findings at this laboratory and support the hipothesis of the one-way cross-reaction.

c. The first serum pool for absorption was adjusted to yield a titer of 16 in both of the slide flocculation tests. The results obtained are presented in table 10. The initial absorption of the trichinosis serum with

Table 8

Pattern of cross absorptions performed with given antigen on serum pools from humans with trichinosis.

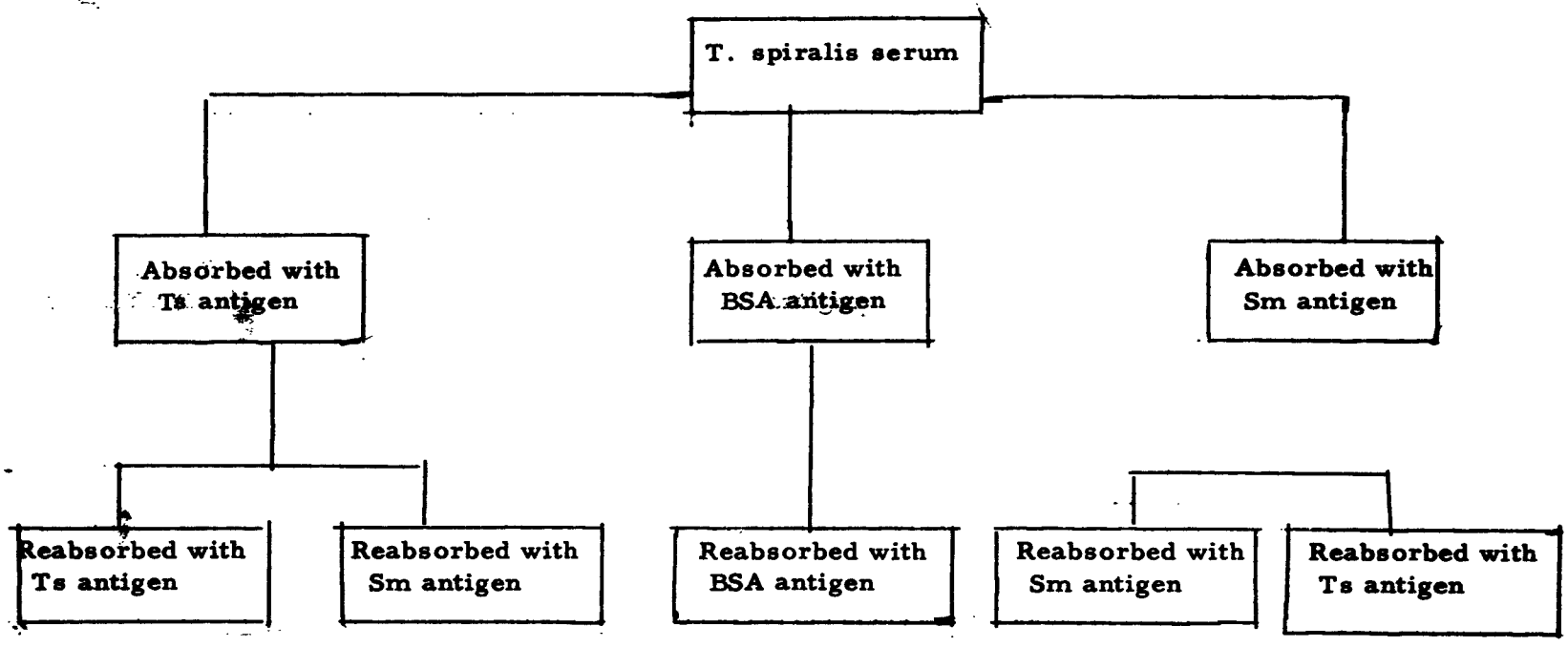


Table 9

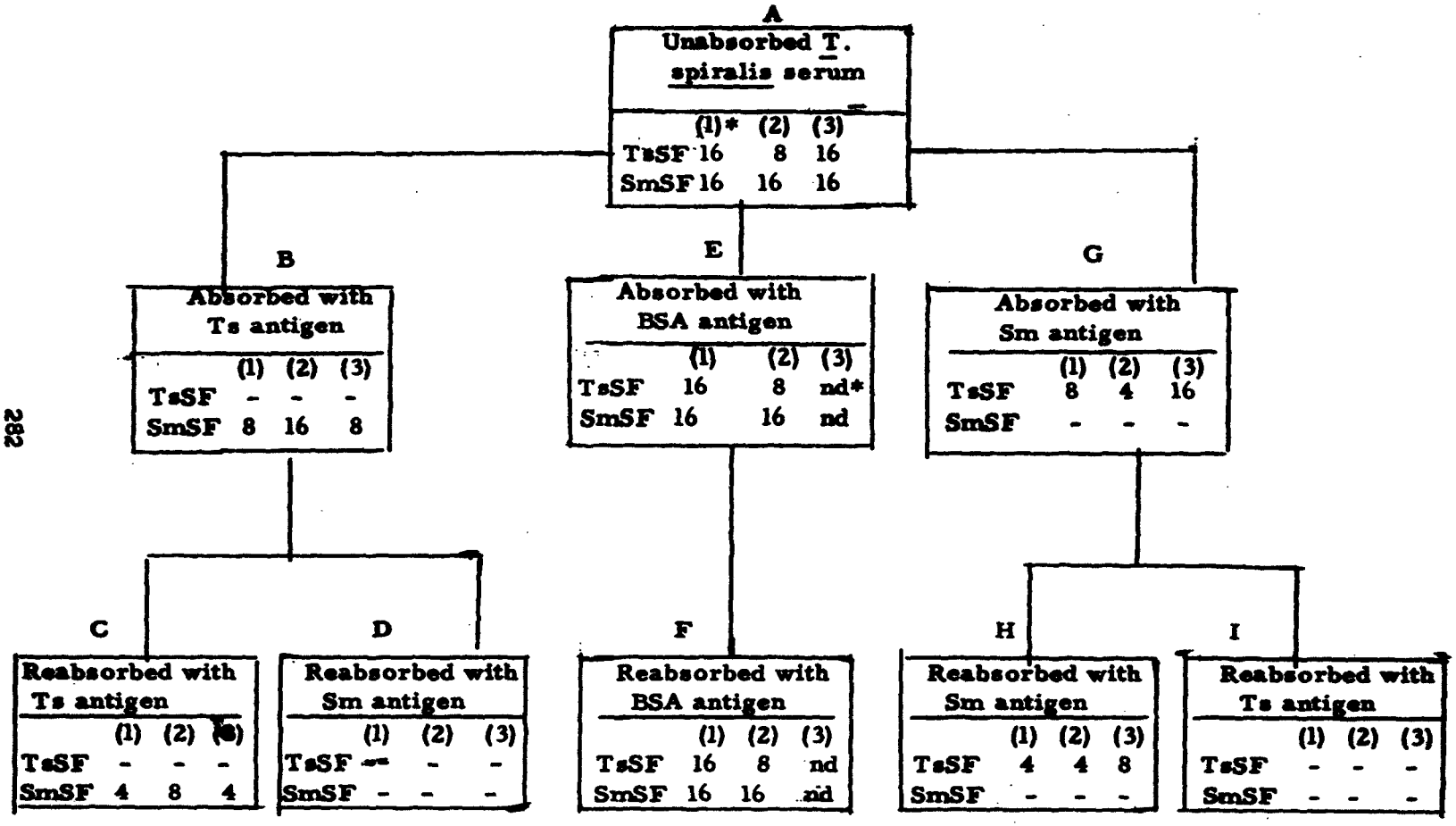
Results obtained in the TsSF and SmSF tests with representative sera from patients with trichinosis or schistosomiasis.

Disease and specimen number		Titer* obtained in given test	
		TsSF	SmSF
Trichinosis	1	4	1
	2	16	16
	3	16	128
	4	64	8
	5	64	64
Schistosomiasis	1	-	4
	2	-	8
	3	-	8
	4	-	16
	5	-	16

*Titer expressed as the reciprocal of the last serum dilution yielding a reaction in the test. The (-) denotes nonreactive.

Table 10

Results obtained in cross absorption studies with serum pools from humans with trichinosis.



*The number in ( ) indicates experiment; the numbers immediately below indicate the titer obtained in the given test.

either TsSF or SmSF (Box B or G) antigen removed all of the demonstrable antibodies reacting with the particular antigen. However, only a portion of the antibodies reacting with the heterologous antigen were removed. Reabsorption with the original antigens (Box C or H) also failed to remove all of the antibodies reacting with the heterologous antigens.

d. Absorption and reabsorption of aliquots of the serum pools with the BSASF antigen having the capacity to remove anti-BSA antibody from immunized rabbit sera, did not alter the titer of the serum pool (Box E or F) thereby indicating that the partial absorption of the heterologous antibody by an antigen (i.e., Ts antibody by SmSF antigen) was not a function of the procedure used.

e. Absorption of a second serum pool with an original titer of eight in TsSF and 16 in SmSF showed essentially the same results (Table I0). The antibody reactive with a given antigen was always removed by the absorption with that antigen. However, the antigen removed some of the heterologous antibody. In instances where the titer was not lowered to the next dilution, partial removal of antibodies was indicated in the reading of the quantitative test. Absorption experiments of a third serum pool gave essentially the same results obtained in the first two experiments.

10. The development of Dirofilaria uniformis Price in Anopheles quadrimaculatus Say. Although studies have been made on life cycle of Dirofilaria uniformis and of various factors influencing its development of Anopheles quadrimaculatus, the detailed development of this parasite in the mosquito and the pathological changes which accompany it are unknown. The present study was planned to determine details of development of D. uniformis and some of the accompanying changes.

a. Mosquitoes were fed on an infected rabbit and then placed in a separate cage. At various stages, either mosquitoes were removed and dissected to determine the development of the larvae, or fixed, sectioned, and stained.

b. Mosquitoes prepared for dissection were anesthetized with chloroform and legs and wings were removed. Two small holes were made in the second segment of the abdomen and insect saline was forced in and out of the abdomen while it was pressed and released to wash larvae and fluid from the hemocoele. The head was removed and set aside. The abdomen was clipped

on both sides near the posterior end. The tip of the abdomen was grapsed with fine forceps and pulled gently to the rear while the body was held firmly. The gut was drawn out into a small pool of saline. The abdomen was separated from the thorax and both parts placed in separate drops of saline. Each part was examined carefully and teased apart to determine the presence of developing larvae.

c. Most mosquitoes prepared for sectioning were fixed at intervals after mosquitoes completed their blood meal. For those mosquitoes fixed at intervals of less than 20 seconds, timing was started as soon as the mosquito began feeding. Entire mosquitoes were sectioned serially and stained with hematoxylin-eosin.

d. On the day after feeding on the infected rabbit, many microfilariae, sheathed and unsheathed could be seen in the blood meal taken from the gut of the mosquito. Some development had begun and some prelarvae were intermediate between the microfilarial and fully developed first larval stage. After 54 hours (third day) no sheathed microfilariae were observed. In the blood meal, prelarvae in various stages of development were present and some pigmented encapsulation had occurred. A few prelarvae were actually in the wall of the gut but most had escaped into the abdomen and some had developed into first stage larvae. Larvae were not free in the hemocoele but were imbedded in the tissues. All larvae had reached the first stage by the end of the fourth day (79 hours) and larvae had become inactive. The majority of the first stage larvae apparently were imbedded in the fat body.

e. On the fifth day (100 hours) a few second stage larvae were present. By the seventh day most larvae had reached the second stage and development appeared to have taken place only in the tissue of the fat body, most frequently in the subcuticular region of the abdomen. Some of the larvae showed activity, suggesting their approach to the third stage.

f. The third stage larvae were found on the eighth day, although most of the larvae were still in the second stage. A few first stage larvae were found which appeared to be developing slowly.

g. By the ninth day about half of the larvae had reached the third stage and some began to migrate within the tissue and in the homocoele (table 11).

Table 11

Development of larvae of Dirofilaria uniformis in Anopheles quadrimaculatus fed on an infected cottontail rabbit with 272 microfilariae in 20 cm of blood.

Total number	Day	Gut lumen	Head	Thorax	Abdomen	Gut wall	State of larvae Mf	Pre	1st	2nd	3rd
	1	Mic[illegible]f. w. & w/o sheaths					Many				
	2	many mf. few pre-larvae		1	8		Many	9			
42	3	19			24	1	2	36	4		
86	4			10	76				86		
50	5			6	44				42	8	
91	6			17	74				12	79	
105	7			8	97					105	
65	8			7	58				4	54	7
	9		3	14	86					59	44

11. Production of a film on the motility of microfilariae. (In cooperation with the Television Section, WRAMC, and Department of Motion Picture Services, WRAIR). The differential diagnosis of microfilariae is difficult and often impossible. In canine filariasis, Dirofilaria immitis is highly pathogenic while other commonly observed species are generally considered innocuous. A rapid, simple method for identifying D. immitis is by the motility of its microfilariae. This film was produced to demonstrate the use of motility in the differentiation of D. immitis from other species occurring in the peripheral blood of dogs.

a. D. immitis motility in freshly prepared wet blood mounts of whole blood is undulating and nondirectional. The microfilariae seldom move from the microscopic field but thrash about with broad undulation and loosely coil in place.

b. Dipetalonema spp. in fresh blood mounts have serpentine, progressive, directional motility alternating with tight coiling and undulating in place. It is usually difficult to keep the microfilariae in the microscopic field.

12. An improved staining tray for blood films. (In cooperation with the Division of Instrumentation, WRAIR). Although many investigators have recognized the advantages of staining thick and thin blood films with the film down, no apparatus designed for this procedure is generally available. A staining tray was designed to accommodate 24 slides with the blood films down, utilize a minimum of space and require minimum handling of the slides. Since in any mass staining procedure the transfer of malaria parasites to films from uninfected individuals has been reported, an experiment was outlined to test the transfer of parasites using the tray.

a. The tray was constructed of transparent lucite with a base 7 x 13 1/2 inches by 1/4 inch thick. The sides were made of strips 1/4 x 5/16 inch by 13 1/2 inches long; one end was made of a strip 1/4 x 5/16 inch by 6 3/8 inches long; the other end was 1/4 x 1/2 inch and beveled to the center of the well; all sides were mounted on the base forming a 1/4 inch deep well. A 1/4 by 3/16 inch strip was mounted lengthwise on the base to divide the well into two equal chambers slightly wider than the length of a microscope slide. On either side of the chambers strips 1/16 inch thick x 3/16 inch wide were mounted lengthwise on the base to hold the slides for staining. Drains 1/4 inch in diameter were drilled at the inside of the corners of the two chambers at the opposite end from the bevel and a gutter was cut along the end of the drain hole.

b. When the tray was placed on a slight angle and slides were placed on the 1/16 inch strips in each compartment, so that they did not touch, the staining solution poured on the beveled end of the tray flowed to the first slide, filled the space under the slide, then flowed to the next slide, and continued in this manner until all slides were in contact with the stain. Excess stain from the last slide flowed into the gutter and out the drains. In order to prevent excess stain from running out of the compartments, drain holes were plugged with small rubber stoppers. Washing the slides proceeded in a similar manner without touching the slides. When slides were washed for sufficient time, they were removed and stood on end to dry.

c. Using the same batch of stain, slides were stained on the tray and by other common methods and the results compared.

d. To determine the possibility of transfer of malaria parasites during staining, 60 thick blood films from a normal mouse and 60 from a mouse infected with Plasmodium berghei were prepared. The infected mouse had greater than 20 percent of its blood cells infected with P. berghei and the slides were placed on the tray during staining so that all films were face down and those from the normal mouse alternated with those from the infected mouse. After staining was completed, films from the infected mouse were examined for staining quality and those from the normal mouse were examined for parasites.

13. Quantitation of malaria parasitemias. The number of parasitized erythrocytes in a given volume is a datum of fundamental importance in malaria research which is necessary for precise dosimetry and as an index of degree of infection. With very high parasitemias, counts have previously been determined indirectly by calculation from the red cell count and the percentage parasitized erythrocytes determined from thin blood films. This latter determination is rendered grossly inaccurate when there is distortion of erythrocyte size such as is the case in P. berghei infections.

a. A method for direct staining and counting of parasitized erythrocytes in a hemocytometer chamber has been developed. The blood is diluted in a solution of formalin, glycerol, and toluidin blue buffered at pH 7.2. A theoretical 90% confidence limit is insured by increasing the volume of blood examined when parasitemias are low. Estimation of parasitemias by this method are uniformly higher than those estimated by the indirect method suggesting that in the latter case the unequal distribution of parasites on the thin smear biases the results.

14. Esterase histochemistry of Plasmodium berghei. Studies have been initiated to examine the rodent malaria parasite Plasmodium berghei for esteralytic enzymes. Since organophosphorus compounds are often inhibitory to these enzymes, preliminary studies on the effect of this agent on the infectivity of the parasite were conducted. Because of the intimate relationship between the parasite and the host cell, histochemical methods are being employed to detect enzymatic activity.

a. These experiments have demonstrated that blood parasitized with P. berghei has a reduced infectivity after in vitro treatment with 10^{-2} M diisoprophyl fluorophosphate (DFP). This is characterized by a four-day delay in the onset of parasitemia as compared to controls. DFP at a concentration of 10^{-3} M does not cause this effect. In an effort to define the enzyme systems being attacked, a histochemical test for nonspecific esterase employing alpha-napthyl acetate as substrate and Fast Blue as the indicator reagent was applied to the parasites. It was found that methanol fixed thin smears were unsatisfactory for the procedure, resulting in nonspecific staining of all blood formed elements. After formalin fixation, however, staining was limited to leukocytes and parasites with no reaction in the normal erythrocytes. The parasite activity cannot be inhibited by 10^{-2} M DFP, 3 mg/ml sodium fluoride, saturated quinine, saturated acridine, saturated 9-aminoquinoline, 10^{-2} M chloroquine, or 10^{-2} M primaquine.

15. Soluble antigens of Plasmodium berghei. Experiments have been performed to determine necessary conditions for the extraction of soluble antigenic components of P. berghei in quantities suitable for gel diffusion studies. The efficacy of 0.5% saponin and saturated digitonin in 0.15 M sodium chloride and of water as agents for lysis of the host cell and of water, 0.15 M sodium chloride, 0.25 M sucrose and 0.05 M barbiturate-hydrochloric acid buffer (pH 8.25) as extractants of the isolated parasites have been studied. Trials of various devices for disrupting the isolated parasites have been made. The study of antigens evolved from these investigations is now in progress.

a. Digitonin was found to be unacceptable as a lytic agent because of its aggregating effect on red cells troma. Water is unacceptable for this purpose because it lyses the parasites as well as the host cells. Saponin has neither of these disadvantages. Of the four protein extractants evaluated, the alkaline barbiturate-hydrochloric acid buffer results in the highest protein concentrations. Among the several disrupting technics employed, the use of sound waves (20 kc) was most effective. Based on these trials a technic resulting in preparations with protein concentrations of approximately 5 mg/ml has been developed. Mice and rabbits immunized with the isolated parasites or the soluble preparation yield antisera which gave strong reactions in gel diffusion tests with the soluble antigens. Preliminary immunoelectrophoretic studies have revealed nine components.

Summary and conclusions:

1. Rats developed an acquired resistance to Schistosoma mansoni following previous injections with whole worm homogenates. This resistance was manifested by a significant reduction in the number of developing worms from the challenging exposure and was observed whether the rats received three or 21 inoculations of worm homogenates. Resistance to superinfection was also observed when one-day old rats were exposed to a primary infection. In three out of four experiments, rats inoculated with albumin developed a significantly increased resistance to S. mansoni comparable in some instances to that developed by the animals which received worm homogenates.

a. Serologic studies indicated that the rats which received worm homogenates developed detectable levels of fluorescent antibodies earlier than their normal controls. Similarly, some rats which received albumin developed detectable antibodies earlier than the controls.

2. The findings indicate that the use of irradiated cercariae to immunize experimental animals against schistosomiasis represents a valuable additional tool which is destined to assume an increasingly important role. They should not, however, be interpreted to say that the findings to date lead us to foresee means for practical immunizing procedures for schistosomiasis in humans.

3/ Exposure to gamma radiation interfered with ability of schistosomes to reach maturity in mice. Since the death and disintegration of schistosomula stimulated a considerable degree of inflammation, different dosage levels provided contrasting pathological situations. At 50,000 rep cercarial dermatitis with ulceration and marked vasculitis was the main pathological feature at 50,000 rep, numerous granulomatous foci were seen in the lung, and at 2,500 rep, foci of liver cell necrosis and liver granulomata occurred. Irradiation at 2,500 rep appeared to be the best dose since the less heavy concentration of parasites in a single organ produced the least amount of objectionable pathological changes.

a. The host tissue responses to attenuated cercariae were strikingly similar to those observed following a primary exposure of an abnormal host to nonattenuated cercariae and those observed in the normal host which had become resistant to a secondary infection following a primary exposure to schistosome cercariae.

4. A single intravenous injection of purified eggs of Schistosoma mansoni had no effect on the number, size, sex ratio, and egg production of adult worms arising from a standard challenge with 200 cercariae. Nevertheless, tissue changes in the lung and spleen indicative of sensitization were still detectable 50 days following challenge, and these included enhancement of

pulmonary arteritis, and of splenic hyperplasia and eosinophilia. Fluorescent cercarial antibodies appeared in the serum after egg injection and previous to cercarial challenge.

a. On critical analysis of these findings and of the literature it is concluded that the role of the egg in resistance to Schistosoma is negligible, and the role of the schistosomulum is paramount.

b. The findings present an interesting example of dissociation between the phenomena of sensitization and resistance.

5. The monkeys receiving 1,000 S. mansoni cercariae died at 40 and 50 days respectively. The average number of worms was 323 per animal (32% development). The number of eggs passed was not as great as those passed by the rhesus monkey. The gross pathology was very severe in the livers of both animals. Histological and pathological studies on the tissues of these animals are now in process. The remaining animals in the study are still under experimentation.

6. By use of this system, the number of infected snails on hand has been more than doubled as compared to former methods without an appreciable increase in space. By placing 25-35 snails per pan and changing the water weekly, 375-525 positive snails per unit can be kept on hand at all times. These, when shed twice weekly, should produce approximately three million cercariae per week per unit.

7. Although the single observations suggests that S. mansoni antigen is excreted in the rabbit the presence of host substances which react with normal serum in serological tests makes the detection of small traces of a specific antigen extremely difficult. Further pursuit of the problem would therefore seem unprofitable at the present time.

8. A final attempt to immunise rats by this means is planned but the experiment has been delayed pending the accumulation of sufficient antigen to increase the numbers of injections from two to 12. It is anticipated that the antigen shall be available within the next month so that the experiment can be completed.

9. When Trichinella antiserum was absorbed with Trichinella slide flocculation test antigen the homologous antibody was removed but the antibody reactive against cercarial slide flocculation test antigen remained. The converse was also true in that absorption of the Trichinella antiserum with schistosome antigen removed antibody serologically reactive with the schistosome antigen but failed to remove antibody reactive with Trichinella antigen.

10. The development of the larvae of Dirofilaria uniformis in the fat body of Anopheles quadrimaculatus is apparently similar to that described for Loa loa in Chrysops silacea by Lavoipierre. More details will be elucidated when the examination of the serial sections of infected mosquiteoes, killed and fixed at various intervals after exposure, has been completed.

11. If blood preparations are carefully prepared on clean slides, covered with a cover glass, and examined soon after preparation, no difficulties should arise in differentiating microfilariae of D. immitis from those of other species found in the peripheral blood of dogs.

12. Thick and thin blood films placed on the tray were more evenly stained and had less debris, dust, and stain particles than films stained with the film up or in staining jars.

a. No parasites were found on the 60 normal thick blood films stained in the presence of films from an infected mouse with many parasites of P. berghei.

13. The problems which limit the usefulness of this method are: (1) insufficient morphological definition of immature forms and (2) aggregation of parasitized erythrocytes in the presence of low protein concentration (i.e., washed cells). Efforts to improve the technique so as to obviate these problems are continuing.

14. Experiments will continue to determine the effect of other inhibitors and of combinations of inhibitors. Attempts to demonstrate proteolytic activity histochemically will also be made. Protease has already been demonstrated in this parasite by other workers; at least one molecular species of this enzyme is DFP labile.

15. These studies have demonstrated that antigenic analysis of plasmodia by immunoelectrophoresis is practicable. Investigations of the specificity of the reactions, presence or absence of lipid and carbohydrate components in the antigens, and the effect of time and method of storage (0° C shell frozen and lyophilized) are now in progress.

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ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 806, Military Preventive Medicine

Task 01, Communicable Diseases (Schistosomiasis and other parasitic diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Experimental Pathology
Division of Special Activities

Department of Medical Zoology
Division of Communicable Disease
and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: D. C. Biggers, Capt., MC

Assistant: John I. Bruce

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Schistosomiasis and other parasitic diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Author: D. C. Biggers, Capt., MC

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Chernin and his co-workers at the Harvard School of Tropical Public Health have reported the rearing of snails in a germfree state. The species used was a Puerto Rican strain of Australorbis glabratus, an intermediate host for Schistosoma mansoni. Although there was no reproduction in the germfree state, the animals could be maintained in the axenic culture until adult size was reached.

It is the purpose of this present work to grow axenically A. glabratus to be used as an invertebrate germfree model and adapted to the germfree techniques already in use at WRAIR. More specifically, the initial studies underway are aimed at: 1) comparing the dynamics of the intestinal epithelial turnover in conventional and germfree snails with the now established differences in dynamics between the intestinal turnover in germfree and conventional mammals; and, 2) studying, histologically, the formation of "granulomata" in response to injection of killed miracidia, as part of a larger study of immune responses in invertebrates.

Progress thus far has consisted of establishing methods and obtaining baselines. Materials and supplies have been assembled; malacologic techniques have been studied and practiced; a histologic atlas has been prepared; a visit to Boston was made for consultation with Drs. Chernin and Pan and direct observation of their axenic culture techniques; a stock of Puerto Rican A. glabratus has been established and a technical assistant is being trained.

Pilot experiments comparing histological differences in germfree and aquarium-raised snails reveal distinct morphologic differences in the mid-intestine, consisting of "heaping-up" of cells and pseudo-stratification in the germfree.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Schistosomiasis and other parasitic diseases)

Description:

Chernin and his co-workers in the Harvard School of Tropical Public Health have grown in axenic culture Australorbis glabratus, an intermediate host for Schistosoma mansoni. The intent of this present work is to establish at WRAIR a group of germfree A. glabratus, adapted to the germfree techniques presently used here. Morphologic differences have been observed by us between the intestinal tract of germfree snails obtained from Harvard and aquarium-raised snails. However, this must be confirmed by further and more extensive study. As a result, we must establish a source of germfree snails. An immediate future use of these animals is a planned study to investigate the differences, if any, between morphologic responses of germfree and conventional A. glabratus to experimentally introduced miracidia of S. mansoni. The model, after its establishment, will serve as a needed example of germfree invertebrate life.

Progress:

Initially, conventional A. glabratus were obtained from the Department of Medical Zoology, WRAIR, and sacrificed. The shelled animals were fixed in formalin, embedded in paraffin and sectioned. The preparations, stained with H&E and PAS, were studied with principle focus upon the gastrointestinal histology. From 10 specimens, multiple longitudinal sections were taken through the entire animal to demonstrate all levels of the tortuous intestinal tract. The microscopic anatomy of the different levels of the tract was described in detail and recorded. As well, color photomicrographs were taken. This data was combined into an atlas of normal gastrointestinal histology.

The next step was to compare the gastroenterologic histology of conventional aquarium-raised snails with that of germfree snails. Sections of germfree snails were obtained from Harvard and the intestinal tracts examined in detail. The findings were compared with same areas in conventional aquarium-raised snails of comparable shell diameters. Decided differences were noted in the intestinal

tracts in this pilot experiment. Most striking was the appearance of the mucosa of the mid-intestine which was altered by "heaping-up" of epithelial cells, pseudostratification of cells between the "heaped-up" areas, increased numbers of "mucin"-producing cells, and an apparent increase in numbers of mitotic figures in the germfree animals. The mucosal epithelium of conventional animals was low columnar to cuboidal with few mitotic figures and few "goblet cells". These findings are suggestive of a marked alteration due to the germfree state, but must be considered inconclusive due to the few animals (4 germfree snails) studied and the lack of application of more dynamic parameters such as nuclear tagging with tritiated thymidine and rigid controls.

For the continued investigation of this and other phenomena, it is necessary to establish a source of germfree A. glabratus. Chernin and his associates in Boston were the first (and only) group to culture the snail axenically, and although they are no longer pursuing this line of work, a visit was made to their laboratory and detailed consultation was obtained from them in techniques, equipment, and elaboration upon methods which they had reported in the literature.

The equipment has been assembled and a stock supply of A. glabratus has been established now in our laboratory. A technical assistant is being trained in maintenance of the stock, malacological techniques and techniques of micromanipulation.

In the immediate future we are prepared to attempt the axenic culture of these snails with the aid of the Department of Germfree Research and Department of Medical Zoology.

Summary and Conclusions:

A difference has been found between the intestinal tract of germfree and conventional aquarium-raised Australorbis glabratus, an intermediate host for Schistosoma mansoni, which consisted of pseudostratification, increased mitotic activity and "heaping-up" of epithelial cells in the germfree animals. These changes suggest an alteration in the epithelial turnover rate of the intestinal epithelium in these animals.

This finding necessitates the attempt to substantiate the suggested tendency by quantitative methods of histologic study and dynamic studies using nuclear tagging with tritiated thymidine. Also, an invertebrate model is much needed in the investigation of

cellular immune responses as modified by a germfree environment. Through such studies, further knowledge may be gained of the host-parasite relationships in A. glabratus infected with the miracidia of S. mansoni.

Therefore, methods, materials and information have been gathered in preparation for the establishment of germfree A. glabratus at WRAIR.

List of Publications: None

ANNUAL PROGRESS REPORT

Project 3A 012501 A 806, Military Preventive Medicine

Task 01, Communicable Diseases (Serodiagnosis of Parasitic Diseases)

Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C. Department of Medical Zoology Department of Serology Division of Communicable Disease and Immunology
Period Covered by Report:	1 July 1962 through 30 June 1963
Principal Investigators:	Robert I. Anderson, Lt. Col., MSC Carter L. Diggs, Captain, MC Ralph E. Duxbury, BS Karl H. Fife, Jr., MS Silvio H. Sadun, ScD H. Kenneth Sleeman, PhD Andre J. Toulissant, MS*
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Reports Control Symbol:	MEDDH-288
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ABSTRACT

Project 3A 0 12501 A 806	Title: Military Preventive Medicine
Task 01	Title: Communicable Disease (Serodiagnosis of Parasitic Diseases)
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
Period Covered by Report:	1 July 1962 through 30 June 1963
Authors:	Robert I. Anderson, Lt. Col., MSC Carter L. Diggs, Captain, MC Ralph E. Duxbury, BS Earl H. Fife, Jr., MS Elvio H. Sadun, ScD Maurice J. Schoenbechler, BS Lois A. Simonton, BS H. Kenneth Sleeman, PhD Andre J. Touissant, MS Joseph S. Williams
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

Plasma card tests were developed and evaluated for the rapid serodiagnosis of schistosomiasis and trichinosis; results obtained were comparable with conventional tests. Economy, rapidity and simplicity of the card tests render them suitable for epidemiologic investigations.

Fluorescent antibody technics for serodiagnosis of filariasis, African trypanosomiasis, malaria and leishmaniasis were developed with experimentally infected animals. Evaluation of the procedures in human infections are being performed and encouraging results have been obtained to date.

Attempts are being made to isolate, purify and fractionate complement fixation test antigens from African trypanosomes and malaria parasites. Difficulties encountered with trypanosome antigens, possibly due to antigenic differences between culture forms and those of natural infections are under investigation.

The antigenicity and chemical components of excretions and secretions (ES) from Schistosoma mansoni cercariae are being investigated. The ES are antigenic in vitro and in vivo, contain protein, carbohydrate and lipid.

The feasibility of a single hemagglutination test for field identification of mosquito blood meals was demonstrated up to 32 hours after ingestion.

Recently developed serodiagnostic tests were made available for diagnosis by a central unit, which determines feasibility of the tests, trains individuals, and is available for rapid expansion if required.

BODY OF REPORT

Project 3A 0 12501 A 806 Title: Military Preventive Medicine

Task 01 Title: Communicable Diseases (Serodiagnosis of Parasitic Diseases)

Description: The purpose of these studies was the continuing development and evaluation of reliable procedures for the serodiagnosis of parasitic diseases. Serological methods for the laboratory diagnosis of many parasitic diseases are essential since the causative agent may be difficult or impossible to demonstrate.

Progress:

1. A plasma card test for the rapid serodiagnosis of schistosomiasis

A fluorescent antibody technic which made possible the use of dried blood collected by finger puncture instead of serum was previously developed in this laboratory. This technic permits rapid collection of specimens under adverse conditions and allows easy transport of specimens. However, the need still existed for an efficient field testing procedure which will permit the prompt collection of data without the time required to mail specimens to a central laboratory and to receive the results. This need was particularly acute in many of the endemic areas where health officers frequently lost contact with infected people before they received the results of a serological examination.

A technic was developed based on studies conducted by other investigators for the development of field tests for syphilis and other treponematoses. The plasma, separated from three drops of finger blood by use of a rapid, simple device, is tested on a plastic coated card surface with a stable antigen suspension containing charcoal. The procedure was evaluated with patients under field conditions and the findings were compared with those obtained with the fluorescent antibody test performed on dried blood samples collected from the same patients. Additional tests were performed in the laboratory with unheated serum specimens. The results obtained with the two serological tests are summarized in Table 1. The findings with specimens from schistosomiasis patients illustrate the relative sensitivity of the two procedures. The findings with specimens from allergic patients and from healthy individuals provide an index of the relative specificity of the two tests. No significant difference in sensitivity and specificity of the two tests was observed.

Table 1. Results obtained with the fluorescent antibody test (FA) and the schistosome-plasma-card test (SPC) in different areas

Location	Diagnosis	Schisto. endemicity	Number persons tested	Results of FA Pos.	Doubt.	Neg.	Results of SPC Pos.	Doubt.	Neg.
Geneva,Switz.	Healthy	None	11	ND*	ND	ND	0	0	1
Rome, Italy	Hypersens.	None	25	2	2	21	0	1	24
Witkoppen,S.A.	Schisto.	High	34	34	0	0	31	3	0
Witkoppen,S.A.	No schisto	High	10	2	0	8	1	0	9
Joburg,S.A.	No schisto	None	7	ND	ND	ND	0	0	7
S.Manne,S.A.	No schisto	None	57	2	12	43	1**	4	52
Barberton,S.A.	Schisto.	Very High	78	78	0	0	78	0	0
Barberton,S.A.	No schisto	Very High	2	0	0	2	0	0	2
Bukandwe,Tang.	Schisto.	Very High	77	77	0	0	77	0	0
Bukandwe,Tang.	No schisto	Very High	3	2	0	1	2	1	0
A. Chini,Tang.	Unknown	High	63	33	9	21	50	8	5
TOTAL			367	230	23	96	240	17	110

* Not done

** Also positive by FA and intradermal tests - admitted going swimming in endemic area.

As indicated in Table 2, a close correlation of results was obtained with the two tests. The SPC (schistosome-plasma-card) test was also performed at the Walter Reed Army Institute of Research laboratories on blood serum obtained from venipuncture which is maintained in a serum bank for comparative evaluation of testing procedures. The 102 serum samples used for this comparison were from several diagnostic categories as described (Table 3). The results indicate that the SPC test has a satisfactory sensitivity and specificity with unheated serum. Attention should be called to the fact that serum specimens from filariasis and leishmaniasis patients were obtained in areas of high endemicity for schistosomiasis, and that all tests described for serodiagnosis of schistosomiasis usually react with trichinosis antisera. Antigen stability studies were also conducted. Aliquots of the antigen were distributed in several 1 ml. vials

which were sealed and stored at room temperature (22-27°C.). Once each month for a total of four months, one vial of antigen was opened and used to test 10 sera of known positive reactivity and 10 of known negative reactivity. In each test the 10 reactive sera gave positive results and the 10 nonreactive sera gave negative results.

Table 2. Correlation of results obtained with the fluorescent antibody test (FA) and the schistosome-plasma-card test (SPC)

		Results of FA			
		Pos.	Doubt.	Neg.	Total
Results of SPC	Pos.	221	7	12	240
	Doubt.	3	5	9	17
	Neg.	6	11	75	92
	Total	230	23	96	349

Table 3. Results obtained with SPC test on serum bank specimens

Diagnostic status	Number of specimens	Results of SPC Pos.	Doubt.	Neg.	Source
Schistosomiasis	29	27	0	2	Brazil; Puerto R.
Trypanosomiasis	4	0	0	4	Uganda
Onchocerciasis	4	0	0	4	Uganda
Syphilis	22	0	0	22	USA
Trichinosis*	12	8	0	4	USA
Healthy	22	0	0	22	USA
Filariasis	7	6	0	1	Permambuco, Brazil
Leishmaniasis	2	1	0	1	Minas Gerais, Brazil

* Biologic false positive reactor with Schistosoma antigens.

2. Antigenicity of Schistosoma mansoni cercarial excretory and secretory products

All available serological procedures for schistosomiasis, with the possible exception of the circumoval precipitin test (COP), while being excellent diagnostic tools, fail to indicate the efficacy of treatment and in addition there is some doubt about the value of the COP test for this purpose. However, the possibility exists that the excretions and secretions of the parasite may serve satisfactorily as antigens in the complement fixation test and also serve as *in vivo* antigens in the host. If this is true, then the alteration of the metabolic processes of the parasite by chemotherapy may result in a reduction of CF titers when the excretions and secretions are used as antigens. The possibility also exists that this type of antigen may be simpler than the complex somatic antigens normally extracted from the whole parasite. In this event, chemical and physical characterization of the excretory and secretory products might lead to better defined antigen(s) and a clearer understanding of the antigen.

Therefore experiments were initiated to determine if the excretory and secretory products of the cercarial stage of *Schistosoma mansoni* passed *in vitro* and *in vivo* antigenicity. As the natural habitat of the cercariae is water, it was possible to collect the products in distilled water, thereby eliminating foreign proteins from the study. The secretions and excretions of the cercaria in water were concentrated initially by pre-evaporation at 3-6°C and later by dialysis against 15% polyvinylprrolidone (PVP) also at 3-6°C. The concentrates were either processed and tested as such, or frozen at -20°C and later pooled. The pooling of specimens served a two-fold purpose in that it provided a more representative sample of the secretory and excretory products, and also allowed the collection of sufficient material for the combined serological and chemical testing.

The results obtained with fresh concentrates and frozen pooled concentrates differed in some respects. The extent and type of these differences will be discussed in the test in which they occurred.

The secretory and excretory concentrates were found to be reactive as antigens in complement fixation (CF) tests with sera from humans with *S. mansoni* infections. However, the antigenic activity of the concentrates in CF tests was minimal, and the products from 100,000 cercaria in 1 ml. of solution were needed for a strong reaction. The solid material realized from 100,000 cercaria was less than 1 milligram.

Double diffusion in gel using undiluted antigen against 1:1 through 1:8 dilution of antisera as well as undiluted antiserum against 1:1 through 1:8 dilutions of antigen yielded negative results. Since these tests are comparatively insensitive, it is possible that the antigen was not sufficiently concentrated.

Studies on the stability of the antigenic substances in the cercarial excretions and secretions revealed that they were stable to refrigeration for weeks, and that they could be stored for long periods of time at -20°C without deterioration. In contrast, the antigenic activity in CF test was markedly reduced upon lyophilization. Heating the solution (pH 7.3) at 100°C for 10 minutes resulted in a reduction of anticomplementary activity which was reflected in a loss of overall antigenic reactivity.

High speed centrifugation of the concentrate (100,000 G for 1 hour) resulted in diverse results depending whether the specimen was frozen or not prior to treatment. If not frozen, centrifugation gave a heavy precipitate. When tested by the CF procedure, neither the reconstituted precipitate, the supernatant solution, or the combined precipitate and supernatant (pH 7.3) were active as antigens. On the other hand, high speed centrifugation of the frozen pool resulted in a very slight precipitate, which had no antigenic activity, and the supernatant solution which retained most of the original antigenic activity. However, the high speed centrifugation did result in some loss of anticomplementary activity with 5C'H50. The observed difference noted between the frozen and non-frozen specimens during centrifugation could result from a physical change in the antigenic substance brought about by freezing and thawing, possibly the release of the reactive component from a complex molecule. Qualitative chemical analysis revealed only one difference between the precipitate and supernant solution resulting from the high speed centrifugation of the fresh concentrate. That was the presence of lipid in the supernatant solution, but not in the precipitate. The serologically active substance derived from the supernatant of the frozen pool also contained lipid.

The serologically active material given off by the cercaria was not dialyzable through cellulose membrane, which showed it to have a molecular weight over 50,000. The dialyzed solution contained protein, carbohydrate,and lipid, however, it has not been ascertained if all three are necessary for antigenic activity. Paper chromatograms of acid hydrolysates (2N HCl for 24 hours) revealed the presence of 10-12 amino acids (glutamic acid and alanine were tentatively identified). Other paper chromatograms of acid hydrolysates (2N HCl for 8 hours) also revealed the presence of carbohydrate and lipid which migrated as a single band. The ratio of protein to carbohydrate in the frozen pool was 4:1.

Antisera to the S. mansoni cercarial secretions and excretions were prepared in rabbits by injecting intramuscularly the frozen concentrate in an equal amount of Freunds complete adjuvant. Three weekly injections produced antisera with a titer of over 384.

3. Development of a plasma charcoal card test for trichinosis

Rapid plasma card tests for syphilis and schistosomiasis have been recently developed. The card test in schistosomiasis gave comparable results to those obtained with other serological technics and permitted tests to be carried out in the field and to be completed within a few minutes on plasma obtained by finger puncture. The purpose of the current study was to determine whether these procedures could be adapted to a plasma card test for trichinosis.

Serum specimens were obtained from humans with a diagnosis of trichinosis based on clinical evidence and positive reactions in other serological tests. Sera from individuals with well documented cases of other parasitic infections, from syphilitic patients, and from healthy persons undergoing extensive medical examinations for admission to a military academy served as controls.

The antigen employed for the charcoal plasma card test was essentially the same as that used in the slide flocculation except that following the washing procedure the antigen-cholesterol-lecithin crystals were resuspended in a solution containing charcoal. The tests were performed on printed cards, without the depression or well which is normally used in glass slide flocculation test slides. A volume of 0.05 ml. of unheated serum or plasma was pipetted into the appropriate circle and spread with the tip of the pipette so that the entire area of the circle was covered. One drop (1/60 ml.) of the antigen containing the charcoal was added to each circle and the card rotated on a slide rotating machine (100 rpm) for 8 minutes at room temperature. The results were read immediately with the naked eye and recorded as reactive or non-reactive. The non-reactive results were easily distinguished as the antigen migrated to the center during rotation and appeared more or less as a single mass. Reactive sera did not contain the central accumulation of charcoal and appeared as if black pepper had been sprinkled on them. The appearance of the reaction did not change appreciably during or after drying if the cards were allowed to dry on a flat surface, and the cards provided thus a permanent record. The same technic could be performed if the cards with tear drop shaped depressions were rocked by hand provided that 0.03 ml. of unheated serum or plasm and one drop (1/70 ml.) of antigen were used.

The results obtained with the charcoal plasma card test are presented in Table 4. Of the sera from the 21 persons with trichinosis all were reactive. Of the 67 sera tested for specificity only 3 yielded a reaction.

Table 4. Sensitivity and specificity of the charcoal card test

Diagnostic Category	Number of persons tested	Results R	-
Trichinosis	21	21	0
Normal	24	1	23
Syphilis	25	2	23
Other parasitic*	18	0	18

*Included sera from infections with the following parasites: Onchocerca volvulus (8), Wuchereria bancrofti (4), Strongyloides stercoralis (2), Trypanosoma cruzi (2), and Leishmania donovani (2).

4. Fluorescent antibody studies of filariasis

Studies on the possible adaptation of the fluorescent antibody technic to the diagnosis of filariasis infection were initiated using Dirofilaria uniformis infections in rabbits and D. immitis infection in dogs as sources of antisera. Initially, microfilariae separated from the blood of infected cottontail rabbits were used as antigen. However, when less than 50% of known positive sera reacted in the indirect test using microfilariae, it was decided to utilize the larval stage instead. Better results were obtained with 7-10 day old larvae dissected from Anopheles quadrimaculatus mosquitoes which had fed previously on an infected rabbit.

Subsequent to publication of the FA technic for diagnosis of schistosomiasis by Sadun, et al (Exper. Parasit. 1961, 11:117-120), the method was applied to the serological diagnosis of Brugia malayi infections by Chowdhury and Schiller (Bull. Calcutta Sch. of Trop. Med. 1962, 10:97-99) who also reported greater success with larvae than with microfilariae. They noted, in addition, that larvae gave a brighter fluorescence when broken or squashed than when intact. This observation was confirmed in the present study.

5. The use of a hemagglutination test for the identification of erythrocytes infested by mosquitoes

At the request of the World Health Organization, studies were conducted to determine the feasibility of using a single hemagglutination test for field identification of the origin of mosquito blood meals. At present the World Health Organization personnel engaged in malaria studies in remote areas depend on results of precipitin tests performed on samples sent to European laboratories.

Antisera for testing were obtained from rabbits immunized with red cell stroma from guinea pigs, cattle, and humans. Erythrocytes were dissected from the stomachs of Anopheles quadrimaculatus one to 32 hours after they had been allowed to feed on guinea pigs and humans. To complete the test, a drop of serum was added to the freed cells either on microscopic agglutination slides or on cards. After rotation by hand or rotating machine for about three minutes, the results were read with the naked eye.

Consistent positive results were obtained when guinea pig erythrocytes from the mosquito stomach were placed in contact with anti-guinea pig cell sera from rabbits; normal rabbit sera gave no hemagglutination reaction. Antihuman cell sera from immunized rabbits reacted with human erythrocytes dissected from mosquito stomachs; no reaction was obtained with antiguinea pig cell sera or normal rabbit sera (Table 5). Anti-bovine cell sera obtained from rabbits failed to react with guinea pig or human erythrocytes ingested by mosquitoes. These results were in agreement with those previously reported by other investigators in France.

Table 5. Results of hemagglutination tests of mammalian blood ingested by mosquitoes

Species	Hours after mosquitoes fed						
	1	2	4	6	8	24	32
1. Guinea pig erythrocytes							
Guinea pig hemagglut.	+	+	+	+	+	+	+
Normal rabbit sera	-	-	-	-	-	-	-
2. Human erythrocytes							
Human hemagglutinations	+			+		+	
Guinea pig hemagglut.	-			-		-	
Normal rabbit sera	-			-		-	

6. Fluorescent antibody studies in African trypanosomiasis in experimental animals

Studies on the development and evaluation of a fluorescent antibody (FA) test in African trypanosomiasis were continued. The indirect fluorescent antibody technic was used to stain the various stages in the life cycles of T. rhodesiense and T. gambiense. Attempts were also made to determine whether organisms which are not pathogenic for man such as T. lewisi could be used as a source of antigen for the serological diagnosis of African trypanosomiasis.

The indirect FA technic was used throughout these experiments. The test procedure was carried out by applying to the antigen preparation a few drops of 5% formalin and rhodamine-bovine-albumin (RBA) solution to fill a circled area on a slide. After 10 minutes of fixation the test area was washed three times with phosphate-buffered saline (PBS). One-tenth ml. of approximately diluted test serum was added and allowed to react for 10 minutes. After washing with PBS, the test area was exposed to previously titrated labeled antirabbit globulin for five minutes. Following a final rinsing with PBS, a drop of buffered glycerol was placed on the slide and surmounted with a cover glass, then the slide was blotted gently to remove excess fluid.

In the course of preliminary experiments the trypanosome and proventricular forms were used fresh from blood or culture, respectively, or allowed to dry on the slides. It soon became obvious that the parasites were washed away during staining or that the reactions were unsatisfactory. When the smears were fixed with 0.5% formalin, 0.1% HCl or methyl alcohol, the results were also poor. Organisms fixed with greater concentrations of formalin up to 10% gave better results. Optimal results were obtained when the organisms were fixed in 5% formalin-RBA solution. A marked degree of fluorescence was observed in the trypanosome forms in the presence of immune sera by the indirect FA technic. This contrasted vividly with the relative lack of fluorescence with normal sera in the same system. When organisms were exposed to formalin-RBA they assumed a reddish coloration which contrasted with the specific yellow-green fluorescence.

To determine whether comparable results could be obtained by using either trypanosome forms from blood smears or proventricular forms from cultures, nine sera from rabbits infected with T. rhodesiense and four sera from rabbits with T. gambiense were tested together with sera from normal controls. In general, brighter fluorescence and more consistent results were obtained with trypanosome forms from blood and therefore, only these forms were used in the experiments which followed.

In a preliminary attempt to evaluate the sensitivity of the FA technic as a diagnostic test for African trypanosomiasis, 29 sera from rabbits infected with T. rhodesiense and 23 from normal rabbits were evaluated by the FA test using T. rhodesiense organisms as antigen. Also, 22 sera from rabbits infected with T. gambiense and 26 sera from normal rabbits were tested using T. gambiense as antigen. The results (Table 6) indicate that both tests are highly sensitive in rabbits.

Table 6. Results of sensitivity studies in the fluorescent antibody test for trypanosomiasis in rabbits

Serum specimen	Number tested	Parasite antigen	Reactions in FA test		
			Positive	Doubtful	Negative
Trypanosomiasis rhodesiense	29	T. rhod.	29	0	0
Normal	23	T. rhod.	0	2	21
Trypanosomiasis gambiense	22	T. gamb.	21	1	0
Normal	26	T. gamb.	0	3	23

In order to obtain some information on the reproducibility of the results with these tests, pools of T. rhodesiense and T. gambiense and of normal control sera were each divided into aliquots and tested at different times with different lots of trypanosomes and antiglobulins. As indicated in Table 7, only one of the 32 specimens from normal sera gave a positive reaction (titer 1:1) and none of the 32 specimens from proven infections was negative.

Table 7. Results of repeated fluorescent antibody tests of same sera with different lots of trypanosomes and antiglobins

Serum specimen	Parasite antigen	Number of times tested	Number of times given titer obtained								
			0	1	2	4	8	16	64	256	1024
Trypanosomiasis rhodesiense	T. rhod.	24	0	0	0	0	0	4	9	10	1
Normal	T. rhod.	24	24	0	0	0	0	0	0	0	0
T. gambiense	T. gamb.	8	0	0	2	4	1	1	0	0	0
Normal	T. gamb.	8	7	1	0	0	0	0	0	0	0

To determine how soon following infection antibodies are present in rabbits in sufficient amounts to be detected by the FA test, four animals were inoculated with T. rhodesiense and four with T. gambiense organisms. The size of the inoculum ranged from approximately 40,000 to 5,000,000 organisms. All animals were bled at weekly intervals. Two weeks after infection antibodies were detectable in all rabbits regardless of the size of inoculum. Results indicated that the initial appearance of fluorescent antibodies and the titer reached seemed to be independent of the number of organisms inoculated.

In order to obviate the need of maintaining laboratory animals with organisms pathogenic for man, sera from rabbits infected with T. rhodesiense and T. gambiense were tested using T. lewisi as antigen. The results indicated that extensive cross reactivity existed with this species and that reactivity of the sera occurred at similar titers without obvious reduction in the intensity of fluorescense.

7. Fluorescent antibody test for the serodiagnosis of African and American trypanosomiasis in humans

Human infections with African and American trypanosomiasis constitute important clinical and public health problems in large regions of the world. An unequivocal diagnosis of trypanosomiasis is ofter difficult to obtain since the clinical picture is not always well defined and the organisms frequently cannot be recovered by blood examinations. Consequently, there is a need for reliable, rapid and inexpensive procedures which could provide the basis for an adequate diagnosis of these infections, especially during the latent and chronic phases of the disease.

The current report summarizes efforts to develop a fluorescent antibody (FA) technique as a reliable and practical laboratory test for the diagnosis of African and American trypanosomiasis in humans. Attempts were made to determine the degree of cross reactivity of different trypanosome species and to determine whether blood smears dried on absorbent paper could be used in the serological diagnosis of trypanosomiasis.

Human sera from well documented trypanosomiasis infections in endemic areas were obtained. The diagnosis of trypanosomiasis in the patients was established by the recovery and identification of organisms from the blood or spinal fluid. To determine the specificity of the FA test, control sera from individuals with viral, bacterial, and parasitic infections other than trypanosomiasis were used. To test whether the presence of auto-antibodies would give rise to false positive reactions, specimens from patients with lupus erythematosus were included. Normal sera were obtained from healthy donors who were undergoing physical examinations as candidates for appointment to the U. S. Military Academy.

The results obtained with the FA test using T. rhodesiense as antigen are summarized in Table 8. The findings with sera from T. rhodesiense patients illustrate the relative sensitivity of this technic. The findings with sera from patients with other conditions and healthy individuals provide an index of the specificity of this test. Extensive cross reactions were obtained with sera from trypanosomiasis patients and particularly with those from patients with T. gambiense. The results obtained with T. gambiense antigen are summarized in Table 9. Once again extensive cross reactions were obtained with sera from trypanosomiasis patients. No significant difference in sensitivity or specificity of the results obtained with T. gambiense and T. rhodesiense antigens was observed. The results obtained with T. cruzi antigen are summarized in Table 10. With this organism, also, extensive cross reactions were observed with other sera from trypanosomiasis patients.

A comparison of results of FA tests performed with dried blood smears and serum from individuals infected with trypanosomiasis and from normal controls (Table 11) indicates that qualitative determinations for antibodies are closely correlated.

Table 8. Results obtained in the fluorescent antibody test using Trypanosoma rhodesiense as antigen (Human sera)

Diagnostic status	Number tested	T. rhodesiense antigen		
		Positive	Doubtful	Negative
Trypanosomiasis:				
T. rhodesiense	82	60	18	4
T. gambiense	7	5	2	0
T. cruzi	27	9	14	4
Other infections & disorders:				
Schistosomiasis	38	0	1	37
Trichinosis	14	0	0	14
Malaria	10	1	2	7
Leishmaniasis	4	1	0	3
Onchocerciasis	3	0	0	3
Echinococcosis	10	0	0	10
Opisthorchiasis	1	0	0	1
Fascioliasis	2	0	0	2
Leprosy	6	0	0	6
Infectious mononucleosis	1	0	0	1
Syphilis	14	0	1	13
Lupus erythematosus	2	0	0	2
Healthy controls:	55	0	6	49

Table 9. Results obtained in the fluorescent antibody test using *Trypanosoma gambiense* as antigen (Human sera)

Diagnostic status	Number tested	*T. gambiense* antigen Positive	Doubtful	Negative
Trypanosomiasis:				
T. gambiense	20	19	0	1
T. rhodesiense	68	54	12	2
T. cruzi	30	15	13	2
Other infections & disorders:				
Schistosomiasis	40	0	3	37
Trichinosis	16	0	1	15
Leishmaniasis	7	0	0	7
Malaria	10	3	1	6
Onchocerciasis	3	0	0	3
Echinococcosis	10	0	0	10
Opisthorchiasis	1	0	1	0
Fascioliasis	2	1	0	1
Leprosy	6	0	0	6
Infectious mononucleosis	1	0	0	1
Syphilis	14	0	1	13
Lupus erythematosus	3	0	0	3
Healthy controls:	69	1	2	66

Table 10. Results obtained in the fluorescent antibody test using *Trypanosoma cruzi* as antigen (Human sera)

Diagnostic status	Number tested	*T. cruzi* antigen Positive	Doubtful	Negative
Trypanosomiasis:				
T. cruzi	36	33	3	0
T. rhodesiense	5	3	1	1
T. gambiense	7	5	2	0
Other infections & disorders:				
Schistosomiasis	42	0	15	27
Trichinosis	15	0	1	14
Leishmaniasis	4	1	0	3
Malaria	10	0	2	8
Onchocerciasis	3	0	0	3
Echinococcosis	10	0	1	9
Opisthorchiasis	1	0	0	1
Fascioliasis	2	0	0	2
Leprosy	4	0	2	2
Infectious mononucleosis	1	0	0	1
Syphilis	13	1	3	9
Lupus erythematosus	3	0	0	3
Healthy controls	56	0	7	49

Table 11. Comparison of results obtained in the FA test for trypanosomiasis with serum specimens and dried blood smears from the same individuals

Specimen Number	Diagnostic Status	Parasite Antigen	FA reactions with	
			Serum	Dried Blood
1	Trypanosomiasis rhodesiense	Trypanosomiasis rhodesiense	2+	2+
2	"	"	2+	2+
3	"	"	2+	3+
4	"	"	2+	1+
5	"	"	2+	2+
6	"	"	2+	1+
7	"	"	1+	1+
8	"	"	1+	1+
9	"	"	2+	2+
10	"	"	2+	2+
11	"	"	2+	2+
12	"	"	2+	2+
13	"	"	-	1+
1c	Healthy	"	-	-
2c	"	"	-	-
3c	"	"	-	-
4c	"	"	-	-
5c	"	"	-	-
6c	"	"	-	-
14	T. gambiense	T. gambiense	3+	3+
15	"	"	3+	3+
16	"	"	3+	3+
17	"	"	3+	3+
1c	Healthy	"	-	-
2c	"	"	-	-
3c	"	"	-	-
4c	"	"	-	-
5c	"	"	-	-

8. Isolation, purification and fractionation of complement fixation test antigens from trypanosomes

Although effective physico-chemical methods have been developed for isolating specific complement-fixing antigens from cultured T. cruzi (Fife and Kent, Am. J. Trop. Med. & Hyg. 9:512, 1960), relatively little attention has been given to the development of reliable complement fixation tests for the serodiagnosis of African trypanosomiasis. This report deals with preliminary studies on the preparation of antigens from cultured T. rhodesiense, applying the methods successfully used for fractionating T. cruzi.

T. rhodesiense (Wellcome TC strain) was cultured on a diphasic medium (Tobie, et al., J. Parasitol. 36:48, 1950), harvested, washed in saline, and dried from the frozen state. For the preparation of antigen, the dried organisms first were extracted twice with anhydrous ether in the cold and the delipidized organisms then were extracted with borate-buffered salt solution, pH 8.0. The saline extract constituted the crude antigen. A portion of the crude antigen was subjected to chloroform-gel fractionation and the resultant protein and carbohydrate fractions were lyophilized along with the remaining unfractionated extract. Each antigen was evaluated in complement fixation tests with sera from cases of African sleeping sickness.

Initial evaluation of the antigens was conducted with two sera from documented cases of Rhodesian sleeping sickness; one was from a patient with advanced disease as evidenced by trypanosomes in the CSF, the other a case of early infection with no CNS involvement. Both sera showed some reactivity with the unfractionated antigen but this was of relatively low order. Essentially no reactivity was obtained with the protein or carbohydrate fractions. Since sera from both early and advanced cases gave essentially the same results, it appeared unlikely that the test sera were responsible for the unanticipated findings. Therefore, consideration was given to the possibility that the unexpectedly low degree of reactivity was due to a peculiarity of the lot of organisms used as source of antigen. Thus, extraction and fractionation was performed on a different lot of organisms. Results of tests with the latter preparations, however, were essentially the same as those obtained with the initial antigens; reactivity again was exceedingly low.

9. Fluorescent antibody studies in Leishmaniasis

Successful adaptation of the indirect fluorescent antibody technic to the laboratory diagnosis of trypanosomiasis suggested the possibility of using similar methods in diagnosing Leishmania infections.

Preliminary tests were carried out on microscope slides. Leptomonad forms from cultures of L. donovani (Khartoum strain) were used as the source of antigen. Smears were made on slides with a loopful of culture material, allowed to dry, and fixed with a 5% formalin-rhodamine bovine albumin (1:20) solution. The indirect method reported for the diagnosis of trypanosomiasis in humans was followed. The results of preliminary studies are summarized in Table 12.

Table 12. Studies of preliminary results using the Fluorescent Antibody test for Leishmaniasis in human sera

Diagnostic status	Number tested	Reactions in FA test Positive	Doubtful	Negative
Leishmaniasis	13	10	1	2
Other parasitic diseases				
Chagas disease	25	0	13	12
Trypanosomiasis rhodes.	3	2	0	1
Trichinosis	1	0	0	1
Echinococcosis	4	0	0	4
Fascioliasis	1	0	0	1
Total	34	2	13	19
Nonparasitic				
Syphilis	3	0	1	2
Leprosy	5	0	3	2
Healthy controls	19	0	1	18

10. Serodiagnosis of Malaria by Complement Fixation

During World War II and continuing throughout the period to 1950, considerable research was devoted to the development of complement fixation (C-F) tests for the serodiagnosis of malaria. However, during the succeeding years, general interest in malaria declined in this country and as a result, relatively little, if any, research along these lines has followed. The early studies on development of C-F tests for malaria culminated with the work of investigators from the Department of Serology, WRAIR (Rein, et al., Am. J. Hyg. 49:374, 1949), and their report represents the status of investigations at that time. It was observed that in initial attacks of sporozoite-induced P. vivax malaria, C-F tests detected antibody approximately 5 days following the onset of patent parasitemia and persisted for some 30 days after disappearance of parasites from the peripheral blood as a result of chemotherapy. Patients became seronegative, however, during the prolonged (ca. 9 months) latent period between the primary attack and the first relapse. In contrast to the serologic response during primary attacks, complement-fixing antibodies appeared on the 4th day following

the patent parasitemia of the first relapse and persisted for an average of 125 days even though the practice of initiating chemotherapy on the 5th day after onset resulted in rapid disappearance of the parasites from the circulatory system. Certain advantages as well as limitations of the C-F test therefore were apparent. For example,the C-F test appeared to have little value in early diagnosis since patent parasitemia always preceded the appearance of detectable antibodies. Furthermore, the extended period of seronegativity that occurred between the primary attack and the first relapse seemed to preclude use of the C-F test as a method for predicting the probability of relapse. However, the prolonged persistence of antibodies during the relapse phase made it possible on any given day to detect more infections among the experimental group by C-F than could be demonstrated by parasitological methods. Since the characteristics of late activity (relapse) would be a predominant feature in an endemic population, it was suggested that the C-F test might be of particular value in mass surveys, giving a more reliable index of the malaria experience of the population than would be indicated by examination of thick blood smears.

Since 1950 several excellent purification, isolation and fractionation procedures have been used to advantage in improving C-F antigens for serodiagnosis of other parasitic diseases. Therefore, studies were undertaken to determine whether these procedures had potential value as a means for improving the C-F test for malaria. Plasmodium knowlesi was selected as a means for improving the C-F test for malaria to be used in the initial phases of the studies. This organism was selected because (1) the majority of earlier investigations has been conducted with P. knowlesi and thereby could serve as a reference point for the present studies; and (2) relatively large quantities of parasites could be obtained from experimentally-infected monkeys.

Inocula used throughout the preliminary experiments were prepared from the blood of a monkey exhibiting a 70% parasitemia. Blood from this animal was collected in a mixture of heparin and 20% glycerine, divided into 1.0 ml. aliquants, and stored at -70°C. In the initial experiment, two monkeys each were inoculated intravenously with 1.0 ml. of the thawed inoculum. Thin films were prepared daily and examined for malaria parasites. When patent parasitemia reached 60-70%, the animals were exsanguinated and the blood preserved in modified Alsever's solution. Several methods for isolating the plasmodia from the parasitized cells were investigated. Hypotonic lysis of the erythrocytes with distilled water permitted isolation of the parasites. However, this treatment extensively altered the morphology of the parasites and in many instances appeared to disrupt the organisms. In view of the probability that the antigen(s) are water soluble, it seemed likely that serious loss of antigen would occur and on this basis the method was discarded. The feasibility of using other hemolytic agents was investigated, portions of parasitized blood were treated with saponin and a nonionic detergent Triton X-100. The later, Triton X-100, yielded the larger volume of free parasites and in addition, had relatively little effect on the morphological integrity of the organisms. Thus, it was selected as the

lytic agent for future studies. The nature of these isolation methods makes it impossible to predict accurately the dried weight of parasites obtainable from a single monkey. However, experience thus far indicates that 0.5-1.0 gm of lyophilized organisms should constitute a reasonable yield. Recovery of amounts of this order will permit parallel isolation, purification experiments employing several different procedures. The methods to be investigated in preliminary studies include: (1) Chloroform-gel - used successfully with Trypanosoma cruzi; (2) Preliminary treatment with anhydrous ether, following with buffered saline extraction and subsequent fractionation at the isoelectric point - used for isolation of Schistosoma mansoni antigens; and (3) Alcohol fractionation in the presence of low ionic strength divalent cation - previously used in fractionation of Trichinella spiralis antigens.

Three additional monkeys currently are infected and parasites obtained from these animals will provide material for the proposed antigen fractionation experiments.

11. Fluorescent antibody studies of malaria

The fluorescent antibody test for malaria antibodies developed by the National Institutes of Health has been adopted. Attempts were made to use the simian parasite, Plasmodium knowlesi, as antigen in the test for human malaria antibodies. However, it was found that the reaction was too weak for this purpose. When sera from volunteers with recent experimental infections were tested with P. falciparum as antigen, six out of eight P. falciparum sera were positive while only one out of seven P. vivax sera were positive with the same antigen.

In cooperation with the Pan American Health Organization, a study of filter paper specimens of blood from proven cases of malaria in endemic areas has been initiated. The use of the filter paper method of collection and storage of blood was found to be adaptable to the malaria fluorescent antibody test. All six specimens from P. falciparum patients were positive whereas only 8 of 34 specimens from P. vivax patients were positive. The study will continue with the use of both P. vivax and P. falciparum as antigen.

12. Evaluation of research tests at a diagnostic level

During recent years, military and other investigators have developed numerous serodiagnostic tests for several parasitic diseases. These tests were satisfactory when performed at the research level. However, it is essential that the procedure be performed successfully at a diagnostic level by less highly trained individuals, and the test designated as acceptable for routine diagnosis before the fruits of the research are realized.

Therefore, during the past year a diagnostic section was initiated through the joint effort of the Department of Serology and the Department of Medical Zoology. Members of the Department of Medical Zoology produce the parasitic material; the Department of Serology prepares the antigens and performs the serodiagnostic procedures. The following procedures have been selected as suitable for the program and are currently available upon request: Trichinosis: a slide flocculation test with acid soluble antigen; a complement fixation with ethanol purified antigen; Schistosomiasis: a slide flocculation test with delipidized cercarial antigen; a complement fixation test with delipidized adult worm antigen; Trypanosomiasis: a complement fixation test with a purified protein antigen; a fluorescent antibody test with trypanosome blood forms as antigen. As satisfactory procedures become available for other parasitic infections, they shall be added to the program.

In addition to proving the value of the tests at a diagnostic level, the establishment of the diagnostic section has the advantage of serving as an experienced group that could be rapidly expanded in the event of an emergency.

Because of the combined effort of several members in each of the two departments and because the number of requests for the tests are relatively small, it has been possible to initiate the service without hampering any one individual already existing research programs.

Summary and Conclusions:

1. On the basis of these and other comparative studies it was concluded that the schistosomiasis plasma card test possesses adequate sensitivity and specificity. Because of the economy, rapidity, and specificity of this test and the fact that it provides a permanent record for future reference the procedure may be particularly useful as a screening device for field epidemiologic investigations.

2. To date the results obtained are encouraging. The excretions and secretions of the cercariae have been shown to be antigenic, both in vitro and in vivo. Considerable information into the chemical and physical characteristics of the antigen has been obtained. However, work on the project has been delayed until the existing vacancy of a biochemist has been filled.

3. In view of the simplicity of this procedure the plasma card test appears to be ideally suited for field investigations and for poorly equipped laboratories. Moreover, because of its economy and simplicity of performance, the test also may be useful for screening hogs at the time of slaughter provided that antibodies are detectable as long as viable larvae are present.

4. Current research is directed toward improving the technic. In addition, laboratory rabbits have been immunized by inoculation of D. uniformis adult worm antigen for the purpose of determining the early appearance and development of antibody titer.

5. As indicated in Table 5, the test could be conducted successfully as long as 32 hours after ingestion of blood by the mosquito. However, erthrocytes were more easily freed at six or eight hours after feeding than at earlier or later intervals. It was noted that more cells could be recovered from mosquitoes engorged with human blood than from those with guinea pig blood. Results in all tests were clear-cut; there were not doubtful reactions. It is expected that field trials of this test will be undertaken soon by the World Health Organization personnel in Burma.

6. The data presented here indicate that the fluorescent antibody technic can be successfully applied to African trypanosomiasis in experimental animals. Trypanosome forms from blood of infected rats appeared to give more satisfactory results than proventricular forms from cultures. The FA test appeared to be highly sensitive in rabbits infected with T. rhodesiense and T. gambiense. The reproducibility of results with this test employing different lots of trypanosomes and labeled antiglobulins was good and compared very well with that of other serological tests employing whole organisms. Antibodies in experimentally infected rabbits were detected between one and two weeks after inoculation.

The similarity of observable reactions in the FA test using T. lewisi and African human trypanosomes as antigens is of great interest. It is obvious that the need for using human pathogens would limit the number of laboratories that could routinely perform the test to those in which sufficient precautions can be taken to prevent human infections. Since T. lewisi is nonpathogenic for humans and can be maintained readily in most laboratories this limitation would thus be removed.

7. The results of these studies, in general, suggest that a fluorescent antibody test may provide a relatively simple and reliable procedure for the diagnosis of trypanosomiasis. In particular, the ability to utilize minute amounts of dried blood which can be easily obtained even under the most adverse conditions and mailed to a central laboratory, suggest that this test can be used to great advantage in epidemiologic investigations for trypanosomiasis. Furthermore, since T. lewisi was found to cross react with the human trypanosomes (Williams, et. al., 1963), a parasite which is nonpathogenic for humans and can be maintained readily in most laboratories might be used for these tests.

8. Efforts to isolate complement-fixing antigens from cultured T. rhodesiense by methods previously used for the fractionation of T. cruzi antigens thus far have been unsuccessful. It is suggested that either of two factors account for the anomalous results. First, it is well known that the African trypanosomes (T. rhodesiense and T. gambiense) rapidly undergo marked variation when removed from their natural vector-host cycle. Thus, it is quite possible that the culture strain, having been maintained in vitro for a prolonged period, bears only remote antigenic relationships to the wild strains transmitted by the tsetse, and antibodies produced during vector-transmitted infections therefore may not be capable of reacting with culture strain antigens. Secondly, consideration must be given to the possibility that certain heterologous antigens of the cultured organisms reside in the so-called "exo-antigen" components recently described by British investigators, and being highly soluble were lost when the harvests were washed to free the trypanosomes from medium components. Studies on animals immunized with culture and syringe-passage strains have been initiated in an effort to elucidate the basic causes of these problems.

9. Most of the Leishmania sera tested were from patients in Panama where L. braziliensis is endemic and therefore the results reported are based mainly on a heterologous system. Efforts will be made to extend these observations by using L. braziliensis cultures as antigen and by testing additional L. donovani sera against the homologous antigen. Attempts will also be made to improve the sensitivity and specificity of the test.

10. If the methods used in these preliminary studies provide a means for improving complement-fixing antigens obtained from P. knowlesi, it is proposed that those yielding the best results will be employed in efforts to obtain specific antigens from human Plasmodia.

11. Sera have been collected for a study of the fluorescent antibody production curve in the mouse infected with P. berghei. Fifty infected mice were used as the experimental group and fifty mice injected with normal erythrocytes were used as controls. Serum was collected on every second day from 10 animals by exsanguination for 10 bleedings. Parasitemias and hematocrits were determined on the day of the bleedings. These serological studies have not yet been completed.

12. The initiation of a section for the serodiagnosis of parasitic diseases has led to the evaluation of procedures at a diagnostic level and has made the procedures available upon request. In addition, the program is available for rapid expansion if an emergency develops.

List of Publications:

1. Sadun, E. H., Anderson, R. I., and Williams, J. S.: Fluorescent antibody test for the serological diagnosis of trichinosis. J. Parasit. 48:(Suppl.) 17, 1962.

2. Sadun, E. H., Anderson, R. I., De Witt, W. B., and Williams, J. S.: Quantitative serological reactions in treated humans and in monkeys recovering spontaneously from schistosomiasis mansoni. J. Parasit, 48:(Suppl.) 16, 1962.

3. Sadun, E. H., Anderson, R. I., and Schoenbechler, M. J.: A new slide flocculation test for trichinosis. J. Parasit. 48: (Suppl.) 17-18, 1962.

4. Sadun, E. H. and Biocca, E.: Intradermal and fluorescent antibody tests on humans exposed to Schistosoma bovis cercariae from Sardinia(Italy). J. Parasit. 48: (Suppl.) 17, 1962.

5. Anderson, R. I., Sadun, E. H., Rosen, L., Weinstein, P. P., and Sawyer, T.: The detection of antibodies in eosinophilic meningitis. J. Parasit. 48: (Suppl.) 3-4, 1962.

6. Sadun, E.H. and Biocca, E.: Intradermal and fluorescent antibody tests on humans exposed to Schistosoma bovis cercariae from Sardinia. Bull. Wld. Hlth. Org. 27:810-814, 1962.

7. Sadun, E. H., Anderson, R. I., and Williams, J. S.: Fluorescent antibody test for the serological diagnosis of trichinosis. Exper. Parasit. 12:423-433, 1962.

8. Anderson, R. I.: Relationship of antibody nitrogen to titer obtained in cercarial antigen slide flocculation test for schistosomiasis. Exper. Parasit. 12:434-440, 1962.

9. Sadun, E. H., Anderson, R. I., and Schoenbechler, M. J.: A plasma card test for rapid serodiagnosis of schistosomiasis (SPC), Proc. Soc. Exp. Biol. & Med., 112:280-283, 1963.

10. 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. (In press), 1963.

11. Sadun, Elvio H.: Seminar on immunity to parasitic helminths. VII Fluorescent antibody technique for helminth infections. Exper. Parasit. 13:72-82, 1963.

12. Sadun, E. H.: Introduction by the Chairman of the International Panel Workshop on immunodiagnosis of helminthic infections. Am. J. of Hyg. (In press), 1963.

13. Anderson, R. I.: International Panel Workshop on Immunodiagnosis of Helminthic Infections. Current and potential value of immunodiagnostic tests employing whole organisms. Am. J. of Hyg. (In press) .

14. Jachowski, L. A., Anderson, R. I., and Sadun, E. H.: Serological reactions to Schistosoma mansoni. I. Quantitative studies on experimentally infected monkeys (Macaca mulatta). Am. J. of Hyg. 77:137-145,1963.

15. Sadun, E. H., Anderson, R. I., De Witt, D. B., Jachowski, L. A., and Williams, J. S.: Serological reactions to S. mansoni. II. Quantitative studies in human patients treated with Stibophen. Am. J. of Hyg. 77:146-149, 1963.

16. 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. (In press), 1963.

17. Anderson, R. I., Sadun, E. H., and Schoenbechler, M.J.: Cholesterol-lecithin slide (TsSF) and charcoal card (TsCC) flocculation tests using an acid soluble fraction of Trichinella spiralis larvae. J. Parasit. (In press), 1963.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 806, Military Preventive Medicine

Task No. 01, Communicable Diseases (Laboratory Diagnostic Procedures for Microbial Diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Bacteriology
Department of Serology
Department of Virus Diseases
Division of Communicable Disease and Immunology

Department of Veterinary Microbiology
Division of Veterinary Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Laboratory Diagnostic Procedures for Microbial Diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

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The diverse investigations reported under this task are as follows: (a) Efficacy of a semi-solid medium for the transport of pathogenic enteric bacteria is discussed. (b) Results of initial studies on the effect of lyophilization and the effect of storage at 4°C. of rehydrated lyophilized staphylococcal bacteriophage on lytic activity are presented. (c) New serological procedures for identification of Pasteurella pestis include (1) the Brewer Card Flocculation Test for plague antibodies, and (2) a modification of the Elek test for detection of the species-specific capsular antigen of P. pestis. (d) Excellent correlation was obtained from comparative studies using the Fluorescent Treponemal Antibody (FTA) Test and the Treponema pallidium Immobilization Test on 2702 sera presenting biologic false positive problems.

Effects of certain serum components on the FTA test are discussed. (e) A modified flagellar agglutination test for Salmonella paratyphi B using the Microtiter method is presented. (f) Methods for obtaining increased yields of ascitic fluid from mice, and optimal inoculation schedules for producing high ascitic fluid CF titers in mice to Anopheles A virus are presented. (g) Preliminary evaluation studies on an indirect fluorescent antibody technique for serodiagnosis of toxoplasmosis showed good correlation with the conventional CF test.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Laboratory Diagnostic Procedures for Microbial Diseases)

Description: The purpose of this task is to discover, develop and evaluate laboratory procedures for the accurate and rapid etiologic diagnosis of acute infectious diseases, with particular reference to those of real or potential value for use in field or other specialized military laboratories.

Progress:

1. Viability Studies on Enteric Bacteria in a Semi-solid Transport Medium.

Results of preliminary experiments (Annual Report 1962) on the use of a modified Stuart Transport Medium for the collection and transportation of rectal swabs and fecal specimens have warranted further testing of the medium under conditions of actual usage. Such a study was initiated in 1962 with the SEATO Medical Research Laboratory (U. S. Component), Bangkok, Thailand. Duplicate enteric specimens were collected in the modified transport medium and one of each was mailed to WRAIR where it was examined bacteriologically for the presence of pathogenic enteric bacteria. Similar studies were performed on the other specimen in Bangkok within 24 hours of collection. To date 155 specimens from Thailand have been processed. The average time in transit from Bangkok to Washington, D. C. was 6 days. Actual times between collection and processing at WRAIR varied between 6 and 59 days, although the majority of specimens were processed within 20 days.

WRAIR isolations of salmonellae and shigellae compared favorably with SEATO results on specimens held for 10-12 days, however, too few positive specimens were found by either laboratory to assess the significance of these findings. Isolations from specimens held in transport media for longer periods fell off precipitously, as compared with SEATO results, although Shigella boydii 4 and Shigella flexneri 4 were recovered from specimens held for 20 and 59 days, respectively. Few non-agglutinable vibrios (NAG) survived storage in the medium, which may be due, in part, to their inhibition by Pseudomonas aeruginosa appearing in 26 per cent of the specimens reported by SEATO to contain NAG vibrios. However, in view of favorable reports obtained from independent comparison studies made by Lt. Col. Sidney Gaines, SEATO Laboratory, testing will be continued until a sufficient number of positive specimens are obtained to determine the optimal period of storage permitted by this medium.

2. Bacteriophage Typing of Staphylococci.

a. Investigations on improvement and simplification of staphylococcal bacteriophage typing methodology have shown the Lidwell phage applicator to be vastly superior to methods of manually applying individual phage

suspensions to agar plates (Annual Report 1962). In order to place this valuable test in all Army hospital laboratories, it will be necessary to eliminate two difficult parts of the typing procedure, i.e. propagation and titering of the 23 typing phages, and maintenance of the 23 propagating strains. These can be obviated by phage preparations which may be diluted ready for use with the Lidwell apparatus without additional quality testing by the using laboratory. Necessary control studies are mandatory for production of such lyophilized typing phages which will meet the standards of the International Subcommittee on Bacteriophage typing of Staphylococci. Preliminary investigations were begun to determine the effect of lyophilization, and the effect of storage on rehydrated lyophile suspensions at 4°C. on the lytic titers of staphylococcal typing phages. In an effort to determine the effect of the pre-freezing temperature on phage survival, equal aliquots of a typing phage 29 suspension containing 8.5×10^{10} phage particles per ml. were placed into small lyophile ampules and frozen by one of the three following methods: (a) the temperature was lowered quickly from 8°C. to -70°C. and the material lyophilized for 18 hours; (b) the temperature was lowered from 8°C. to -50°C., then lowered to -70°C. and the material lyophilized for 18 hours; and (c) the temperature was lowered to -50°C. and the material held at -50°C. for 24 hours. Ampules from each group were rehydrated, or thawed, dilutions were prepared and plaque counts determined by spotting appropriate dilutions on a staphylococcal lawn. Plaque counts from these preliminary tests were higher in treatments (c) and (b) than (a). This effect was also demonstrated in routine test dilution (RTD) titers of 10^{-4}, 10^{-4} and 10^{-3}, respectively. Additional tests will be performed using phage 29 as well as each of the other 22 typing phages in order to assess the significance of these results.

b. Lyophilized typing phages were reconstituted and diluted 1:10 through 1:10M with broth. Dilutions were devised so that the log dilution containing the exact routine test dilution (ERTD) was further diluted in an arithmetic series of 10 dilutions. Equal volumes from each dilution of the entire series were placed on the appropriate staphylococcal lawns and the ERTD determined after 18 hours at 30°C. All diluted phage suspensions were stored in a conventional refrigerator at 4°C. and tested over a six month period by spotting each dilution on seeded plates. During this period all but four phage suspensions gradually decreased in titer, as reflected by the ERTD. Loss of original titer varied between 45-99 per cent, indicating that under the conditions of this experiment individual differences in survival potential existed between the phages tested. Four additinnal phage suspensions showed no loss in lytic titer during the first month of storage, four lost more than 50 per cent activity, and six phages lost between 9 and 40 per cent of their original (ERTD) titers. Additional studies to improve quantitation and to devise a better storage medium for phage suspensions are planned.

3. A Card Flocculation Test for Pasteurella pestis Antibody.

A simple, rapid immuno-diagnostic test has been developed which may be well suited to field conditions and eliminate the transportation of sera for plague antibody determinations. This need is particularly acute in

many of the endemic areas where good laboratory facilities are lacking.

Plague capsular antigen was prepared by the method of Baker, et al., 1952. The antigen emulsion for the card flocculation test is prepared by adsorbing the purified capsular antigen onto cholesterol-lecithin crystals, centrifuging and resuspending the sediment with a solution containing charcoal.

Using a capillary tube, 0.05 ml. of unheated plasma or sera is placed on a card especially designed by Brewer. One drop of antigen suspension (1/60 ml.) is distributed to each sample with a specially designed needle attached to a plastic dispensing bottle. Using a separate toothpick for each test sample, the plasma and antigen suspension are mixed and spread until the outlined test circle is covered. The card is then rotated on a rotating shaker (100 rotations per minute) for 8 minutes and read immediately. Specimens showing characteristic clumping are reported as positive and those showing no clumping at the end of the 8-minute test period are reported as negative.

Sera or plasma may be serially diluted with saline on the card so that actual titrations may be performed. Titrations carried out in this fashion appear to correlate reasonably well with the CF test. However, several sera from recovered plague animals yielded negative CF tests but were positive using the card test. In over 100 normal sera obtained from various laboratory animals and personnel there was only one instance of a positive card test. It was subsequently learned that this individual had been recently vaccinated against plague.

Further evaluation of this procedure is in progress. In addition, the stability of the antigen emulsion has yet to be examined.

4. Agar-Gel Test for Serological Identification of Pasteurella pestis.

As a further aid to differentiating between P. pestis and Pasteurella pseudotuberculosis use was made of the precipitating ability of the specific capsular antigen of P. pestis. Suspect strains are inoculated in a single heavy streak across trypticase soy agar or blood agar base plates. A disc previously impregnated with rabbit anti-FI globulin is placed adjacent to the streak and the plate is incubated at 37°C. for 24 to 48 hours. After this period of time anti-capsular globulin diffusing from the impregnated disc has encountered and formed a characteristic line of precipitation with the capsular material diffusing from positive cultures.

Discs are prepared by moistening them with a solution of hyperimmune globulin followed by lyophilization. These discs have remained stable for at least eight months at room temperature. Inasmuch as more than 99 per cent of the wild strains of P. pestis produce capsular material when grown at 37°C., it is likely that this technique, either by itself, or in conjunction with procedures previously described for differentiation of P. pestis from P. pseudotuberculosis, may prove of value in field areas where laboratory facilities are lacking.

5. Evaluation of the Fluorescent Treponemal Antibody (FTA) Test for Syphilis: Comparison with the Treponema pallidum Immobilization (TPI) Test.

A preliminary report describing the FTA procedure and its potential value in differentiating biologic false positive (BFP) from true syphilitic reactions has been presented (Fife, et al., J. Clin. Path. 36: 105, 1961). The present report is an extension of these studies and is based on findings obtained with a significantly larger group of sera. Thus the efficacy of the FTA was evaluated in comparative FTA and TPI tests conducted on 2702 sera submitted to the Department of Serology, WRAIR, for TPI examination. The majority of the sera were from cases presenting diagnostic problems, and, for the most part, represented either true syphilitic or BFP reactors. Therefore, they provided an especially critical measure of the specificity, as well as sensitivity, of the FTA procedure. As was observed in the earlier studies, correlation of FTA and TPI findings was excellent; agreement of test results was obtained with 2519 (93 per cent) of the specimens examined. Moreover, the practice of re-examining all sera that gave divergent results in initial tests improved the correlation still further. Simple retesting of the 183 sera giving contradictory results resolved the differences with 102 specimens and thereby increased the over-all agreement between the tests to 2621 (97 per cent) of the sera evaluated. The preliminary studies suggested that the FTA was less apt to give reversal of reactions than was the TPI, presumably because the former was subject to fewer uncontrolled variables than were inherent in the TPI. However, the present more comprehensive evaluation revealed that reversal occurred with approximately the same frequency in both tests. In addition, it was observed that the majority of reversals occurred with sera containing threshold amounts of antibody (i.e., giving weak reactions in the test in question), and apparently reflected intrinsic day-to-day fluctuations of sensitivity in both procedures. Also, during the course of the current studies it was noted that approximately 18 per cent of the sera submitted for TPI examination were unsatisfactory for TPI testing, either because of nonspecific immobilization (immobilization in the absence of active complement) or because of anticomplementary properties. However, these factors did not interfere with performance of the FTA. Thus, in contrast to the special precautions necessary for collecting sera for the TPI, it is apparent that sera which are suitable for conventional serologic tests (e.g., flocculation and complement fixation tests) also are satisfactory for use in the FTA.

6. Effect of Macroglobulins on the Specificity of the FTA Test for Syphilis.

During the course of the earlier evaluation (op. cit.), review of case histories revealed no particular disease syndrome that was consistently associated with false reactions in either the FTA or TPI. However, the more comprehensive current studies revealed that sera from certain nonsyphilitic patients with rheumatoid arthritis, particularly those with elevated bentonite flocculation titers, reacted in the FTA but not in the TPI. Since the most striking serologic feature of

rheumatoid arthritis is appearance of the so-called "rheumatoid factor", a high molecular weight component of gamma globulin with a sedimentation coefficient of about 19 S, consideration was given to the possibility that this factor, and conceivably other macroglobulins, might predispose false FTA reactions. Therefore, sera from a group of patients with rheumatoid arthritis or other disorders (necrotic cirrhosis, Lannec's cirrhosis, idiopathic thrombocytopenia purpura, etc.) known to lead to elevated macroglobulin levels were examined in FTA and TPI tests. In general, sera giving bentonite flocculation titers greater than 512 reacted in the FTA but not in the TPI. There was one notable exception, however; one rheumatoid arthritis serum with a bentonite titer of 1024 did not react in the FTA. It is suggested that this apparently anomalous result reflects the heterogeneous character of macroglobulins, and indicates that this particular serum contained atypical high molecular weight components that for some unapparent reason failed to mediate the expected reaction in the FTA. Specimens with bentonite titers of 512 or less, on the other hand, generally were nonreactive in the FTA. Ultra-centrifuge studies provided direct evidence concerning the role of macroglobulins in false FTA reactions. Rheumatoid arthritic and syphilitic sera were centrifuged at 100,000 rcf for one hour, and the supernate and sedimented portion of each was evaluated in the FTA and TPI tests. Prior to centrifugation, the rheumatoid sera reacted in the FTA but not in the TPI. However, after centrifugation, the components responsible for reactivity in the FTA were concentrated in the sedimented fraction; the supernates were essentially nonreactive. In contrast, centrifugation did not have a similar effect on the reactivity of the syphilitic sera; there was no detectable reduction of FTA or TPI reactivity in the supernatant fractions. Thus it was evident that little or no syphilitic antibody was sedimented by the employed conditions of centrifugation.

7. A Flagella Agglutination Test using the Microtiter Technique.

Preliminary studies using Salmonella paratypi B were performed to study the immunologic efficiency of newborn rats preliminary to anticipated viral studies in these animals. A need arose to simplify the presently available but cumbersome agglutination procedures, and to devise methods which could use smaller amounts of serum in view of the limitations imposed by these experiments.

A highly-motile phase I strain of S. paratyphi B (Java ETS #5) was passed once through semi-solid agar medium. An inoculum of 0.1 ml. of this material was transferred to 125 ml. of single veal infusion broth and grown at 37°C. for 25 hr. At the end of this period the culture was killed by 0.6 per cent formalin. The flask was allowed to sit at room temperature overnight and checked for sterility.

The broth suspension was centrifuged at 12,000 rpm in the Servall for one hour. The button of cells was then resuspended in an appropriate volumn of 0.15M saline to produce a suspension containing a count of 2.8×10^{-9} bacteria/ml. This count was determined by means of a Coulter counter.

Twenty-five (25) ml. of this suspension was sedimented by low centrifugation at 3500 rpm and resuspended in 1.5 ml. of saline. To this suspension 0.2 ml. of carbolfuchsin (Ziehl-Neelsen Method) stain was added. It was allowed to incubate at 56°C. for 10 minutes.

Following this incubation, the sediment was washed once with saline and repacked by centrifugation. The button was then resuspended in a small volume of saline and washed a second time with 70 per cent alcohol. The suspension was packed and resuspended an additional two times to remove excess stain. Following the last centrifugation the stained button of cells was resuspended in a volume of 5.0 ml. of 0.15M saline.

Serial two-fold dilutions of hyperimmune rabbit serum were prepared in tubes. Quantities of 0.025 ml. amounts of each of the serum dilutions were transferred to the wells of the "V" microtiter plate and 0.025 ml. of the stained bacterial suspension was added to each well. A replicate tube titration was performed using the same serum dilutions and a 1:2 dilution of the stock unstained bacterial suspension.

Both tests were incubated at 4°C. overnight. The following morning the tube titration was read in the conventional manner, and the titer was expressed as the highest dilution of serum giving visible agglutination. The microtiter titration was read by the pattern method. The titer was expressed as the highest dilution of serum giving a clear-cut shield of agglutination. The titer in both cases was the same, namely 1:1280.

The significance of these findings seem to suggest that the conventional flagellar agglutination procedure can be modified by means of this staining procedure for use in the microtiter method. The obvious advantages of this method are conservation of antigen, serum and equipment, as well as a greatly facilitated visual assessment of the end point. These studies may also serve as a basis for the use of other bacteria in this method.

Studies are now in progress to further improve and refine the method.

8. Production of Ascitic Fluid from Mice Immunized with Anopheles A Virus.

A scarcity of reagents to certain arthropod-borne viruses necessitated investigations into volume production methods. In collaboration with the Sub-committee on Serological Reagents, American Committee on Arthropod-borne Viruses, reagents in the form of mouse ascitic fluid were made to Anopheles A virus.

Two to four month old Charles River male swiss albino mice were immunized with Anopheles A virus according to a schedule suggested by the Sub-committee (I - Table 1) and on other schedules (II, III - Table 1). Intraperitoneal and subcutaneous doses were 0.5 ml. and intramuscular doses were 0.1 ml. The adjuvant mixture consisted of 1 part of 10 per cent infected mouse brain in phosphate-buffered saline, 2½ parts of Freund's incomplete adjuvant (Difco) and 1½ parts of a heat-killed

trypticase soy broth suspension of Staphylococcus aureus, strain 18, adjusted to 1 x 10^{10} per ml.

Table 1

Schedule I

Day	Inoculum	Route
1	mouse brain	IP
3	mouse brain	IP
30	adjuvant mix.	IP
35	adjuvant mix.	IP
40	adjuvant mix.	IP
45	adjuvant mix.	IP

Schedule II

Day	Inoculum	Route
1	adjuvant mix.	SQ & IM
10	mouse brain	IP
20	mouse brain	IP
30	adjuvant mix.	IP

Schedule III

Day	Inoculum	Route
1	adjuvant mix.	SQ & IM
30	adjuvant mix.	IP

Only 9 out of 35 or 26 per cent of the mice in Schedule I developed ascites. This occurred between the 40th and 50th day of the schedule, during which time 16 ml. was collected, almost half of it from one mouse over several paracentesis three to four days apart. Ten ml. remained after clotting and centrifugation, barely half the amount of serum that could be obtained from 35 mice. CF antibody in both the ascitic fluid and serum titered 1:512 or greater.

No ascitic fluid was obtained by Schedules II or III. A few mice in Schedule II exhibiting dilated abdomens were found to have masses of a fibrose-adipose material at autopsy.

Since the R.H. strain of Toxoplasma gondii is known to cause ascites, approximately 10 of these organisms were inoculated IP into all mice in an attempt to get reasonable quantities of ascitic fluid for further study, and to utilize the mice already immunized. Ascites was evident approximately seven days after inoculation and death occurred by the ninth day. During this seven to nine day interval, the mice were bled out by cardiac puncture under anesthesia. Holding the animal upright so the abdominal contents float upwards, away from the point of puncture, the ascitic fluid was removed from the peritoneum by drawing it through an 18 gage needle into a syringe. Since this material contains live parasites, the

gravity-drip method is not recommended. A freeze-thaw treatment, usually effective for facilitating the clotting of ascitic fluid, had no effect on the parasite induced fluid.

Yields: Schedule I, 8/30 mice or 27 per cent contributed 14 ml.
Schedule II, 22/27 mice or 80 per cent contributed 35 ml.
Schedule III, 10/30 mice or 33 per cent contributed 22 ml.

The total yield of 71 ml. from 87 mice is somewhat better than the maximum amount of serum that could be obtained. The fluid was centrifuged at 15,000 X G for one hour. All of the undiluted material from the various schedules neutralized between four and five logs of virus. Schedule I was the higher of the three. The CF titers of the ascitic fluid and serum from all schedules were low -(1:32 to 1:64). This may have been due to the time lapse of two to three months after the last antigen dose. All of the parasite-induced ascitic fluids were anticomplementary as high as 1:8. This was completely removed by a double extraction with 50 volumes of acetone. After drying, reconstitution was accomplished with an amount of normal saline effecting a 1:10 dilution of the original material. After this treatment Schedule I titered 1:80, II and III, 1:40. After the parasite-induced ascitic fluid had been stored for 2½ months at -20°C., it was found in another test that anticomplementary activity was minimal (1:2) eliminating the necessity of acetone extraction. Clots were first evident after this storage period.

Freund's complete adjuvant (Difco) was substituted for the incomplete in the adjuvant mixture for mouse inoculation and gave the best results. The efficiency of the complete adjuvant to cause ascites was quickly tested by giving the mixture at the start of the immunization. The doses were given IP on days 1, 5 and 9 and on day 13 most of the mice had dilated abdomens and all contributed in some degree to the pool of ascitic fluid, from 0.5 ml. to 2.5 ml. The group was tapped four times, five to ten days apart giving a cumulative total of 35cc after clotting and centrifugation. This is about four times the maximum 9 ml. of serum obtained from the 12 mice. The percentage of mice with ascitic fluid rapidly diminishes on successive taps. The closely spaced inoculations given within a nine day period only permitted titers to reach 1:64 to 1:128 on the 20th day, then fell to 1:4 on the 40th day. Walter Reed male swiss white mice, four months of age were used in this test.

Lieberman's schedule was modified by using complete rather than incomplete adjuvant. Two IM and SQ injections on days one and three with infected mouse brain only, were followed with the complete adjuvant mixture on days 9 and 13 in Walter Reed female white mice. Three taps between days 17 and 26 provided 40 ml. of ascitic fluid after clotting and centrifugation from 10 mice. This was five times the amount of serum obtained. Ten cage mates marked with picric acid given the first two doses IP contributed only 7 ml. after clotting and centrifugation. The fluids titered 1:32 on the 20th day and when the mice were bled out on the 41st day, the serum titers were even higher, 1:64 to 1:128. This is in contrast to the serum titering 1:4 from an all IP schedule completed four days

earlier, 9 days instead of 13 days. Serum from mice started IM titered a dilution higher. This became significant after the next experiment.

Two groups of 12 mice each (Walter Reed males) were inoculated on days 1 and 4 with mouse brain only. One group received it IM and SQ, the other IP. On days 35, 39, 43 and 48 they were all inoculated with the complete adjuvant mixture. Both groups were tapped seven times between days 55 and 95. A total of 51.8 ml. was obtained from the IM schedule, and 55.6 from the IP schedule, negating the inference in the above paragraph that all IP inoculations are detrimental to ascitic fluid yields. Approximately 35 ml. of fluid remained from each group after clotting and centrifugation. This is about four times the maximum amount of serum from the same mice. Fluid from mice immunized initially IM and SQ reached CF titers of 1:2048 between day 59 and 72, a range of 14 days. The IP schedule only titered 1:512 at one point during this period. Since some of the material had a sticky consistency pipettes were changed in a repeat CF test. This lowered the 14-day plateau only 1 dilution (1:1024). The IP schedule material titered only 256 for approximately the same period.

One dose immune reagents are recognized as the most specific. Antigen was included one time with one of the complete adjuvant inoculations for ascites to occur over the peak of the response. In the experiments completed, however, the CF titers did not go beyond 1:16.

A final schedule is now in progress which will determine the necessity of the heat-killed Staphylococcus aureus in addition to the Mycobacterium sp. already included in the complete adjuvant.

9. Comparison of the Complement Fixation Test and the Fluorescent Antibody Technic in the Serodiagnosis of Toxoplasma gondii Infections.

Studies were conducted to determine the potential usefulness of a fluorescent-antibody technic (FAT) for the serological diagnosis of Toxoplasma gondii infections. The availability of a large number of human serums, positive by complement-fixation test for toxoplasmosis afforded an opportunity to study the sensitivity and specificity of FAT.

One hundred-fifty-eight serums submitted for tests for complement fixation (CF) antibodies for toxoplasmosis were tested with a fluorescent-antibody technic (FAT). Sixty-nine of the serums had significant CF antibodies for toxoplasmosis, the remainder were negative. The FAT employed was an indirect test patterned after methods employed by the Department of Serology, Division of Communicable Diseases and Immunology, WRAIR, for diagnosis of syphilis. Ascitic fluid from infected mice was used as source of antigen. The antigen was deposited in 0.04 to 0.06 ml. amounts in an area of a slide circumscribed with indelible ink, allowed to dry and fixed with acetone. Test serum, diluted in 1/200 was added to antigen in 0.04 ml. amounts. (The dilution of serum for optimum sensitivity and specificity was determined previously.) Slides were then shaken in a humidity chamber at 37°C. at 100 revolutions per minute for 30 minutes. After washing with phosphate buffered NaCl solution, (.01 M., pH 7-7.2) and gentle blotting, 0.04 ml. of a dilution of anti-human globulin goat (or mule) serum

"conjugate" was applied and allowed to mix by shaking again for 30 minutes in the humidity chamber. Slides were gently washed to remove excess "conjugate", blotted dry, mounted in buffered 90 per cent glycerin, and observed for reactivity in an ultra-violet source microscope with a BG12 exciter filter. The "conjugate" used in this study was either anti-human globulin prepared in goat or mule combined for fluorescein isothiocyanate. These conjugates were used in dilutions of 1:500.

All sixty-nine CF positive serums were also positive with FAT. Of eighty-nine CF negative serums, two were FAT positive, the remainder were negative.

At the present time methods for stabilizing the toxoplasma antigen are being studied.

Summary and Conclusions:

1. Preliminary results from a comparison study, conducted with the SEATO Medical Laboratory, on the recovery of pathogenic enteric bacteria from rectal swabs and fecal specimens held for varying periods in a modified Stuart Transport Medium is discussed.

2. Studies on the effect of the lyophilizing process on loss of staphylococcal bacteriophage activity indicate that the pre-freezing temperature may account for a partial loss of lytic activity. Lyophilized typing phages which have been reconstituted and diluted vary in their ability to remain viable (lytic) when stored in the refrigerator.

3. A card flocculation test for Pasteurella pestis antibody is described.

4. An in vitro gel precipitation technique for finite laboratory identification of P. pestis is presented.

5. Evaluation of the FTA test for syphilis with a large group of sera from patients presenting diagnostic problems yielded results that were in essential agreement with those of the less comprehensive preliminary studies. In tests with 2702 sera, FTA and TPI tests showed excellent correlation, agreement being obtained with 2519 (93 per cent) of the specimens. Moreover, agreement could be further improved to 97 per cent simply to retesting sera giving discrepant results in initial tests. Results of the earlier evaluation suggested that the FTA was less likely to undergo reversal of reactions than was the TPI. However, the more comprehensive current studies indicated that this was not the case; reversal of reactions occurred with essentially the same frequency in both procedures, and these usually occurred with sera giving threshold (weak) reactions. Findings obtained under actual test conditions have revealed that the FTA unquestionably is a valuable adjunct to the TPI. Moreover, in view of the high degree of specificity exhibited by both tests, the procedures complement each other unusually well. In our experience, the practice of conducting parallel FTA and TPI tests on all sera submitted for TPI examination has significantly

improved the confidence in the validity of serologic findings, and on occasion has prevented the reporting of erroneous results. In addition, the FTA appears to provide a highly specific and technically feasible diagnostic procedure for reference laboratories not having facilities for performing the more intricate and costly TPI test.

6. Although preliminary evaluation of the FTA revealed no particular disease syndrome that was consistently associated with false reactions, recent studies have shown that sera with elevated macroglobulin levels often react nonspecifically in the test. The high molecular weight components responsible for these overt reactions could be concentrated by centrifugation at 100,000 rcf and efforts now are being made to elucidate the mechanisms of their reactivity in the FTA. Fortunately, sera of this nature are encountered only occasionally and thus do not appear to constitute a problem that seriously limits the usefulness and practicability of the FTA as a diagnostic tool. Furthermore, the present studies showed that valid FTA results can be obtained even with macroglobulinemia sera simply by centrifuging these specimens for one hour at 100,000 rcf and testing the supernatant and sedimented fractions. This treatment has no detectable effect on syphilitic antibody.

7. A method is outlined describing the modification of the conventional flagellar agglutination test by means of staining with carbolfuchsin reagent permitting its use in the microtiter method.

8. Reagents in the form of mouse ascitic fluid were made to Anopheles A virus. The inciting agents, ascitic fluid yields and the methods of immunization are described. A 50 day immunization schedule initiated with intramuscular and subcutaneous rather than intraperitoneal inoculations provided the higher complement-fixation titers. The last four doses given at four to five day intervals with Freund's complete rather than incomplete adjuvant mixed with heat-killed S. aureus produced ascitic fluid in quantities four times the maximum amount of serum obtained from the same animals, 3.0 ml. vs. 0.5 ml. per mouse. Higher CF titers are obtained when these last four doses are given after, not before, a 30 day interval following the initial SQ and IM injections.

9. An indirect fluoresein-antibody technic was found to be highly specific and sensitive for the serodiagnosis of toxoplasmosis in man. Results with these procedures correlated very closely with those obtained with CF tests.

List of Publications and Presentations:

1. Fife, E. H. Influence of macroglobulins on the specificity of the Fluorescent Treponemal Antibody (FTA) test for syphilis. Presented at the World Forum on Syphilis and other Treponematoses, September, 1962. Published in Proceedings of the World Forum on Syphilis and other Treponematoses. In Press.

2. Fife, E. H. Immunofluorescence as applied to the serodiagnosis of syphilis. Presented before the D. C. Branch, Society for Experimental Biology and Medicine, February, 1963.

ANNUAL PROGRESS REPORT

Project: 3A 0 12501 A 806, Military Preventive Medicine

Task No. 01, Communicable Diseases (Plague)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Hazardous Operations
Department of Bacteriology
Division of Communicable Diseases and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: James H. Rust, Jr., Ph.D.
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No.: 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Plague)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. During the past year the Department of Hazardous Operations has furnished acetone killed and dried (AKD) Pasteurella pestis to the Microbiology Division, U. S. Army Medical Unit for chemical fractionation.

2. Human sera (131) and rodent sera (63) from Viet Nam were screened for antibodies to Pasteurella pestis by the micro-complement fixation and micro-hemagglutination tests.

3. Personnel from this laboratory were invited to participate in the study of endemic plague in New Mexico by the State Director of Public Health. During this four week study a six day course on field and laboratory methods for isolation and identification of Pasteurella pestis was presented to Public Health representatives from throughout the State of New Mexico.

4. A technique of demonstrating and isolating avirulent P. pestis isolated from a New Mexican lagomorph.

5. Eighty large animal predators consisting of coyote, fox, skunk, bobcat, raccoon, and others were examined serologically for evidence of Pasteurella pestis antibodies. These studies were undertaken in an attempt to delineate an area which has recently undergone a plague epizootic.

BODY OF REPORT

Project: 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Plague)

Description: This task involves the study of the antigens and properties of Pasteurella pestis, and the associations of this organism with the rodent reservoir, the insect vector, and the human host to elucidate the environmental, and biological mechanisms of infection and resistance.

Progress:

1. Preparation of Acetone Killed and Dried P. pestis.

During the past year the Department of Hazardous Operations has furnished acetone killed and dried (AKD) Pasteurella pestis to the Microbiology Division, U. S. Army Medical Unit for chemical fractionation.

An average of 50 culture bottles of P. pestis strain EV 76 have been processed each week. A total of 140 grams of AKD cells have been made available.

Four recently isolated strains of P. pestis were received from Viet Nam and will be tested for virulence in mice and guinea pigs. Representative subcultures have been included in the department's stock culture collections.

2. P. pestis Antibody in Human and Rodent Sera from Viet Nam

A number of sera from various areas of Viet Nam were examined by the micro-hemagglutination (HA) and micro-complement fixation (CF) test described in the Annual Report (1962).

Serological data from these sera for antibodies to P. pestis are presented in Table I.

All of these sera were screened by the micro-serological CF and HA test, using chemically purified P. pestis capsular material (FI) as the antigen.

Repeated serological observations on animals infected with, or immunized against, P. pestis suggest that complement fixing antibody appears early, e.g. within four or five weeks and then rapidly declines. On the other hand, hemagglutinating antibody appears somewhat later and remains elevated for some time. Therefore, it is not suprprising that the single human convalescent serum has only a relatively low complement fixing titer and no hemagglutinating titer. With this in mind one could speculate from the rodent serology that the Minh Mang area was recently involved in an epizootic while the Phu Loc (A) area experienced an epizootic probably some months previous. It should be noted that about

TABLE I

VIET NAM (PLAGUE) SERA*

No. of Specimens	Species	Area	HA	CF
105	Human	Saigon	2 pos	Neg
25	Human	Phu Bon	Neg	Neg
1	Convalescent plague	?	Neg	Pos
10	Rodent	Phu Loc	2 pos	Neg
8	Rodent	Minh Mang	Neg	1 pos
21	Rodent	Ly Thai To	Neg	Neg
14	Rodent	Nguyen Thien Thuat	Neg	Neg
10	Rodent	Bui Vien	Neg	Neg

* Sera submitted by Major Eugene Feeley, Medical Laboratory Hq., USASGV, APO 143, San Francisco, California

50 per cent of the 105 Viet Namese soldiers had been vaccinated within the last three years and only two showed evidence of plague antibody.

Dates of sera collection and other pertinent information concerning this material is not available and for this reason no absolute conclusions can be drawn from this data. Pending receipt of additional information further studies on these sera are contemplated.

3. Studies in Plague Endemic Areas of New Mexico.

a. A field and laboratory course was presented to about 25 health officials from the New Mexico Department of Public Health. This course covered methods of trapping animals, handling and removing ectoparasites, autopsy of possibly infected animals and laboratory methods in isolation and identification of P. pestis. Laboratory facilities of the State Health Department at Albuquerque, New Mexico were made available for the laboratory aspects of the course.

b. During the period 23 May - 20 June 1962 personnel from Walter Reed Army Institute of Research, working in conjunction with Mr. Bryan E. Miller, Chief, Vector Control Section, New Mexico Department of Public Heath and Mr. John Doll of the New Mexico State Health Department, conducted a follow-up survey of rodent and lagomorph populations in the Sante Fe, Las Cruces and Roswell areas of New Mexico. These areas were chosen since (1) three human cases of plague occurred in the Sante Fe area in 1961 and a few P. pestis isolates were obtained in this area by the California group; (2) plague had been demonstrated in rodents of the Las Cruces area on several occasions in previous years; (3) a severe rabbit epizootic was experienced in Roswell in 1960 with two human cases, and a number of serologically positive animals were collected in this area last year.

c. A total of 791 animals were trapped using both snap and live traps. Unfortunately, the study was severely hampered by lack of serum specimens due to the late arrival of live traps. However, 58 sera were collected and examined by the micro-hemagglutination and complement fixation tests (Annual Report, 1962). All of these sera were negative by the complement fixation test. On the other hand, one of the 19 sera collected in the Roswell area yielded a relatively high hemagglutination titer. This specimen was obtained from a Dipodomys ordii. This information suggests that the plague situation in the Roswell area is rather static since data from 1961 demonstrated 6½ per cent (3 of 43) of the sera were positive by the HA test, while in 1962, 5.3 per cent (1 of 19) were positive.

4. Technique for Demonstrating Avirulent P. pestis from Field Material.

a. Iron salts appear to significantly enhance the virulence of certain avirulent P. pestis strains (Annual Report 1962), therefore, it was decided to use this technique in screening field material for avirulent strains of this organism.

All animals collected in New Mexico were combed for ectoparasites and autopsied. Ectoparasites were pooled according to area and host species, triturated in saline and inoculated onto bacteriological media and into mice. Similarly, tissue (liver and spleen) pools were prepared, triturated in saline and inoculated in the same fashion. Two mice were inoculated intraperitoneally with each pool. In addition, two mice were inoculated with 40 micrograms of ferrous iron (in the form of ferrous sulfate) simultaneously with the pooled materials. It was anticipated that if virulent P. pestis were encountered, all four mice would succumb to plague infection, while if only avirulent strains were present only the mice receiving ferrous iron would die of plague infection. Of some 300 pools examined, no virulent isolates were obtained and only one avirulent strain was found. This was demonstrated to have originally been present in the tissues of a shot Lepus californicus from the Roswell area, and has subsequently been shown to have an LD_{50} in mice of $8x10^6$ (a toxic dose) while a similar titration in white mice in the presence of ferrous iron yielded an LD_{50} of 25 organisms.

b. All of the surviving inoculated mice were bled at the end of 21 days and screened by the micro-serological tests previously described. Four mice from two tissue pools yielded positive complement fixation tests. These four mice represented two mice from each of two pools which had received ferrous iron. Mice not receiving ferrous iron and inoculated with material from the same pools were serologically negative. Attempts to isolate P. pestis from the tissues of these mice by agar culture or by reinoculating tissues into other mice failed.

These data show conclusive evidence of avirulent P. pestis in animals from the Roswell area, reaffirming that this area is certainly endemic for P. pestis.

5. The Role of Predators in Plague Ecology.

The spread of wild-rodent plague over distances could be effected not only by rabbits, as previously suggested, but by the foraging of larger predator animals. In order to test this hypothesis, 80 sera from fox, coyote, skunk, raccoon, bobcat and other predators were secured from the Southwest Rabies Station, Las Cruces, New Mexico. These sera were examined for CF and HA antibodies by the micro-serological techniques described in the 1962 Annual Report. Eighty sera were collected from all areas of the State of New Mexico. Among these were five (one fox, one coyote, two skunk, one raccoon) animals showing serological reactivity in the HA test. These animals were trapped from two contiguous counties suggesting that an active wile rodent epizootic had occurred in this area.

Further studies are underway to determine if antibodies against P. pestis antigens other than FI are present. The implications in using this field technique in delineating locally confined epizootics is obvious.

Summary and Conclusions:

1. A total of 140 gm. of Acetone killed and dried Pasteurella pestis cells have been made available to the Microbiology Division, U. S. Army Medical Unit for chemical fractionation.

2. A number of rodent and human sera from Viet Nam were serologically examined for antibody to P. pestis. The possible significance of those found positive is discussed.

3. A large number of animals (791) was collected from the State of New Mexico and examined for evidence of P. pestis.

4. Utilization of ferrous iron for obtaining an apparent increase in virulence of avirulent P. pestis strains led to the isolation of a strain which otherwise would have been missed.

5. A number of large predator animal sera were serologically examined for P. pestis antibody in attempts to delineate new enzootic plague areas.

ANNUAL PROGRESS REPORT

Project: 3A 0 12501 A 806, Military Preventive Medicine

Task 01, Communicable Diseases (Bacterial Infections)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Bacteriology
Division of Communicable Diseases and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Sylvia G. Cary, M.S.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No: 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Bacterial Infections)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Author: Sylvia G. Cary, M.S.

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

A large number of strains of Mima polymorpha and Herellea sp. isolated from cases of dermatitis and conjunctivitis in Panama and Bolivia are being serologically typed as a part of studies in progress on their classification. Attempts will be made to determine the etiologic role, if any, of these organisms in disease processes and their therapeutic management.

BODY OF REPORT

Project No.: 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Bacterial Infections)

Description: The objectives of this task are to study various bacterial infections and particularly the interplay of microorganisms on each other and the effect these associations may have in complicating the diagnosis of disease and its therapeutic management.

Progress: Serologic typing of a large number of strains of Mima polymorpha and Herellea sp. isolated from cases of dermatitis among U. S. Army personnel in Panama is being conducted at the present time. These strains were collected by Dr. David Taplin, University of Miami Medical School. A second group from Project Pinto, Le Paz, Bolivia has just been submitted by Capt. D. J. Demis, MC, Walter Reed General Hospital, Washington, D. C. All of the latter strains were isolated from children with conjunctivitis. Many of the Bolivian cultures were mixed with Pseudomonas aeruginosa. As a member of the ad hoc Committee under the International Committee on Bacterial Nomenclature, all of the serologically identified strains will be added to the current collection of Bacterium anitratum, Moraxellae, Mimeae under study.

Summary and Conclusions: A large number of strains of Mima polymorpha and Herellea sp. isolated from cases of dermatitis and conjunctivitis in Panama and Bolivia are being serologically typed as a part of the studies in progress on their classification. Attempts will be made to determine the etiologic role, if any, of these organisms in disease processes and their therapeutic management.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 806, Military Preventive Medicine

Task 01, Communicable Diseases (Bacterial infections)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Experimental Pathology
Division of Special Activities

Department of Applied Immunology
Division of Communicable Disease
and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: J. R. Dupont, Capt., MC
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Note: In association with Professor K. Kobari, Tokyo Municipal Komagome Hospital, Tokyo, Japan--Contract #DA-92-557-FEC-34616, Army Medical Research and Development Command, and Dr. N. K. Dutta, Haffkine Institute, Bombay, India.

ABSTRACT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Bacterial infections)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Shigellosis has been studied in humans. Biopsy material was obtained from Japan and in experimental animals. Particular emphasis has been placed on the study of neuropathological changes in Shigella flexneri infection and in a sequential study of the intestinal pathology in acute Shigella in Rhesus monkeys. Routine as well as special enzyme histochemical procedures are being emphasized.

The investigation of the pathology and pathogenesis of cholera is being continued with emphasis on the suckling rabbit and the guinea pig as the experimental model.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (Bacterial infections)

Description:

A sequential biopsy study of patients with Shigellosis was undertaken. A second study of Shigellosis is underway in the Rhesus monkey. Two aspects of the disease are pursued: a) the study of the neuropathology of Shigella flexneri infection and, b) the study of the evolution of the histological lesions in the bowel during the course of the infection. Routine and histochemical tissue techniques are being employed.

The investigation of the morphologic alteration of the suckling rabbit intestine following challenge with cholera vibrios and cell-free cholera filtrates and sonicates is being studied.

Progress:

The sequential biopsy study in humans has been completed and the material presented at national meetings. The work on Shigellosis in the Rhesus monkey is progressing rapidly and will be presented in part by Formal this fall at an international meeting in Yugoslavia. The neuropathological studies in Shigella flexneri infection are nearing completion.

The initial phase of the study of cholera infection in the suckling rabbit has been completed and the material has been presented at the Federation Meetings. The study is now being extended to the pathogenesis of the diarrhea produced by moieties of the vibrio.

Summary and Conclusions:

Cooperative projects in Shigellosis and cholera are being conducted and have stimulated further in-house research of these two diseases.

List of Publications:

1. T. Magnani, K. Kobari and H. Sprinz. The Morphologic Response of the Human Colonic Mucosa to Shigella Infections. Fed. Proc. 22-512, 1963. Presented at the Meetings of the Federation of American Societies for Experimental Biology, April 1963 and the Meeting of the American Federation for Clinical Research, District of Columbia Club, December 1962.

2. H. T. Norris, N. K. Dutta, R. A. Finkelstein, S. B. Formal and H. Sprinz. Morphologic Alterations of the Intestine of Ten Day Old Rabbits Given Intact and Ultrasonically Disrupted Cholera Vibrios or Cholera Endotoxin. Fed. Proc. 22:512, 1963. Presented at the Meetins of the Federation of American Societies for Experimental Biology, April 1963.

ANNUAL PROGRESS REPORT

Project: 3A 0 12501 A 806, Military Preventive Medicine

Task 01: Communicable Diseases (New Drugs and Antibiotics in the Treatment and Control of Communicable Diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Bacteriology
Department of Medical Zoology
Division of Communicable Diseases and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Ernest Bueding, MD*
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project: 3A 0 12501 A 806 Title: Military Preventive Medicine

Task 01 Title: Communicable Diseases (New Drugs and Antibiotics in the Treatment and Control of Communicable Diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Bacteriology
Department of Medical Zoology
Division of Communicable Diseases and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: John I. Bruce, BS
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. Studies on the use of sodium antimony dimercapto succinate (TWSb) as a prophylactic and suppressibe drug in Schistosoma mansoni infections has been continued. Temporary suppression of eggs was obtained even with a single dose of the drug. Three injections of the drug given three weeks apart or five injections at two-week intervals, practically eliminated eggs from the monkeys' stools and greatly reduced the number of worms recovered at necropsy. The prophylactic activity reported previously was markedly reduced when the monkeys were treated on alternate days. Studies involving the use of an acid preparation of TWSb suspended in oil as a repository preparation has been started. A new non-antimonial compound, S-201, is also under investigation.

2. Preliminary studies suggest that the mode of action of TWSb in schistosomiasis is through the inhibition of the phosphofructokinase of the parasite. These studies are being repeated on a larger scale.

3. Studies were continued on the use of antibiotics to eliminate pathogenic staphylococci in the external nares of monkeys to create a bacterio-

logical void after which selected phage types of Staphylococcus aureus were seeded onto the area. Animals were sampled periodically for several months to measure feasibility of displacing normal staphylococcal flora and to determine the duration of successful transplants. All seedings were successful for periods ranging from 15 to 36 days, after which nasal flora either reverted or was colonized by a "wild" strain of staphylococci. Two of six monkeys seeded with S. aureus expired in 18 and 36 days after seeding. At death the animals had high nasal counts of the seeded strains. This study was terminated because of essentially negative results.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 01 Title: Communicable Diseases (New Drugs and Antibiotics in the Treatment and Control of Communicable Diseases)

Description: The object of this task is the evaluation of potential therapeutic agents for the prevention, suppression and treatment of communicable diseases. Furthermore, whenever possible, studies are carried out to determine the mode of action of some of these drugs.

Progress:

1. The prophylactic and suppressive activity of drugs against S. mansoni.

Investigations on the prophylactic and suppressive activity of sodium antimony dimercapto succinate (TWSb) in S. mansoni infections has been continued and extended to include a repository form of this drug. Furthermore, a non-antimonial compound (S-201) which has shown promise in preliminary trials is being investigated.

a. A total of 20 monkeys was exposed to 400 S. mansoni cercariae and divided into seven groups. Those of the first group received TWSb every other day starting two days before exposure. Those of the second group received a single dose of the drug on the 56th day after exposure. Those of the third group received the drug three times once every two weeks starting on the 56th day after exposure. Those of the fourth group received the drug three times, once every three weeks, starting on the 56th day after exposure. Those of the fifth group received the drug five times once every two weeks, starting on the 56th day after exposure. Those of the sixth group received no treatment, but were exposed to the same number of cercariae and served as infection control animals. Those of the seventh group received the drug for five times every other day but were not exposed to S. mansoni cercariae and served as uninfected drug controls.

b. The results of the effect of treatment on the number of living schistosomes found at necropsy have been summarized in table 1. Ninety days after exposure a significant reduction in the worm burden was observed in the animals which received the drug prophylactically (Group I). Eggs in the stools of these animals appeared two weeks later in the untreated controls

Table 1

The effect of TWSb on the numbers of living Schistosoma mansoni found in monkeys after treatment with TWSb.

Group	Monkey number	Rx started (day)	Number of doses	Intervals between treatment (days)	Results of necropsy	
					Number of live worms	Percent recovery of live worms
I	1	-2	5	1	58	14
	2	-2	5	1	63	16
	3	-2	5	1	31	8
II	4	+56	1	-	94	23
	5	+56	1	-	194	48
III	6	+56	3	14	94	23
	7	+56	3	14	59	15
IV	8	+56	3	21	11	3
	9	+56	3	21	16	4
	10	+56	3	21	31	8
V	11	+56	5	14	29	7
	12	+56	5	14	47	12
VI*	13	-	-	-	150	37
	14	-	-	-	117	29
	15	-	-	-	105	26
	16	-	-	-	91	23
	17	-	-	-	90	22
	18	-	-	-	87	22
VII**	19	-	5	1	-	-
	20	-	5	1	-	-

*Untreated controls

**Drug controls

and their number remained lower throughout the experiment. The monkeys which were treated with a single suppressive dose (Group II) showed a marked drop in the number of eggs for a period of two weeks and were negative three weeks after treatment. However, the eggs reappeared in the stools in relatively large numbers the following week (four weeks after treatment). The number of worms recovered from these animals at necropsy was not significantly different from that of the untreated controls. In the monkeys treated with three doses two weeks apart (Group III) there was a partial suppression of eggs but the egg count increased three weeks after the last dose and reached a level similar to that of the untreated controls. The worm burden was not significantly reduced by this therapeutic regimen. More encouraging results were obtained in the animals which received three doses at three-week intervals (Group IV). In this group the passage of eggs was almost completely suppressed and the worm burden was significantly reduced. Likewise, excellent results were observed in those monkeys which received five doses at two-week intervals (Group V). Although the egg suppression was apparently complete in the animals of this group, a few worms were found at necropsy. Egg viability studies conducted each week throughout the experiment showed that miracidia could be recovered from all of the specimens containing eggs.

c. The results of field trials conducted by other investigators with the water soluble form of sodium antimony dimercapto succinate (TWSb) indicated that untoward reactions were observed previously when the drug was administered on consecutive days. Spacing the doses at intervals of several days or weeks reduced the toxicity of the drug but its therapeutic effectiveness was also markedly decreased. These observations have resulted in the preparation of a water and fat insoluble TWSb acid which is slowly absorbed when suspended in oil. Preliminary results involving schistosomiasis of the mouse have shown that the oil suspension can extend the prophylactic effect of the drug from three to thirty hours prior to exposure to infection. The curative activity of the repository form of TWSb proved to be five to ten times greater than the water soluble form. Experiments have now been set up to study the prophylactic and curative activity of the oil suspensions in primates. This experiment is still in progress.

d. Since most of the effective schistosomicidal compounds in use today are antimonials, they are potentially dangerous. A new compound, S-201 (Farbwerke Hoechst AG), has shown encouraging results in preliminary investigations with schistosomiasis in mice, hamsters, and monkeys. This drug is well tolerated and is the first injectionable non-antimony compound. Experiments have been set up to test the curative activity of this compound in primates experimentally infected with S. mansoni. This experiment is still in progress.

2. Effect of TWSb on the concentrations of Hexose phosphate esters in Schistosoma mansoni. Previous investigations have shown that stibophen and potassium antimony tartrate inhibit the activity of schistosome phosphofructokinase in vitro and in vivo. If TWSb had a similar mode of action, this should be reflected in an increase in the subtrate (Hexose monophosphate) and a decrease in the product (hexose diphosphate) of the phosphofructokinase reaction.

a. The concentrations of these phosphate esters was determined in worms from a monkey which had received a subcurative dose of TWSb and the levels of these intermediates were compared with those of control worms from an untreated monkey. It was found that in the worms from the treated animal, the concentration of hexose monophosphate was higher and the concentration of hexose diphosphate was lower than in the worms from the control animal.

3. Suppression of the staphylococcal carier state by topical antibiotics. An attempt was made to microbiologically alter the nasal flora of monkeys by the temporary suppression of the staphylococcal carrier state with topical antibiotics followed by planting onto the nasal mucosa selected strains of staphylococci from stock cultures.

a. The monkey was the experimental subject. Baseline flora were determined for eight weeks after which each animal received three intranasal instillations in 24 hours of a salve containing 5.0 mg of neomycin and 250 units of Bacitracin per instillation. One hour after the last instillation each animal was seeded with strains of S. aureus in trypticase soy broth as indicated in table 2. Controls were "seeded" with sterile trypticase soy broth. All monkeys were sampled periodically for an additional 18 weeks unless death intervened.

b. It was found that original staphylococcal flora could be displaced for brief periods but in no instance for more than 36 days. Both of the monkeys which expired had extremely high counts of the seeded strains of staphylococci in their noses just before and at death; a finding which suggests that this procedure might be hazardous. Autopsy of one of these failed to show any gross abnormalities or sites of infection. The other expired animal had been dead for more than 48 hours and was not autopsied.

Table 2

Results of Nasal Instillation of Staphylococci into Monkeys

Monkey	Phage Type Original Staphylococcal Strain(s)	Seeded with Phage Type	Successful Seeding	Duration of seeded Organism	Comment
1	N.T.*	3C	Yes	36 days	Died in Cage on 36th day
2	N.T.	3C	Yes	20-27 days	Reverted to N.T.
5	N.T.	42E	Yes	20-27 days	Reverted to N.T.
8	N.T.	42E	Yes	8-15 days	Reverted to N.T.
9	N.T. and 42E/47/54	80/81	Yes	15-20 days	Reverted to N.T.
10	N.T.	80/81	Yes	18 days	Died in Cage on 18th day
3	N.T.	Not seeded	N.A.	N.A.	Remained N.T.
4	N.T.	Not seeded	N.A.	N.A.	Remained N.T.
6	47/54	Not seeded	N.A.	N.A.	Retained 47/54 Type

* Non-typable

Summary and conclusion:

1. Intramuscular injections of sodium antimony dimercapto succinate (TWSb) into Macaca mulatta monkeys, experimentally infected with Schistosoma mansoni resulted in suppressing the passage of eggs in their feces. Encouraging results were obtained when three injections of the drug were given three weeks apart or five injections at two-week intervals. This regimen practically eliminated eggs from the monkeys' stools and greatly reduced the number of worms recovered at necropsy. Less encouraging results were obtained with a single dose of the drug or by three doses given two weeks apart.

2. This first exploratory experiment suggests that the mode of action of TWSb is similar to that of the older antimonials, i.e., inhibition of the phosphorfructokinase of the parasite. However, since this was done only on a single test animal and a single control, more experiments along these lines must be carried out in order to confirm this tentative conclusion. Toward that end a second experiment involving five monkeys exposed to 1,000 cercariae each was begun. The experiment is in progress.

3. This study indicated that a single displacement of the nasal staphylococcal flora was not a successful technique in solving the problem of nasal carriers of S. aureus. The death of two of the six monkeys receiving stock strains of S. aureus indicated that this approach is potentially hazardous.

Publications:

1. Bruce, J. I. and Sadun, E. H. 1963. The suppressive activity of sodium antimony dimercapto succinate (TWSb) in experimental infections with Schistosoma mansoni. Am. J. of Trop. Med. and Hyg. 12: 184-187.

2. Noyes, H. E., Evans, J. R., and Serritella, A. A. 1963. A laboratory evaluation of three new penicillins against Staphylococcus aureus. Antimicrob. Agents and Chemotherapeutics. In press.

3. Rosanelli, J. D. and Price, D. L. 1963. Sodium antimony dimercapto succinate (TWSb) in the treatment of human Schistosoma mansoni infections. Am. J. of Trop. Med. and Hyg. In press.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 806, MILITARY PREVENTIVE MEDICINE

Task No. 01, Communicable Diseases (Zoonoses of military importance)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Veterinary Microbiology
Department of Veterinary Pathology
Division of Veterinary Medicine

Period Covered by Report: 1 July 1962 to 30 June 1963

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Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

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ABSTRACT

Project No. 3A 0 12501 A 806 Title: MILITARY PREVENTIVE MEDICINE

Task No. 01 Title: Communicable Diseases (Zoonoses of military importance)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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1. Serological and cultural studies on selected zoonoses were continued on wildlife mammals trapped in Maryland and Virginia. The widespread occurrence of leptospirosis in foxes, skunks, opossums, and raccoons was demonstrated. Rocky Mountain spotted fever antibodies were found in a high percentage of skunks, raccoons and woodchucks, but in few opossums. Influenza antibodies were not disclosed in animals. Relatively few viruses have been isolated from brain, respiratory and intestinal tissues of wildlife. An apparently new pox virus was isolated from the respiratory tract of a raccoon. Homologous antibodies were found in 25 per cent of raccoons.

2. Epidemiological investigations of an outbreak of leptospirosis in troops in the Canal Zone were continued. Seven different serotypes, six of which are new serological entities, were shown to be present in the focus of infection. Serological studies uncovered a large number of inapparent infections in soldiers of two companies in which the outbreak occurred.

The survival of leptospiras in tissue cultures prepared with leptospira-infected kidneys was studied to evaluate the potential infection hazard of tissue culture vaccines. Leptospiras were viable in tissue cultures containing antibiotics immediately after culture processing, but not after 24 hours incubation.

3. Six serologically distinct groups of viruses were found amongst 45 enteroviruses isolated from swine. Relationships to human enteroviruses are being studied.

An attenuated Western Equine Encephalitis strain is currently being evaluated to determine if it can be used for preparation of vaccine.

A new virus was isolated from the tissues of adult rats with CNS disease signs.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: MILITARY PREVENTIVE MEDICINE

Task No. 01 Title: Communicable Diseases (Zoonoses of military importance)

Description:

The major objective is to study those microbial diseases of animals transmissible to man that have potential military significance. Investigations encompass basic studies on the biological and microbiological characteristics of infectious agents, their pathogenesis in various hosts, their distribution in nature, modes and factors in transmission and methods of treatment and control. Specific attention during the past year was given to: a study on the presence of specific zoonoses in mammalian wildlife; study of the potential infection hazard for leptospirosis in vaccines prepared from mammalian kidney tissue cultures; epidemiological studies of an outbreak of leptospirosis in Canal Zone troops; studies on relationship of enterovirus isolated from swine with human viruses; evaluation of an attenuated Western Equine Encephalitis vaccine; studies on a virus causing a CNS disease in laboratory rats.

Progress:

1. Zoonoses in Wildlife:

a. Leptospirosis: Part of the cultural and serological findings were reported in the Annual Progress Report, WRAIR, 1 July 1961 through 30 June 1962. Summaries of data obtained to date are shown in Tables 1 and 2. Included in the tables are findings obtained from 30 foxes trapped in the Southwest section of Virginia. As noted in the previous progress report, a high prevalence of infection in skunks and gray foxes was disclosed. It was apparent from serological findings that opossums and raccoons may also serve as principal reservoirs of infection. The high prevalence of antibodies in raccoons, in contrast to the rare isolate recovered from this species, is noteworthy. A possible explanation is the presence in raccoons of strains that cannot be readily cultivated. Serological findings also provided presumptive evidence of infection with types not recovered in cultures. Four of 26 skunks and 2 of 9 foxes that were leptospiral carriers were serologically negative. Similar observations have been noted by others. In four instances, skunks had agglutinins for serotype grippotyphosa, although icterohaemorrhagiae was isolated from kidneys.

Table 1

Isolation of Leptospiras from Wildlife

Host	No. Examined	Total Positive No.	%	Distribution by Serotypes
Skunk	104	26	25	icterohaemorrhagiae-12, grippotyphosa-4, ballum-1, not typed-9
Opossum	64	3	5	icterohaemorrhagiae-1, ballum-1, not typed-1
Raccoon	97	1	1	icterohaemorrhagiae-1
Woodchuck	10	0	0	
Feral cat	5	0	0	
Gray fox	16	5	31	pomona-5
Red fox	14	4	29	pomona-4
Muskrat	1	0	0	

Table 2

Prevalence of Leptospiral Agglutinins in Wildlife

Species	No. Tested	Positive No.	%	Distribution of Predominant Reactions
Skunk	101	63	62	grippotyphosa-22, icterohaemorrhagiae-10, autumnalis-7, ballum-2, djasiman-2, javanica-1, andamana-2, multiple-17
Opossum	60	9	15	grippotyphosa-3, autumnalis-2, ballum-1, icterohaemorrhagiae-1, andamana-1, multiple-1
Raccoon	94	47	50	autumnalis-24, icterohaemorrhagiae-10, pomona-2, grippotyphosa-2, canicola-2, borincana-1, javanica-1, australis-1, multiple-4
Woodchuck	10	0	0	
Cat	5	0	0	
Muskrat	1	0	0	
Gray fox	16	11	69	andamana-3, autumnalis-4, pomona-1, wolffi-1
Red fox	14	10	71	andamana-4, autumnalis-5, pomona-1

b. Rickettsiosis: Sera from 260 of 271 animals trapped at Aberdeen, Maryland, were examined for the presence of complement-fixing antibodies for the Rocky Mountain spotted fever (RMSF) group of rickettsia. These tests were conducted in cooperation with the Department of Rickettsial Diseases, Division of Communicable Diseases, WRAIR. Part of these findings were presented in the WRAIR Annual Progress Report, 1 July 1961 through 30 June 1962. A summary of completed test results is shown in Table 3.

Approximately 15% of the skunks, 18% of the raccoons, and 40% of the woodchucks had significant antibody titers for RMSF group. Prior to this study, the high prevalence of antibodies for RMSF in raccoons, skunks, and woodchucks had not been demonstrated. These findings extend the information on significant wildlife reservoirs for RMSF group of rickettsia. Attempts will be made to isolate rickettsia from tissue samples collected from the wildlife.

Table 3

Presence of CF Antibodies for RMSF Group of Rickettsia in Sera of Wild Animals Trapped at Aberdeen, Maryland

Host	No. Examined	Positive No.	Positive %	Dist. of Positive Reactions by CF Titer 1:5	1:10-1:20	1:40-1:80	1:160
Skunk	102	15	15	6	8	1	0
Raccoon	94	17	18	6	3	6	2
Opossum	54	1	2	0	0	1	0
Woodchuck	10	4	40	3	0	1	0

c. Examination for Influenza Antibodies: A total of 286 sera from 100 skunks, 93 raccoons, 56 opossums, 30 foxes and 7 woodchucks were examined for the presence of hemagglutination-inhibition (HAI) antibodies against influenza virus types A and B, employing standard techniques. No HAI antibodies were detected in any of the sera. Within limitation of test procedures, no evidence of occurrence of human influenza viruses in various wildlife mammals was observed. The sera will be tested for parainfluenza antibodies.

d. Screening of Wildlife for Viruses: Sections of brain, liver, spleen, intestine, lung, and trachea were collected from 104 skunks, 91 raccoons, 64 opossums, 30 foxes, 10 woodchucks, and 1 muskrat for virological study. Initially, upper respiratory tract tissues from each of 25 raccoons, 3 skunks, 15 opossums, and 5 woodchucks were cultured for viruses and an isolate was obtained from 1 raccoon. Subsequently, to expedite the screening of the large number

of specimens, various tissues from 3 to 5 animals were pooled and tested. To date, brain, intestine, and respiratory tract tissues from approximately 100 skunks, 50 raccoons, 55 opossums, 10 woodchucks, 30 foxes and 1 muskrat have been cultured on one or more tissue culture cell lines. At least three passages were made on the respective cell lines, including a screening on agar overlay tissue culture medium (Delbecco plaque technique) before a culture was considered to be negative. A virus was isolated from the pool of respiratory tract tissues from 5 raccoons and is presumably the same virus isolated in initial studies, since this pool contained the tissue from which a virus was originally obtained. Unidentified non-bacterial agents (possibly virus) have been isolated from nine separate pools of intestinal tissues from skunks. The remaining samples tested were negative. Further attempts to isolate viruses are continuing. At least five different culture cell lines will be utilized in addition to embryonated eggs and weanling or suckling mice. The relative paucity of virus isolates from samples processed to date is noteworthy in view of the rich viral flora disclosed in monkeys, man, pigs, cows, and other animals. The findings in wildlife species studies parallel those reported for dogs--a species that apparently has a limited viral flora.

e. Characteristics of a Raccoon Virus: The raccoon virus was initially isolated on monkey kidney tissue culture after ten days incubation at 37°C, at which time a characteristic cytopathogenic effect (CPE) was noted. Subsequent passages in monkey kidney tissue culture and other cell lines resulted in an increase in the virus titer, and a more rapid development of CPE, viz., in 2 to 3 days. Inoculation of chorioallantoic membrane (CAM) with the agent produced minute, discreetly embedded lesions resembling those produced by Herpes simplex or Herpes simiae (Monkey B).

Since the agent was first isolated in monkey kidney tissue culture, its possible identity with the Monkey B virus was considered. This was ruled out by failure of agent to cross react with Herpes simplex antiserum in complement-fixation tests. A 20% suspension of CAM from infected egg embryos hemagglutinated susceptible chicken erythrocytes in dilution levels up to 1:32 to 1:64. Hemagglutination was inhibited specifically by anti-vaccinia rabbit serum. The heterologous hemagglutination-inhibition titer (HAI) was 1:320, the homologous HAI titer of the vaccinia antiserum was 1:640. On the basis of these findings, the isolate was identified to be a member of pox group of viruses. Further evidence that the virus originated from raccoons was obtained by the demonstration of a high HAI titer (1:2560) in the animal from which this isolate was obtained. Significant HAI antibody titers were disclosed in 23 of 92 raccoon sera. A summary of the HAI tests in raccoons employing the isolate, vaccinia, and monkeypox as test antigens is shown in Table 4.

Table 4

Presence of Hemagglutination-Inhibition Antibodies for Pox Viruses in Serums from 92 Raccoons

Antigen	No. Positive	Distribution of Positive Reactions by Titer 1:80	1:160	1:320	1:640	1:1280	1:2560
Raccoonpox	21	5	8	3	4	0	1
Vaccinia	3	0	0	1	1	1	0
Monkeypox	0	-	-	-	-	-	-

Serological findings in raccoons provided evidence of widespread infections in this host. It also provided ancillary evidence that the raccoon isolate was not a vaccinia or monkeypox virus. The raccoon virus could also be differentiated from variola virus in that it produced consistent vesicular skin lesions in rabbits after successive passages and caused death in mice that were inoculated either intraperitoneally or intracranially. Relationships to other pox viruses are now being studied.

2. Leptospirosis:

a. Study of an outbreak in troops at Ft. Kobbe, Canal Zone: Some of the findings of an epidemiological investigation of an outbreak of leptospirosis that occurred in troops at Ft. Kobbe, Canal Zone in November 1961 were reported in the previous annual report. During the course of the investigation, leptospiras were isolated from two of eight patients, from a sample of river water in the locus of infection, and from the kidney and/or urine of 15 wildlife. With few exceptions, two or more isolates were obtained from each source. It was apparent from preliminary cross-agglutination tests that seven different serotypes were present. Representative strains were selected for definitive identification studies employing agglutinin-adsorption technics. On the basis of test results, six new serotypes were disclosed. Five were related to members of the cynopterus, hyos, bataviae, icterohaemorrhagiae, and hebdomadis groups respectively; the sixth gave low titer cross-reactions with some members of the australis group, but with no other serotypes. The seventh representative strain was identified to be a member of the pomona serogroup. The identification of isolates from the various hosts is shown in Table 5.

Dual infections with members of the hyos and pomona groups were bacteriologically verified in a spiny rat (Proechimys sp) and also in a Licmys sp. Changes in cross-agglutination reactions of one isolate provided evidence of a mixed infection in a third animal (Proechimys sp). The marked diversity of types at a small focus of infection provided unequivocable evidence that Panama is an area of multiple leptospirosis.

Table 5

Source & Identification of Serotypes Isolated in Panama

Representative Type	Distribution by Source: Man	Proechimys sp.	Liomys sp.	Philander sp.	Dedelphys sp.	Stream H_2O	Total
cynopterus (strain 188)		2	1	1			4
hebdomadis (strain 285)		5				1	6
hyos (strain Bravo)	1	1*	1*				3
icterohaemorrhagiae (strain 390)	1	1**					2
bataviae (strain 320)		1					1
unclassified new (strain 214)					1		1
pomona (strain 299)		2*	1*				3

* Two different serotypes isolated from each of species as shown.
** Isolate originally had serological affinities with serotype pomona.

Paired sera obtained from approximately 200 soldiers in two companies in which the outbreak of leptospirosis had occurred were tested for the presence of leptospiral agglutinins. In addition to eight recognized cases, significant antibody titers were found in nine soldiers. In retrospect, evidence of 16 human infections was obtained, approximately half were inapparent infections.

b. Viability of leptospiras in kidney tissue cultures: Kidneys from hamsters and a dog which were infected experimentally with leptospiral serotype canicola were processed for tissue culture. Tissues from infected animals were taken just prior to expected time of death, at which time they contained profuse numbers of leptospiras. Tissues were minced, washed, trypsinized and suspended in a maintenance medium according to conventional methods. Each ml. of growth medium contained 100 units penicillin, 100 micrograms dihydrostreptomycin and

0.25 micrograms fungizone. Tissue culture suspensions were placed in culture tubes and prescription bottles and incubated at 37°C. in a stationary position. Examinations for the presence of leptospiras were made on samples obtained immediately after cultures were prepared and after cultures were incubated 1, 2, 3, and 6 days. For each examination, two to three tubes were pooled, examined microscopically, and cultured on leptospiral medium. Samples taken on 3rd and 6th days of incubations were inoculated also into hamsters.

Four different pools of infected hamster kidneys and one infected dog kidney were processed and examined. Viable leptospiras could be seen in tissue cultures immediately after their preparation. On microscopic examination, immobile leptospiras could be seen after 24 hours incubation, but not thereafter. No leptospiras were recovered from tissue culture samples taken 24 hours after their preparation. Loss of viability of leptospiras was probably due to action of antibiotics present in tissue culture suspensions.

3. Virological Studies:

a. Enteroviruses of swine: The isolation of 45 viral agents from the feces of pigs before and after these animals were exposed to lethal or sublethal doses of radiation were reported previously. During the past year, pure cultures of porcine virus were obtained in tissue culture, by the agar overlay (plaque) technique. Hyperimmune sera to 42 of these 45 viral agents were prepared in rabbits for use in neutralization studies. To date, serological typing has revealed at least six distinct antigenic types with several subtypes in each group. Four distinct plaque types were ascertained by the plaque technique. Correlation between plaque types and serological properties has not been completely determined. Comparative morphological and serological studies on viruses obtained from pre- and post-irradiated pigs are underway to determine if differences in plaque or serological characteristics could be attributed to radiation effects. In many of these tandem samples taken from pigs before and after irradiation, no difference in serological and morphological types could be determined. However, from three pigs, the agents isolated from pre- and post-irradiation samples fell into different serological groups. Further elucidation of this finding is now in progress. The presence of at least three serological types has been ascertained from the same sample of two pigs. Attempts are being made to separate viruses in mixed cultures by utilization of plaque tissue culture techniques. Comparative studies of the biochemical, serological, and morphological characteristics of isolated viruses and their relationships to other known ECPO strains are in progress. Termination of this project has been delayed due to difficulties in separating the mixed cultures.

b. Evaluation of an attenuated strain of WEE: An attenuated strain of Western Equine Encephalitis virus (WEE) B628 TC 00218 cl 15 P1-1 CE was obtained from Dr. Harold A. Johnson, Rockefeller Institute, Berkeley, California. This strain was found to be virulent for guinea pigs, weanling mice and rabbits; however, it had a low virulence for suckling mice, 1 day old chicks, and chick embryos characterized by a prolonged survival time. Observations on the virulence of this virus for larger animals (e.g. burros) are now being made. Its potential usefulness for the preparation of an attenuated vaccine is being evaluated.

c. Characteristics of a virus isolated from rats: In the autumn of 1961, three adult white rats of the Sprague-Dawley strain were presented with CNS signs. These signs included circling, incoordination, tremors, and a torticollis. However, the animals were not visibly disturbed by sudden noises or when twisted by the tail. No gross lesions were observed upon necropsy. Bacteriological examinations of the middle ear canal, spinal fluid, and brain tissue were negative. In addition, no significant findings were obtained from parasitological and hematological examinations.

At necropsy, specimens of brain, lung, liver, and intestine were collected for virological examination. Suspensions of these specimens were prepared in phenol red base broth containing 0.5% lactalbumin hydrolysate, and 1000 units of penicillin and 1000 micrograms streptomycin per milliliter. The suspensions were centrifuged and the supernatant fluid was collected and stored at -70°C. Stored suspensions were found to be free of bacterial, leptospiral and pleuropneumonia-like organisms in cultural examinations.

An agent was readily isolated from all tissues in suckling mice and adult hamsters. However, in suckling rats, the agent was successfully isolated from the intestinal material only. No isolations were made in adult rats, weanling mice, suckling guinea pigs, or in the rabbit. Pools of virus material were made from brain suspensions of suckling mice and suckling rats and stored at -70°C. for further study.

The agent (designated MHG) was found to be infective for 1-2 day old suckling mice when administered either intracranially (IC), intraperitoneally (IP), by intranasal instillation, or per os; suckling rats could be infected by IC route of inoculation only. The agent initially produced signs of disease in hamsters, but in later passages, no signs of disease were seen, nor could the agent be recovered from inoculated animals. Weanling and adult mice and rats were refractory to infection.

The characteristic signs of disease in rats and mice were paralysis of one or more limbs, disorientation, ruffled fur, arching of back and emaciation. Depending on the exposure dose, the incubation period varied from seven to nine days. The mortality rate in animals

given undiluted virus suspensions was usually 100%. The infectivity titer of tissue suspensions ranged from 10^3 to 10^4. Permanent paralysis was common in animals which survived infection. Paralyzed mice remained sensitive to external stimuli--an indication that sensory nerves were not affected.

On necropsy, hydrocephalus and edema of the brain were frequently seen. Lesions similar to those found in poliomyelitis, e.g., destruction of neurons in the gray matter of the cord or brain stem, were revealed by microscopic examination of tissue sections. Necrosis of neurons, neurophasia and gliosis were the most constant and striking findings. Perivascular cuffing and meningeal infiltration by cells, although present, were rather minimal. No inclusion bodies could be demonstrated.

The agent could pass filters with average pore diameters of 100-300 millimicrons. Neither treatment with ether or sodium desoxycholate affected the viability of the agent.

The agent has been serially passed five times in suckling mice and rats. An increase in virulence has been noted with successive passages. The LD_{50} in mice inoculated IC with ten-fold dilutions of brain suspension was found to be $10^{-4.9}$ per 0.01 ml. The agent can multiply in tissue cultures of human embryonic diploid brain cells. It could not be established in tissue cultures prepared from kidneys of mice, cows, rabbits, rhesus monkeys, rats and hamsters, horses, nor from mouse embryo, Hela and AV-3 cell lines. Neither lesions nor death was produced in chick embryos inoculated with the isolate; however, fluids from embryonated eggs were infective.

The virus did not have hemagglutinating properties when tested with human O, guinea pig, sheep, goose, rat, and rooster erythrocytes. It could not be neutralized with antisera of the following agents: Monkey B, Venezuelan Equine Encephalomyelitis, Western Equine Encephalitis, Coxsackie A_1, A_2 and A_3 pools, 747 hepato-encephalitis virus, Nelson Agent, and reovirus (Echo 10).

Attempts to produce MHG hyperimmune serum in rabbits and guinea pigs were unsuccessful. However, by repeated IP challenge, hyperimmune sera were produced in suckling rats and suckling mice which had survived an initial IC challenge with the virus. The immune sera for rats had neutralization antibody (NA) titers of 1:32; significant NA titers were not detected in mice. Complement fixing antibody titers of 1:32 to 1:64 were seen in both rat and mouse anti-MHG sera. Results of a serological comparison between Theiler's GDVII virus and MHG are shown in Table 6. MHG was serologically distinct from Theiler's GDVII virus.

Table 6

Serological Comparison between Theiler's GDVII and MHG Virus by the Complement-Fixation Test

Antigen	Hyperimmune Serum CF Titer			Normal Rat	Normal Mouse	AC Control Saline
	Mouse Anti-GDVII	Rat Anti-MHG	Mouse Anti-MHG			
GDVII(SMB)*	64	4	0	0	0	0
MHG(SRB)*	ND	32	32	4	0	0
MHG(SMB)	8	64	64	16	0	0
Normal SMB	0	0	0	0	0	0

* Code: SMB = suspension of mouse brain; SRB = suspension of rat brain

Summary and Conclusions:

1. Serological and cultural studies on selected zoonoses were continued on 396 wildlife mammals (104 skunks, 64 opossums, 97 raccoons, 10 woodchucks, and 5 feral cats) trapped at Aberdeen Proving Ground, Maryland and 30 foxes trapped in Virginia. A high prevalence of leptospiral infections was disclosed in skunks, opossums and raccoons. Leptospiras were isolated from approximately 25%of the skunks, 5% of the opossums, and 30% of the foxes. Although only one isolate was obtained from 97 raccoons, significant leptospiral antibody titers were found in approximately 50% of the animals. It was evident from cultural and serological findings that multiple leptospiral types occur in Maryland, and that the fox, skunk, opossum and raccoon species are important reservoirs of infection. Complement-fixing antibodies for Rocky Mountain spotted fever group of rickettsia were found in sera of 15% of 102 skunks, 18% of 94 raccoons, 2% of 54 opossums and 40% of 10 woodchucks. Prior to this study, the extensive occurrence of such antibodies in raccoons, skunks, and woodchucks had not been known. None of the wildlife species had hemagglutination-inhibition antibodies against influenza viruses. To date, attempts have been made to isolate viruses from brain, respiratory and intestinal tissues from approximately 100 skunks, 50 raccoons, 55 opossums, 10 woodchucks, 30 foxes and 1 muskrat. Unidentified agents, possible viruses, have been recovered from nine separate pools (3-5 animals per pool) of intestinal tissue from skunks and 1 virus was isolated from the respiratory tissues of a raccoon. The relatively few successful isolations were surprising in view of the rich viral flora disclosed in man and many domestic animals.

The isolate from the raccoon was identified to be a member of the pox group of viruses. It was differentiated from vaccinia, variola and monkeypox viruses. Widespread occurrence of infection in raccoons was demonstrated by disclosure of significant hemagglutination-inhibition antibodies in approximately 23% of sera from 92 raccoons.

2. Epidemiological studies of a human outbreak of leptospirosis at Fort Kobbe, Canal Zone were continued. Serological studies were conducted on leptospiral isolates obtained from two patients, and water and wildlife associated with the natural focus of infection. Seven different serotypes were disclosed, six of which represent new serological types, the seventh was identified as a member of the pomona group. Five of the types were in the hyos, bataviae, icterohaemorrhagiae, cynopterus, and hebdomadis groups, respectively. A sixth new serotype shared no major agglutinogens with any of the known serotypes. In follow up serological studies in troops in which the outbreak occurred, significant antibody levels were disclosed in nine soldiers in addition to eight previously diagnosed patients. In retrospect, approximately half of the infections were subclinical. Findings affirmed previous observations that Panama comprises areas of multiple leptospirosis.

Tissue cultures were prepared from kidneys of hamsters and a dog which were infected with a virulent strain of leptospiral serotype canicola. Viable leptospiras were seen in tissue culture suspensions at the time of their preparation, but not after 24 hours of incubation of kidney cultures. Loss of viability of leptospiras was attributed to antibiotics present in tissue cultures. On the basis of observations, there is no leptospiral infection hazard in administration of vaccines prepared from animal kidney tissue cultures if antibiotics are present in tissue cultures.

3. Studies on enteroviruses isolated from pigs before and after exposure to radiation have continued. To date, six serologically distinct groups of virus have been defined. Further characterization and identification studies are in progress.

Studies to evaluate the potential usefulness of an attenuated Western Equine Encephalitis strain for the preparation of a vaccine were initiated.

A virus, designated MHG, was isolated from tissues of adult rats with CNS disease signs. It passes Millipore filters with pore diameters ranging from 100-300 millimicrons. MHG was resistant to ether and desoxycholate treatment. It produced CNS infections in suckling mice and rats, but not in adults or weanling animals. It has no hemagglutinating properties and is not serologically related to Monkey B virus, Coxsackie viruses, Western Equine Encephalitis, Venezuelan Equine Encephalomyelitis, hepato-encephalitis or to Theiler's GDVII virus. Studies to date indicate that it is a new virus.

Publications:

1. McConnell, S. J., Herman, Y. F., Mattson, D.E., Huxsoll, D. L., Lang, C. M., and Yager, R. H. Protection of Rhesus Monkeys Against Monkeypox Disease by Vaccinia Virus Immunization. Journal of the Am. Vet. Med. Assoc. In Press.

2. Acha, P. N., Alexander, A. D., Santamarina, G., Rubin, H. L., and Yager, R. H. Serological Studies on Leptospirosis in Guatemala. Am. J. Trop. Med. & Hyg. In Press.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 806 MILITARY PREVENTIVE MEDICINE

Task 01, Communicable Diseases (Role of mesenchymal tissues in the control of infection)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Hematology
Division of Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: Colonel William H. Crosby, MC

Assistants: Captain Roger A. Ewald, MC
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 806 Title: MILITARY PREVENTIVE MEDICINE

Task No. 01 Title: Communicable Diseases (Role of mesenchymal tissues in the control of infection)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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It was demonstrated that iron-dextran injected intraperitoneally in rats is taken up by macrophages which carry it across the intestinal serosa into the lamina propria. Other materials were injected intraperitoneally to ascertain whether or not they are handled in similar fashion.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: MILITARY PREVENTIVE MEDICINE

Task No. 01 Title: Communicable Diseases (Role of mesenchymal tissues in the control of infection)

Description:

An investigation of serosal "absorption" by the small intestine.

Progress:

Three days following intraperitoneal injection of iron-dextran in rats, ferritin was found in the tissues of the small intestine, but at ten days it was no longer there. No ferritin was found in the stomach or colon at either time. When iron was given intravenously it did not accumulate in the small intestine.

A loop of duodenojejunal intestine was brought outside the peritoneal cavity prior to intraperitoneal injection of iron. No ferritin could be recovered from this herniated loop of gut although it was recovered from the intestine adjacent to the loop. This indicated that the iron came through the serosal surface of the gut.

It is concluded that the ferritin which is present in the small intestine following intraperitoneal injection of iron-dextran is derived from iron or iron-dextran which penetrates the serosal surface of the intestine. Histological studies indicate that the iron is carried into the intestinal tissues by diapedesis of iron-laden phagocytes. Iron-dextran injected into the base of the tail appeared as ferritin in the terminal ileum.

It is suggested that the small intestine may be an excretory organ for debris-laden phagocytes.

Futher work on the mechanism by which macrophages remove debris from the peritoneal cavity and the possibility that the small intestine is an excretory organ for debris laden macrophages has been in progress. Lipid material, killed tubercle bacilli, India ink, and heparinized and clotted blood injected intraperitoneally were phagocytized but no diapedisis through the small intestinal serosa has been demonstrated by tissue sections and special staining techniques. An investigation of the "serosal" absorption of live virulent bacteria will be studied by the fluorescent antibody technique.

Summary and Conclusions:

It was demonstrated that iron-dextran injected intraperitoneally in rats is taken up by macrophages which carry it across the intestinal serosa into the lamina propria. Other materials were injected intraperitoneally to ascertain whether or not they are handled in similar fashion.

List of Publications:

1. Thirayothin, P. and Crosby, W. H.: The distribution of iron injected intraperitoneally. Evidence of serosal "absorption" by the small intestine. J. Clin. Invest. 41: 1206, 1962.

2. Thirayothin, P. and Crosby, W. H.: A possibility that the small intestine may be an excretory organ for debris. Conf. on Intestinal Malabsorption and Allied Hematologic Problems, San Juan, Puerto Rico, March 20-22, 1962. Am. J. Digest Dis. 17: 975, 1962.

ANNUAL PROGRESS REPORT

Project 3A 12501 A 806 Military Preventive Medicine

Task 02, Acute Respiratory Diseases (Acute Virus Infections of the Respiratory Tract)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Departments of Virus Diseases, Epidemiology, Rickettsial Diseases and Gastroenterology
Divisions of Communicable Disease and Immunology, Preventive Medicine, and Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

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Security Classification: UNCLASSIFIED

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ABSTRACT

Project 3A 12501 A 806 Title: Military Preventive Medicine

Task 02 Title: Acute Respiratory Diseases (Acute Virus Infections of the Respiratory Tract)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Efforts to study the ecology of virus-caused respiratory disease in military populations have been continued. These studies are summarized under 6 major and 14 minor categories. The factors influencing the dissemination of adenovirus in recruit populations were studied. The epidemiology of rubella in these same populations was determined. The study of the biological and immunological characteristics of rubella virus showed it to be unique among human virus pathogens. The occurance of new variants of Type A Influenza virus, Winter 1963, and its significance in the production of disease in military populations is described. The decay of infectivity of several common viruses in artificial aerosols was studied. Search for antiviral substances to common respiratory viruses in naturally occurring human nasal mucus was commenced. Defects in alveolar gas exchange in primary atypical pneumonia and morphologic changes in the small intestine during acute respiratory disease are described. An apparently new hemadsorbing virus of murine origin has been studied.

* Division of Biological Standards, National Institutes of Health
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BODY OF REPORT

Project 3A 12501 A 806 Title: Military Preventive Medicine

Task 02 Title: Acute Respiratory Diseases (Acute Virus Infections of the Respiratory Tract)

Description: Purpose: to define the etiology and ecology of virus caused respiratory disease in military populations and its differentiation from bacterial disease; to devise and evaluate means for precise diagnosis, control and/or prevention of disease.

Progress:

1. Factors Influencing Respiratory Disease in Recruits.

a. Laboratory studies have continued in support of the field investigations commenced in recruits at Fort Dix, New Jersey, during Winter, 1962. Etiology and epidemiology of acute respiratory disease were studied in 5 basic training companies which formed in February 1962. Etiology of disease occurring in approximately 1300 men was sought. Five hundred of these (Co. B 2 T/R and Co. G 3 T/R) were studied in greater detail. Throat swabs and washings were obtained from each man during every week of training as well as at each dispensary visit and hospital admission. Blood samples were obtained as soon as companies were formed, again in the 3rd week of training, and at the end of the eighth week in order to follow the sequence of dissemination of common respiratory viruses and bacteria among recruits of newly formed companies, and if possible to assess environmental and microbiologic factors influencing these transmissions. During the period of study, no recruits were immunized against adenovirus disease.

b. Etiology: Results of bacteriologic investigations were reported in the last annual report (Project 6X61-01-001, Task 02, pp. 368-371). Throat washings were studied either fresh or after having been frozen at -70°C for varying lengths of time up to 6 months after collection. Throat washings from hospitalized patients were inoculated into primary Rhesus monkey kidney and HEp 2 cells. All cases admitted from each of the two "intensive study" companies were examined; only a sample of admissions from the other three companies were studied. Virological techniques used were similar to those described in the annual report 1961-1962 (Project 6X61-01-001, Task 02, pp. 371-372).

(1) Patients from all five companies yielded adenoviruses Types 4 and 7 in approximately equal numbers. Nineteen strains of Herpes Simplex virus were also recovered. From Table I, it can be seen that all but one of the adenovirus strains were recovered during the first four weeks of training. In Co. B 2 T/R, all isolates were made in the first three weeks, while in G 3 T/R, no isolates were made in week one, and all but one isolate was made in weeks two through four. Interestingly,

Table I. Total Adenovirus Isolations from Hospitalized Patients.

Company	Week of basic training								Total
	1	2	3	4	5	6	7	8	
G 3T/R	0/11*	8/22	35/41	11/15	1/6	0/3	0/4	0/0	55/102
B 2T/R	3/7	14/25	27/31	0/2	0/1	0/1	0/10	0/0	44/77

*Strains recovered/total hospitalized patients

Co. B spent one week more in the company forming area than Co. G; thus, the high isolation weeks correlate best with week on post, rather than week of training. Recovery of adenoviruses from apparently well men of two companies showed a similar pattern (Table II).

Table II. Recovery of Adenoviruses from Non Diseased Recruits.

Company	Week of basic training								Total
	1	2	3	4	5	6	7	8	
G 3T/R	0/238*	23/223	59/223	59/207	7/212	-	-	-	145
B 2T/R	1/235	45/224	69/212	5/226	0/53	0/57	0/50	0/44	120

*Strains recovered/specimens obtained

(2) Isolations of adenovirus were divided between Types 4 and 7 with both types occurring in the same company during any single week. However, the breakdown,units into platoons,yielded no single pattern. (Table III) In platoons 2 and 4 of B 2T/R, for example, it appears that two separate peaks of disease occurred - Adenovirus Type 4 in the 2nd week and Type 7 in the 3rd week. Other patterns are less striking, but obviously differ from one another. When data are combined by company, however, these are lost in the overall pattern of the company.

Table III. Distribution of Adenovirus Disease by Serotype based upon Virus Isolations

Company	Week of Basic Training									
	1		2		3		4		5	
G 3T/R	T4	T7	T4	T7	T4	T7	T4	T7	T4	T7
Plat 1	0	0	6	2	14	7	16	6	0	2
2	0	0	2	4	13	12	15	8	1	1
3	0	0	2	4	4	16	3	7	0	0
4	0	0	4	2	7	8	7	7	0	4
Totals	0	0	18	12	38	43	41	28	1	7
B 2T/R										
Plat 1	0	0	5	1	13	9	0	0	0	0
2	1	0	20	2	9	12	0	3	0	0
3	1	0	6	3	17	6	0	4	0	0
4	0	2	12	7	6	15	0	1	0	0
Totals	2	2	43	13	45	42	0	8	0	0

c. Persistance of Adenoviruses In Throats of Recruits. In 58 instances, the same adenovirus was recovered from the same individual on 2 or more separate occasions. The distribution of adenoviruses by type was approximately equal (30 Type 4 and 28 Type 7). The duration of time between virus isolations was 7 days or more in 24 instances and as long as 16 days in one case (Type 7) and 9 days in another (Type 4).

d. Incidence of Adenovirus Infection in Study Companies. Based upon serological data obtained by following men through the entire basic training cycle, the incidence of adenovirus infections was approximately equal in each company (Table IV). The distribution of these 349 adenovirus

Table IV. Incidence of Adenovirus Infections in Study Companies.

Company	Strength	Numbers CF rise	% with Evidence Infection
B 2T/R	240	180	75
G 3T/R	240	169	70.4

infections by clinical manifestations and infecting serotype is indicated in Table V. Among those infections which were considered as "proved", type 4 virus was implicated slightly more frequently than type 7. The same rough

Table V. Distribution of Adenovirus Infections by Serotype and Clinical Manifestation

Company	Number of patients with "Proved" Infection[1] (Total)			Hospitalized			"Carrier"[3]	
	T4	T7	Unk[2]	T4	T7	Unk	T4	T7
B 2T/R	79	46	55	28	14	5	11	18
G 2T/R	67	61	41	27	22	10	9	13
Totals	146	107	96	55	36	15	20	31

Code: [1]Proved Infection - Isolate with 4x or greater CF rise
[2]Unknown - 4x or greater CF rise without isolate
[3]Carrier - Adenovirus isolate without CF rise

proportion of type 4 to type 7 infections was reflected in those patients hospitalized with overt disease. However, fewer strains of type 4 than type 7 virus were recovered from recruit "carriers". This group is of special interest. All but 8 of 51 cases in which adenoviruses were recovered were without clinical illness, and these 43 individuals (8.8% of the total test population) might not be recognized under ordinary circumstances. The fact that 7 of these "carriers" harbored adenoviruses without evidence of disease for up to 2 weeks adds significance to this initial observation that recruit carriers for adenovirus indeed do exist. While the precise role played by these carriers in the dissemination of adenoviruses among recruits could not be determined from this study, the significance of the carrier state to the epidemiology of this infection is obvious, and should be studied further.

e. Dissemination of adenoviruses. By surveying the two companies at weekly intervals it was anticipated that a pattern of dissemination of virus from a single or multiple sources could be documented. With this in mind, individual floor plans of the barracks of the 2 companies were laid out. Weekly isolations from survey and hospitalized men were plotted. Even though grouping of one type of adenovirus in certain areas of the barracks is suggested, no specific statement can be made at this time as to how real this grouping of cases is. Further information especially regarding susceptibility of neighboring recruits to the agent in his area may yield useful data.

f. Multiple Infection. Of interest in the past has been the problem of dual or sequential infections of individuals with more than one agent in any period of time, (see Annual Report of 1960-61, Project 6X61-04-001, Task 1, p. 28). In 1961 a number of agents other than adenovirus were implicated in the etiology of ARD on the basis of serology as well as isolation data. In several instances an individual had more than one isolate made or a serologic rise to more than one agent noted during the 8 week training period. In no case, however, was more than one adenovirus isolated from a single patient either simultaneously or on subsequent hospitalization. In 1962, however, adenovirus types 4 and 7 and Herpes Simplex were the only viral agents isolated. No hemadsorbing agent could be identified. None of the specimens were inoculated into embryonated eggs, but there was no serological evidence of influenza in the community at the time. With the availability of survey gargles in this study, a total of 18 individuals had isolate of both adenovirus type 4 and 7 during the study period. Of these, 3 had both agents isolated from a single specimen. This considerable number is hardly likely to be a laboratory artifact (see below). The isolation of 2 separate adenoviruses from a single individual was noted in consecutive weeks in 11 men and separated by 2 weeks or more in 6 men. No pattern of initial occurrence of one agent could be defined. In addition to the above, one survey throat wash yielded both adenovirus type 7 and rubella virus. Eleven other additional men yielded Herpes Simplex virus on at least one occasion separate from an adenovirus isolate. Four were associated with adenovirus type 4, and 7 with adenovirus type 7. The Herpes isolate occurred prior to the adenovirus isolate in 6 instances and subsequent to the adenovirus isolate in 5 cases.

g. Neutralization Tests. In view of the new information concerning isolation of more than one type of adenovirus and two or more isolations of the same type from single individuals, the question arose as to whether it was possible to determine the spectrum of adenovirus immunity by serotype in individual persons by neutralization test. Serial sera from 18 men with type 4 isolates, 16 who had type 7 isolates, and 17 who had both type 4 and type 7 isolated either simultaneously or on separate occasions were studied. Neutralizing antibody was measured against 4 - 16 $TCID_{50}$ of adenovirus types 4 and 7. Sera were inactivated first at 56° for 30 minutes and virus and serum were allowed to incubate at room temperature for 1 hour before inoculation of the test. Of 51 patients tested, 11 had measurable neutralizing antibody to either type 4 or type 7 in the pre bleeds (drawn during the first week on post). Among the 18 who yielded type 4 virus, the antibody response to type 4 was greater than that to type 7 in all but 4 instances. Likewise, among the 16 who had type 7 isolates, antibody response to type 7 was greater than that to type 4 in all but 3 instances. Among the 17 with dual isolates, 4 had greater antibody rises to type 4, 10 had greater rises to type 7 and 3 had equal antibody rises to both. Table VI outlines the geometric mean fold rise in neutralizing antibody in these 3 groups.

Table VI

Geometric Mean Fold Rise in Neutralising Antibody

Virus Recovered	No. Tested	Antibody Titer To Type 4	To Type 7
T4	18	83	13
T7	16	32	167
Dual	17	51	102

There is a marked difference between the homologous and heterologous increase of the sera of patients from whom type 4 and type 7 were isolated separately. The difference is less striking in the 17 with dual infections.

2. Characteristics of Rubella Virus.

a. In earlier studies propagation of rubella virus was observed in primary cultures of African green monkey kidney (GMK) by its interference with the cytopathologic effect (CPE) of ECHO virus type 11 (ECHO 11). Rubella virus was destroyed by heating at 56°C, exposure to ether and chloroform and was filterable through 300 but not 100 mu millipore filter. In addition to GMK cultures, growth was demonstrated in primary rhesus monkey kidney (RhMK) and human embryonic kidney (HEK) cultures. No differences were apparent in the gross or microscopic appearance of inoculated and uninoculated cultures prior to ECHO 11 challenge. The interference technique in infected GMK cultures has been used to extend observations on the properties of rubella virus.

b. Infectivities of various preparations of rubella virus have been determined by titration of its associated interference, endpoints being expressed as the interfering $dose_{50}$ (IND_{50}). Maximal, and most reproducible endpoints are attained after at least 10 days of incubation of infected cultures at 36°C. In such experiments serial 10 fold dilutions of virus are made in Hanks BSS. Two to four GMK cultures are inoculated with 0.1 ml of each dilution. Titrations are incubated at 36°C in stationary racks for periods of 5, 10 or 15 days prior to challenge with ECHO 11 virus. At challenge, the supernatant culture fluids are replaced with 1 ml of fresh medium containing approximately 1000 $TCID_{50}$ of ECHO 11; cultures are observed daily for CPE until controls show complete destruction (2-4 days after challenge). Titration endpoints when challenged after 5 days incubation are lower than those challenged at 10 and 15 days; further, interference was incomplete at terminal virus dilutions. In contrast, challenge after 10 and 15 days yield equivalent and readily determined

followed by a decreasing rate from 5 to 10 days, and stabilization between 10 and 20 days. Curves for cell associated and supernatant virus are very nearly equivalent. Data given in Table VIII show that this type of curve was also found for each of 3 other lines previously shown to support rubella virus propagation. Despite very nearly identical growth curves, the interference phenomenon varied in its expression and onset of appearance for each of the cell lines tested.

Table VIII

Growth Curves of Rubella Virus[1] and ECHO 11 Interference In Several Cell Cultures

Tissue Culture Tested	1st Day of Complete Interference With ECHO 11	Rubella Virus Titers (Log_{10} Units) On Indicated Day of Incubation				
		1	5	10	15	20
GMK	2	1.0[2]	2.5	3.0	3.3	3.5
BSC-1	4	0.5	2.3	3.0	3.3	3.5
Rabbit Kidney	NT[3]	1.3	2.7	3.3	3.3	3.0
WI-26	None in 20 Days	<0	2.5	2.7	3.5	4.0

[1] M33 strain in 20th GMK passage, 100 - 1000 IND_{50} inoculum.

[2] Titrations on clarified tissue culture supernates, 4 GMK cultures/dilution.

[3] NT = not tested.

f. Inoculation of suckling mice or adult guinea pigs with rubella virus did not result in recognizable clinical disease. Attempts to recover virus from serum, or suspension of lung, and spleen in animals sacrificed 6, 16 and 25 days following intranasal and intraperitoneal inoculation have been unsuccessful. Similarly, it has not been possible to recover virus from suspensions of mouse brain 7 days after intracerebral inoculation.

g. Observations have been made on the response of rubella virus to a number of physical and chemical manipulations. Early studies on thermal stability showed the virus to be destroyed within 30 minutes by heating at 56°C. More recent experiments have shown steady decreases in

titer of infectious virus with incubation at 37°C. In these tests virus preparations in tissue culture medium clarified by centrifugation were incubated; at successive intervals samples were removed and held at -60°C. until collection of the final specimen before titrations were performed. A steady decrease in infectivity titers of 0.3 to 0.4 log units/hour was observed. No virus was detected in any specimen after 24 hours of 37°C. incubation. In contrast rubella virus IND_{50} titers are stabile for 24 hours at 4°C, 3 months at -60°C, and in the dried state for 5 months. Exposure to a variety of chemical agents including ether, chloroform and cesium chloride reduced titers to undetectable levels. Incubation with 1:10,000 aqueous thiomerosal had no effect on virus titers, nor did addition of 10 or 100 mcg/ml of tetracycline to tissue culture medium. In two experiments, rubella infectivity was unaffected when the pH of the virus preparation was adjusted to pH 7.0 or 8.0 with Na_2HPO_4-KH_2PO_4 buffers but decreased from 10 - 1000 fold at pH 6.0. Incubation at 37°C did not increase the decrease in infectivity beyond that predicted from thermal inactivation.

h. At present tissue culture neutralization test is the only method available for measurement of serologic response to immunization or infection with rubella virus. Reciprocal neutralization tests with antisera prepared by immunization of rabbits with several rubella virus strains failed to show significant differences in virus strains with the limitations posed by the test technique (see below, Serologic studies). Each of 5 virus strains was tested against its own antiserum and the prototype M33 serum; in addition the M33 prototype virus strain was tested against each of these 5 antisera. No 4 fold or greater differences from the prototype occurred in any test with these 5 strains tested against the prototype antisera, nor were greater than 2 fold differences in serum titers observed when prototype and homologous viruses were tested. Further evidence for the homogeniety of strains recovered is provided by (1) the demonstration of neutralizing antibody responses to M33 virus in all patients tested from each epidemic, and (2) the neutralization of all of over 100 strains of rubella virus recovered from 1960-1963 by the prototype M33 serum. In addition to viruses tested previously (Herpes simplex, Influenza A and B, Para-influenza types 1, 2, and 3, Respiratory syncytial, and an SV-5 like virus) M33 antiserum failed to neutralize 10 - 100 $TCID_{50}$ of SV-40 (vacuolating virus) and rubeola virus.

3. Development of Serologic Tests for Rubella. Before the epidemiology, pathogenesis or prophylaxis of rubella could be studied critically, an accurate, sensitive test for antibody to rubella virus was required. At present, the only available test is that for neutralizing antibody, performed in cell cultures. While this test is complex, time consuming, and therefore not practical for extensive serological surveys, it has proven to be reliable for studies on a moderate scale.

a. Standard tests for neutralizing antibody were performed in African green monkey kidney tissue cultures (GMK) as follows: Rubella virus, as 19th or 20th passage culture fluids, was centrifuged, buffered to pH 7.2 - 7.3, fortified with 1% bovine plasma albumin (BPA), and

stored at -60°C in sealed glass ampules. Diluent for virus and sera was Hanks balanced salt solution (BSS) containing 1% BPA. Animal and human sera, mouse ascitic fluids and gamma globulin samples used in the test were inactivated at 56° or 60°C for 30 minutes. Serial two-fold dilutions of the material to be tested were mixed with an equal volume of test virus and incubated along with control virus titrations at 37°C for 1 hour. Immediately thereafter 0.1 ml of each dilution of the serum-virus mixtures, appropriate serum controls and control virus titrations were inoculated into 2 - 4 GMK tube cultures. After 5 days incubation, supernatant fluids from each of the cultures were replaced with fresh maintenance medium containing 100 - 1000 $TCID_{50}$ of ECHO 11. Cultures were observed daily thereafter until destruction of control cultures was complete (2 - 4 days). The interfering $dose_{50}$ (IND_{50}) was determined by the Reed Muench method. Antibody titers were expressed as the highest initial dilution at which rubella virus interference was clearly inhibited. Production of ECHO 11 hemagglutinin for human red cells, type O was frequently assessed to confirm observed enterovirus CPE; high hemagglutinin titers corresponded to more obvious CPE. This objective measure of interference often was helpful to determine end-points in antibody titrations. Rarely, sera containing high titers of ECHO 11 antibody required the use of another challenge enterovirus; usually Coxsackie virus, group A, type 9, was employed in these instances.

b. During development of this standard test a number of variables were evaluated for their effect on serum antibody titer. Measurable human and animal antibody was consistantly found to be of lower titer than that commonly attained by man or animals to other viruses, rarely reaching levels of 1:128 even when tested against as little as 10 IND_{50} of virus. Serum titers were dependent on dose size; for example, each 10 fold increase of test rubella virus between 10 - 1000 IND_{50} resulted in a 4 fold decrease in titer of hyperimmune rabbit antibody, or a 4 - 8 fold decrease in human convalescent antibody. With less than 10 or greater than 1000 test IND_{50}, low titered (1:2 - 1:4) neutralizing "antibody" was not reproducible and difficult to interpret. Time of incubation before challenge was also critical, since delay beyond 5 days resulted in decrease in detectable antibody titer, and frequently "washed out" low titered antibody. Experiments with hyperimmune rabbit antibody suggest that incubation of virus-serum mixtures for 1 hour at 37°C results in more complete neutralization than incubation for up to 18 hours at 4°C before inoculation. Animal antisera prepared by inoculation of GMK grown rubella virus contain both complement fixing and agglutinating antibodies (for rabbit red cells diazotized with control GMK fluids). That this antibody fails to affect the specificity or titer of the sera in the GMK neutralization test system is suggested by two findings: first, these antisera do not inhibit Influenza, para-influenza, respiratory syncytial, or rubeola viruses in GMK culture systems, and second, preliminary studies of rubella virus neutralization by these sera in a primary heterologous cell line (bovine embryonic kidney) or in a continuous diploid line of GMK (BSC-1) have failed to show significantly altered antibody titers.

c. Attempts were made to evaluate the reproducibility of the standard neutralization test. Ten sera obtained from military recruits prior to rubella infection (1, 3, 5, 9 and 10, Table IX) and in convalescence from overt rubella (2, 4, 6, 7, 8) were coded and tested three different times against 10 - 30 IND_{50} of virus. The tests were read by two individuals without knowledge of the number of arrangement of seronegative and positive sera. These tests showed no errors in the determination of presence or absence of rubella antibodies. Four fold variations were noted in 2 of the five sera containing antibody (2, 4);

Table IX

Rubella[1] Neutralizing Antibody Titers in 10 Human Sera

Test No.	Serum No.[2]									
	1	2	3	4	5	6	7	8	9	10
1	<2	8	<2	8	<2	8	16	8	<2	<2
2	<2	16	<2	16	<2	8	16	8	<2	<2
3	<2	32	<2	32	<2	16	32	8	<2	<2

[1]M33 Strain, 19th GMK passage, 10 - 30 IND_{50}.

[2]Sera numbered 1 and 2, and 3 and 4 represent acute, convalescent serum pairs.

these were drawn 2 and 5 weeks after onset of infection and may represent a lesser avidity of antibody in convalescent specimens during this stage. This point deserves further study.

d. Virus dilution-constant serum neutralization tests were found to be of less value for antibody studies. These differed from the standard serum dilution test in that a single 1:2 dilution material to be tested for antibody was incubated with serial 10 fold dilutions of rubella virus. Incubation and inoculation procedures were similar. Although the test was sensitive in detecting low levels of serum antibody, higher levels did not produce a correspondingly greater increase in log neutralization indices (LNI). Thus in 15 acute convalescent serum pairs tested by both methods and in which 4 - 32 fold rises were detected by the standard serum dilution method, LNI's varied from 0.5 to 2.0 with 10 of 15 between 1.0 and 1.5. There was no correlation of fold antibody increase and the LNI. Because of this, the low LNI values obtained, and the lesser usefulness of this test for serologic survey work, use of this test has been discontinued.

e. In addition to tests of patient and immune rabbit sera a number of other materials have been tested for rubella antibody, including commercial lots of human gamma globulin and sera from normal animals of various species. No evidence for rubella antibody was found in sera from 8 rhesus monkeys which had been housed here for more than 3 years. None of 7 rabbits, 1 burro or 3 fetal calf sera contained antibodies, although one of three calf sera showed significant rubella neutralization. All of 6 lots of human gamma globulin from various commercial sources as well as 2 National Institutes of Health standard lots, neutralized rubella virus in approximately equal titer (1:160/0.1 ml).

f. Attempts to produce rubella antibody in adult male WS mice by immunization and subsequent harvest of tumor induced ascitic fluid were unsuccessful. Immunization of a young male burro at weekly or biweekly intervals for 4 months with a rubella virus preparation in Freunds incomplete adjuvant produced low neutralizing antibody titers (1:4 vs 30 IMD_{50} virus dose). Death of the animal following a surgical procedure obviated further study of immunologic response.

g. Production of rubella complement fixing or agglutinating antigens has been unsuccessful.

4. Epidemiology of Rubella.

a. During investigation of respiratory disease at Fort Dix, New Jersey, February - March 1962 (see section 1, Factors Influencing Respiratory Disease in Recruits, this report) clinical "rubella" occurred in recruits of study companies. These patients presented with polymorphous rashes of brief duration, posterior auricular and posterior cervical adenopathy, and little or no fever or debility. Study of throat washings and swabs collected from these patients suggested that rubella virus played the major etiologic role. Thus of 92 patients with exanthems, 75 yielded rubella virus (82%). Of this same group only 6 (6.5%) yielded beta hemolytic streptococci, group A. This finding was in contrast to experience of the preceding year in which the same figures for a similar group of 79 patients showed 45% and 32% respectively for rubella virus and group A streptococci.

b. From 6 - 22 February 1962 all patients admitted to Walson Army Hospital with diagnoses of rubella were interviewed and examined. During this time 66 recruits were admitted with rubella from the 2nd, 3rd and 4th training regiments. Recruit companies in training at this time represented the 2nd - 8th weeks; from 1 - 9 cases occurred in each company. The distribution of onset of these cases by week of training and the approximate rate for each week is shown in Table X. The rate of rubella for the entire post during the month of February 1962, 64.1/1000/annum (174 cases), is roughly equivalent to the low recruit rates in the 2nd and 6th weeks. The highest rates in the 7th and 8th training weeks were approximately 60 times the post rates. The pattern

Table X

Rates of Overt Rubella in Recruits
Fort Dix, N.J. 6-22 Feb. 1962

	Week of Training							Total
	2	3	4	5	6	7	8	
No. cases 2, 3, 4 TR	3	9	9	0	4	20	21	66
No. Co's. in training	5	6	5	5	6	5	5	
Strengths	1200	1440	1200	1200	1440	1200	1200	
Rate/1000	2.5	6.3	7.5	0	2.8	16.7	17.5	

of disease shown in Table X, with the disease occurring in two waves, one early, the other late in the training cycle, was observed in each of the three regiments. This is probably determined by the incubation period's effect upon the pattern of introduction and transmission of disease.

c. Serial blood and throat washing specimens along with clinical data from the 246 men of company B, second recruit training regiment as described previously were studied. The distribution of susceptibles and the numbers of those with evidence for rubella virus infection are given in Table XI. More than 10% of recruits failed to demonstrate rubella antibody when their sera were screened against 10 - 50 IND_{50} of virus. Of this group of 30 recruits, 4 were hospitalized with clinical rubella; rubella virus and neutralizing antibody increases were shown in all 4. All of the remaining group of 26 without demonstrable antibody were infected. All 26 had 4 fold or greater increases in neutralizing antibody; 23 of the 26 had rubella virus recovered from 1 or 2 consecutive survey throat washing specimens. Thus the ratio of overt rubella to subclinical infection was 1:6.5, and the secondary attack rate for infection in this population 100%.

Table XI

Rubella Virus Infections
Co B 2TR 3 Feb.-28 Mar. 1962

Platoon	Strength	Number Susceptible[1]	Overt Infection[2]	Subclinical Infection[2]
1	62	9	3	6
2	62	5	1	4
3	61	7	0	7
4	61	9	0	9
Totals	246	30(12.2%)	4	26

[1]Criteria for susceptibility: neutralizing antibody on arrival at Fort Dix $<1:2$ vs 10 - 50 IND_{50} rubella virus.

[2]Criteria for infection:
a) recovery of rubella virus and/or
b) 4 fold increase in antibody titer from $<1:2$.

d. Rubella virus epidemiology was further studied by a survey of 242 sera selected by donor age from the departmental serum file. These sera, originally submitted for diagnostic study were from military personnel and their dependents suspected of having a variety of clinical diagnoses. They were collected from 1954 - 1963; the majority of sera were obtained from personnel residing in the United States. All sera were tested for antibody to both rubella and rubeola viruses. Rubella neutralization tests were performed as described above. Sera were considered negative if no antibody was demonstrated in a 1:2 dilution of serum. Tests for rubeola antibody were made by the hemagglutination inhibition technique (HAI) with 1:5 to 1:160 dilutions of kaolin treated, monkey red cell adsorbed sera.

Table XII. Presence of Immunity to Rubella and Rubeola

Disease	Percent with Antibody at Indicated Age (Years)							
	0-.41	.41-.92	1-4	5-9	10-14	15-19	20-24	≥ 25
Rubella	77	8	34	39	53	89	80	100
Rubeola	77	42	50	82	100	100	100	100
Total Tested	13	12	58	39	49	28	20	23

Antibody to both viruses was present in 70 - 80 percent of infants less than 6 months old. In each instance decrease in frequency of those with antibody was observed in the 6 - 11 month age group. Subsequently frequency increased until 100% showed evidence of infection with both agents. While donors represent no specific population with regard to time or geography, the rate of acquisition of rubeola HAI antibodies paralleled those observed for the same virus in countries with high urban population density. In the present study the rate of increase in rubella antibody lagged behind that of rubeola; all persons were apparently immune to measles by age 10 to 14. In contrast comparable incidence of immunity to german measles was not attained until after age 24. This suggests that the transmission potential of rubella is significantly less than for measles. Further, since 100% of susceptible recruits can be infected in 8 weeks of basic training, the relatively slow rate of increasing immunity to rubella in children, suggests a distinct difference in transmission rates for the 2 populations.

5. Plaques by Rubella Virus in Grivet Monkey Kidney Cell Cultures.

a. Studies were undertaken to develop plaques by rubella virus in agar-overlaid cultures of African green monkey (Cercopithicus aethiops) kidney cells (GMK) in 4 oz prescription bottles. The principle employed uses the phenomenon of interference by rubella virus with the cytopathogenic effect of enteroviruses. After innumerable failures, rubella plaques were finally produced.

b. The agar-overlay medium comprised the following constituents by volume: 50% of a three gram percent stock of noble agar (Difco) in distilled water; 10% of a ten-times concentrated stock of 199 medium without phenol red and sodium bicarbonate; 10% of inactivated (60°C for 30 minutes) chicken serum; 3% of a 7.5 gram percent stock of sodium bicarbonate in distilled water; 1% of a 20mM stock of aluminum chloride in distilled water; and 26% of distilled water. Aluminum chloride was incorporated to prolong the viability of the cell cultures under agar. Monolayers were stained with a 1:1000 aqueous solution of neutral red. The M33 strain of rubella virus in the nineteenth or twentieth GMK passage and the Lansing strain of polio type 2 virus were used in all experiments.

c. Following a primary overlay of 9 ml of agar-medium, each rubella infectedGMK culture underwent a preliminary 5-day incubation period at 37°C. It was then challenged with 3.15 plaque forming units (pfu) of polio type 2 virus which was incorporated in a second overlay of 3 ml of agar-medium. Each GMK culture underwent a further 5-day incubation period at 37°C. Neutral red was added to each culture two days after the polio challenge. Plaques, representing islands of rubella-infected cells, became apparent at the termination of the second incubation period.

d. Three types of controls were included in each experiment to determine the effectiveness of the polio challenge, and the quality of the uninoculated and rubella-infected GMK monolayers. They comprised

a set of uninfected cultures, a set of cultures inoculated with 100 interfering doses (IND_{50}) of rubella virus and a set inoculated with 3.15×10^6 pfu of poliovirus type II. The uninfected and rubella-infected cultures each received a second overlay of 3 ml of agar medium without poliovirus. The polio challenge controls were only infected at the second overlay in the same manner as the plaque producing cultures. Rubella plaques were never recorded unless the polio challenge controls showed complete destruction of the GMK monolayers.

e. Difficulties encountered during the preliminary phases of the study were due to: the inadequacy of the agar-medium to support GMK monolayers for prolonged incubation period, certain batches of chicken serum which appeared to cause toxic degeneration of the monolayers, and possibly to the age of the donor animal. By increasing the content of certain lots of chick serum in the agar-medium coupled with the incorporation of aluminum chloride, these difficulties were to a great extent overcome.

f. The Lansing strain of poliovirus type II was arbitrarily selected as the challenge virus because large quantities were available. In the early studies, it was placed on the surface of the agar of each overlay and allowed to diffuse to the membrane. Since it was impossible to distribute the small volume of inoculum evenly over the agar surface, the amounts of virus reaching the different areas of the monolayer varied. Several attempts to challenge cell sheets following removal of the overlay were equally unsatisfactory. Some cellular detachment occurred during the challenge period and degeneration of the monolayers followed the second agar-overlay. Only by incorporating the poliovirus in the second overlay, without removing the first, were monolayers evenly challenged.

g. The production of plaques with diameters ranging from 3 to 5 mm could only be achieved by a primary 5-day incubation period prior to challenge with poliovirus type II. A second 5-day incubation period was also necessary for the total destruction of all except the rubella-infected cells.

h. These cell islands represented rubella plaques because similar phenomena were not observed in the three controls and because they could be reproduced in all tests. As a further verification, a preliminary plaque neutralization test was performed. A hundred-fold reduction in the titer of rubella virus, as measured by plaque production, was apparent 4 to 5 days after challenge.

i. Studies are in progress to improve and evaluate the plaque technique in the quantitation of infective rubella particles and plaque reduction as an index of virus neutralization. The method will also allow plaque purification of rubella virus strains and investigation of variation in plaque morphology.

6. Analysis of Influenza, Winter 1963, and Study of New Antigenic Variants. During study of clinical specimens submitted for confirmation of the clinical diagnosis of influenza, January - March 1963, a new variant of A_2 influenza virus was recovered. Fifty strains of influenza virus have been recovered from military populations and patients in VA hospitals in Eastern United States (Table XIII). All strains were recovered in embryonated eggs on initial or second passage; all have been identified by hemagglutination inhibition (HI) test with rooster

Table XIII

Recovery of Influenza Viruses From Outbreaks in Eastern U.S.

AREA	TOTAL SPECIMENS TESTED	# ISOLATE
Washington, D.C. Area	63	32
Martinsburg, West Va., VAH	18	6
Ft. Bragg, N.C.	8	3
Ft. Dix, N.J.	2	2
Ft. Jackson, S.C.	8	3
Navy PMU #2, Norfolk, Va.	5	2
Temple, Texas, VAH	3	1
Memphis, Tenn., VAH	10	1
Totals	117	50

antisera. Four strains initially recovered in this laboratory, and A_2Japan 170/62 (received from Dr. J. A. Morris, NIH) were subsequently adapted to rhesus monkey kidney (RhMK) cultures.

a. Information had been received of widespread outbreaks of influenza in what is considered a relatively well immunized population (see Table XIII). This prompted further investigation as to any differences which may exist between the 1963 strains and earlier prototype Asian strains of influenza.

b. Paired sera were available from 126 individuals with clinical influenza from the installations listed in Table XIII. Of these 77 pairs were tested by HI against both the 1957 strain of Asian influenza and the 1962 strain (egg adapted). All sera were treated with Periodate immediately before testing. 58.4% (or 45) of those tested against 1957 strain showed 4 X or greater rise in antibody whereas 63.6% (49) tested against the 1962 strains showed significant increases in antibody. Of the 32 individuals with influenza isolates tested in this group of 77

Table XIV

Antibody Response to A_2Japan 170/62

Paired Sera Tested	126
4 X or > Rise to HI	83 (65.8%)
4 X or > Rise to CF	76 (60.2%)
Paired Sera with Isolate	**42**
4 X or > Rise to HI	35 (83.3%)
4 X or > Rise to CF	31 (73.8%)
4 X or > Rise to HI and/or CF	39 (92.9%)

23 or 71.8% showed 4 fold or greater rise in antibody to the 1957 strain and 26 or 81.2% showed similar rise to the 1962 strain. With this evidence that the 1962 strain was a more satisfactory test antigen, all further tests were performed using this latter antigen. Of the 126 pairs of sera tested 83 (65.8%) demonstrated 4 fold or greater rise in HI antibody (see Table XIV). Of the 42 individuals with isolates and paired sera, 35 (83.3%) demonstrate a significant rise in HI antibody. Complement fixation antigens were prepared in embryonated eggs to one of the 1963 strains (A_2DC 327/63) and the sera were tested against this antigen as well. Results are tabulated in Table XIV. It can be seen that the CF test was not quite as sensitive for demonstrating significant antibody responses, yet 4 individuals with isolates showed CF antibody rises without significant HI antibody increases. No explanation is available for the 3 patients with isolates who failed to develop either CF or HAI response.

c. Antigenic Analysis. Rooster antisera were prepared in the standard manner against 5 of the 1963 strains and the A_2Japan 170/62 strain. These 6 antisera together with those against the 1957 strains when tested in reciprocal HI tests clearly demonstrate that the current strains are different from the older Asian strains, (Table XV), as well as earlier A Influenza strains. In repeated tests, newly recovered viruses were regularly inhibited by rooster antisera to the 1957 Asian strains, whereas similarly prepared antisera to 1962 and 1963 viruses react weakly, if at all, with the two 1957 viruses tested. These data suggest that the newly recovered strains are more closely related to A_2Japan 170/62 than to other A strains, including the 1957 viruses.

TABLE XV

INTERPRETIVE SUMMARY, CROSS REACTIONS OF CURRENT A INFLUENZA STRAINS WITH OTHER VIRUSES

Rooster Antisera	Prototype Antigens							Current Antigens					
	Pr-8	FM-1	FW-1-50	FLW-1-52	A Haw 303-56	A_2 Jap 305-57	A_2 For 313-57	A_2 Jap 170-62	A_2DC-302-63	A_2FB-322-63	A_2DC-327-63	A_2DC-301-63	A_2FD-382-63
A Pr-8 34	++++[1]	0	0	0	0	0	-	±	0	0	0	-	-
A FM-1 47	++	+++	+++	+	+	0	-	0	0	0	0	-	-
A FW-1-50	0	++++	++++	++	++	0	-	0	0	0	0	-	
A FLW-1-52	±	+++	+	++++	+++	0	-	±	0	0	0	-	
A Hawaii 303-56	0	++	±	+++	++++	0	-	0	0	0	0	-	-
A_2Jap. 305-57	0	±	0	0	++	++++	+++	++++	+++	++++	++++	++++	+++
A_2For. 313-57	0	0	0	0	0	++++	++++	+++	++	++	+++	++	-
A2Jap. 170-62	-	-	-	-	-	±	-	+++	+++	+++	+++	+++	++
A_2DC 301-63	0	0	0	0	0	±	-	+++	++	+++	++++	-	-
A_2DC 302-63	0	0	0	0	0	±	±	+++	+++	+++	+++	+++	++
A_2FB 322-63	-	-	-	-	-	±	-	+++	+++	++++	+++	+++	-
A_2DC 327-63	-	-	-	-	-	0	-	+++	+++	+++	++++	-	+++
A_2FD 382-63	-	-	-	-	-	+	-	+++	+++	+++	+++	-	+++

[1] ++++ = 1:1600 - 1:6400
+++ = 1:400 - 1:800
++ = 1:200
+ = 1:100
± = 1:50
0 = < 1:50
- = not tested

d. Other New Antigens. Recent reports have been received concerning the appearance of a distinctly new strain of influenza Type B. This strain was isolated in Taiwan in 1962 and egg passage material was received here for analysis. Rooster antisera were prepared to this strain B-Taiwan 2/62 and tested by hemagglutination inhibition against older strains of B Influenza. (See Table XVI.) It can be seen that rooster antiserum prepared against the B-Taiwan 2/62 strain has virtually no inhibitory effect against any of the earlier strains. Of considerable interest in this study was the finding that the antibody prepared against strain B-Va. 301/55 was extremely broad and afforded protection against older as well as newer strains. Further studies are in progress to define this phenomenon more specifically.

Table XVI

Interpretive Summary
Cross Reactions of B Influenza Strains

Rooster Antisera	Antigens									
	B-Lee 40	IB 1-50	B-Va. 301/55	B-Maryland 1/59	B-Canada 380/61	B-Miami 2/61	B-Georgia 9/61	B-Arizona 1/61	B-Ann Arbor 1/61	B-Taiwan 2/62
B-Lee 40	++++	0	0	0	0	0	0	0	0	0
IB 1-50	+	+++	++	0	0	±	±	±	0	0
B-Va. 301/55	++	+++	++++	+++	+++	+++	+++	+++	+++	+++
B-Ann Arbor 1/62	+	++	+++	++++	++++	++++	++++	++++	++++	+
B-Taiwan 2/62	0	0	0	0	+	+	+	±	±	++++

++++ = 1:1600
+++ = 1:400-800
++ = 1:200
+ = 1:100
± = 1:50
0 = 1:50

e. Neutralization Tests on Asian 1963 Strains. The data reported above on rooster antiserum suggested a distinct difference between the newer Asian strains and the 1957 prototypes. Corroboration of this hypothesis has been obtained by tissue culture neutralization tests in Rhesus monkey kidney cells. One strain of A_2Japan 305/57 has been adapted to tissue culture and the newer strains already referred to were adapted for these tests.

(1) The rooster sera prepared against the newer Asian strains were heat inactivated at 56° for 30 minutes prior to use. Antigens were used at 100 - 1.000 $TCID_{50}$ as measured by hemadsorption. Results of a cross neutralization test can be seen in Table XVII. There is virtually no cross neutralization between the 1957 and newer strains. This test appears to be even more specific than the hemagglutination inhibition.

Table XVII

Cross Influenza Neutralization Reactions

Rooster Sera	Antigens					
	A_2Jap 305/57	A_2Jap 170/62	A_2DC 327/63	A_2DC 302/63	A_2DC 317/63	A_2DC 342/63
A_2Formosa 313/57	640[1]	10	20	20	40	20
A_2Jap 170/62	10	160	80	160	320	160
A_2DC 327/63	20	20	160	320	320	160
A_2DC 302/63	<5	20	40	80	160	80

[1] reciprocal of antibody titer against 100-1000 HAD doses$_{50}$ of virus; titers /0.05 ml serum.

(2) Human Sera. The preliminary data thus far have suggested that the tissue culture neutralization test can be a specific method of differentiating infection with antigenic variants of Asian influenza. Attempts to demonstrate this phenomenon in human sera are now underway. Specifically being tested are sera drawn from recruits before and after immunization against influenza (prior to 1963).

Also under investigation are the paired sera from the 1963 outbreak. This approach may be very helpful in determining the efficacy of prior influenza vaccination in preventing infection with newer antigenic strains.

7. Studies of Artificial Virus Aerosols.

a. As part of the continuing studies to define the transmission of respiratory pathogens, a preliminary study of the stability of viruses in aerosols was undertaken. The principal goals were: first, to determine how long common viral respiratory pathogens survived in an aerosol; second, to determine the effect of relative humidity on survival of aerosolized viruses. Viruses were aerosolized by means of a Vaponefrin nebulizer into a 200 liter tank rotating constantly at 4 rpm. Samples were collected in tissue culture media with "all glass impingers" (Ace Glass Co., Vineland, N.J.).

b. The following viruses were aerosolized and were successfully recovered: adenovirus types 4 and 7; rubella, influenza A_2, para-influenza 3, Coxsackie A9, ECHO 11, rhinovirus. Two viruses (adenovirus type 7 and para-influenza type 3) were studied in more detail to determine the effect of relative humidity on survival rates. Temperature was constant at 74°F. Table XVIII lists the mean titers (expressed as $\log_{10}$ of the $TCID_{50}$) for adenovirus type 7 at intervals after aerosolization.

Table XVIII

Decay of Adenovirus type 7, in Aerosols

Trials	R.H.	Titers at Indicated Times						
		pre[1]	5 min	1 hr	2 hr	3 hr	4 hr	24 hr
Total[2]	12-100%	7.4	2.7	2.1	1.8	1.3	1.4	0
High RH[3]	> 70%	7.5	3.2	2.8	2.5	2.2	2.2	0.3

[1] Log_{10} of $TCID_{50}$ in HEp 2 cell cultures.

[2] represents nine trials.

[3] represents three trials.

It can be seen that there is a slower loss of infectivity at a high relative humidity. Preliminary results suggest that there is another, less marked, increase in survival rates at relative humidities below 30%.

c. For comparison, the survival pattern of para-influenza type 3 is presented in Table XIX. With this agent better survival was noted with very low humidities, as opposed to the findings with adenovirus. It was noted

Table XIX

Decay of Para-influenza Virus, Type 3, In Aerosol

Trials	R.H.	Titers at Indicated Times						
		pre[1]	5 min	1 hr	2 hr	3 hr	4 hr	24 hr
Total[2]	18-100%	7.2	3.2	2.9	2.0	1.4	1.2	pos[4]
Low RH[3]	<20%	7.2	3.5	3.7	2.7	2.5	2.5	0.2

[1] Log_{10} of $TCID_{50}$ in monkey kidney cell cultures.

[2] represents six trials.

[3] represents three trials.

[4] mean is less 1.0 $TCID_{50}$ but greater than 0.

that in the presence of high relative humidities (greater than 80%), no viable virus could be recovered at 24 hours and only small amounts at 4 hours. Therefore a series of four trials at relative humidities exceeding 80% was done. Virus was recovered after 8 hours, but a mean recovery of less than 1 $TCID_{50}$ was made at 12 hours. There does not appear to be a diphasic survival rate curve for this agent.

8. Aerosol Sampling of Naturally Acquired Respiratory Infection.

T

a. The exact mode of transfer of infections from one person to another during outbreaks of acute respiratory disease has been a matter of speculation. Direct proof of a true aerosol spread or of droplet contact, the two main routes for respiratory transmission, is lacking. An attempt was made, therefore, to recover virus from air in the immediate vicinity of patients with naturally acquired respiratory infections.

b. Cases of acute upper respiratory disease were selected from Walson Army Hospital, Ft. Dix (recruits, children), the Pediatric Clinic and ASO of WRGH. Table XX lists the clinical diagnoses. Patients were studied during the acute phase of the illness by means of one to five glass impingers (AGI, Ace Glass Company, Inc., Vineland, N.J.), each of which samples 12.5 liters of air per minute. Total sampling times varied from 15 to 30 minutes per patient, the impingers being placed from 6 inches to 4 feet from the face during normal breathing or forced coughing by the patient. Throat washings or swabbings were then obtained. Virus isolation techniques included several cell culture systems

(HEp 2, monkey kidney, continuous diploid human lung) and embryonated eggs.

Table XX

Attempts to Detect Viruses In Natural Aerosols

Clinical Diagnoses of Patients	
Recruit ARD	23
Recruit Rubella	4
Nonspecific URI	11 (children)
Influenza	6
	44

c. As shown in the accompanying Table XXI, 23 virus isolations were made from throats of 44 patients tested. Only a single aerosol sampling was found to contain virus, that being the specimen taken from a child who had Para-Influenza type 3 in the pharyngeal secretions.

Table XXI

Attempts to Detect Viruses In Natural Aerosols

Virus Isolations from Throat Washings	
Adenovirus Type 4	12
Herpes simplex	1
Rubella virus	1
Respiratory syncytial virus	2
Para-influenza type 3	1
Unidentified hemadsorption virus	1
Influenza A_2 (variant)	5
	23

d. It is concluded that refinement of sampling techniques is required before quantitative recovery of exhaled virus is possible. Nevertheless, the ability to isolate viruses from natural aerosols has been demonstrated.

9. Studies of Nasal Antibody to Common Respiratory Viruses.

a. As part of an investigation of the physiology of the respiratory tract in health and disease mechanisms for local defences against invading organisms have come under study. The initial investigation has been to define the nature of antiviral substances in respiratory mucus.

b. Nasal secretions were collected from normal subjects by means of saline washes with or without stimulation by sodium carbonate. The nasal washings (NW) were shaken vigorously with glass beads to emulsify the mucous threads and then cleared by centrifugation.

c. Two or more nasal antibody determinations were performed on 15 normal subjects in attempts to determine the relationships of nasal to humoral antibody. Table XXII summarizes the results of neutralization tests with 6 different viruses. Although the number of

Table XXII

Relationship of Serum Titer to Presence of Nasal Antibody[1]

Serum Titer	Virus					
	Polio 1	Adeno 4	A_1 Flu	B Flu	Para 3	Para 1
<1:16	0/3[2]	0/1	0/2	0/2	1/1[3]	-
16-32	-	-	0/2	0/3	2/3	2/3
64-128	2/4	1/2	1/5	1/2	3/3	-
256 or >	5/8	2/2	-	0/1	-	-

[1] NW protein not standardized

[2] Number with antibody/total number tested

[3] Serum titer 1:8 in this patient

determinations is small several correlations are apparent. First, nasal antibody is not found in the absence of serum antibody. With Polio 1 and Adenovirus type 4 increasing serum titers are associated with greater percentage of positive nasal washes. This correlation is not clear for Influenza and Para-influenza viruses, although too few cases have been studied to make final conclusions.

d. A recognized variable requiring elucidation is the significance of the protein content of the nasal washings in terms of antibody status of the specimen. Many of the specimens recorded in Table XXII had

less than 10 milligrams protein (sulfosalicylic acid method) per 100 ml. Of considerable interest, however, is the fact that of 12 subjects with serum titers of 1:64 or greater against poliovirus type 1, seven had nasal antibody despite protein concentrations of less than 20 mg %. In one patient with a serum titer of 1:32 to 1:64 no nasal antibody was demonstrable even when the specimen was concentrated to 150 to 220 mg % protein.

e. Table XXIII presents data which suggests that the antiviral substances in nasal mucus are specific. In patient #2 in whom polio antibody is lacking in NW, Para 3 antibody is present, the serum titers being equal (1:32) for both viruses. Patient 13 lacks Para 3 NW antibody despite a serum titer of 1:32. He has NW polio antibody. Other properties

Table XXIII

Antibody Studies of Standardized Nasal Washings[1]

Patient #	Neutralizing Antibody			
	Poliovirus 1		Para-influenza 3	
	Nasal Wash	Serum	Nasal Wash	Serum
1	+[2]	1:32	+	1:32
2	0	1:32	+	1:32
4	+	1:256	+	1:128
6	0	1:8	-	-
8	+	1:512	+	1:8
12	+	1:128	+	1:128
13	+	1:256	0	1:32
17	+	1:64	+	1:64

[1] concentrated to approximately 150 mg % protein

[2] + = neutralizing antibody present
0 = neutralizing antibody absent

of the NW "antibody" against poliovirus type 1 are (1) resistance to heating at 56°C for 30 min., (2) stability when subjected to 3 cycles of freezing and thawing, and (3) destruction by boiling for 10 minutes.

f. Further studies of nasal antibodies are planned to investigate their relationship to immunity. Immunoelectrophoretic techniques have been utilized in an attempt to relate neutralizing antibody with protein fractions, but these analyses are incomplete at the present time.

10. Pulmonary Function in Viral Pneumonia.

a. Viral pneumonia is an epidemic disease of military recruits which results in considerable loss of time from duty (a 2 to 3 week period of hospitalization is the average) although fatality is rare. During the past year two reports in the literature have demonstrated abnormalities in alveolar capillary gas diffusion following certain viral pneumonias. A preliminary study of adenovirus pneumonia in recruits was therefore undertaken to determine what pulmonary function parameters, if any, are disturbed in this condition.

b. Recruits with pneumonia were selected from the wards of Walson Army Hospital, Ft. Dix, N.J. after bacterial etiology had been ruled out and physical and roentgenographic signs of pulmonary involvement were confirmed. During the convalescent phase of illness, seven patients were transfered to Walter Reed General Hospital for pulmonary function studies. Virus isolation attempts were made during the acute illness; serologic studies (complement fixation and neutralization) for adenoviruses were performed on acute and convalescent serum.

c. The pulmonary function parameters which were studied are indicated in Table XXIV.

Table XXIV

Tests of Pulmonary Function

Vital capacity, 1 second vital capacity
Functional residual capacity
Residual volume
Total lung capacity
Maximum mid expiratory flow
Maximum breathing capacity
Carbon monoxide diffusion (DL co)

d. Results described in Table XXV are preliminary only in that further serologic studies are in progress. Six of the seven patients were shown to have adenovirus infection at the time of the acute illness. Four of the six yielded adenovirus type 4 from the throat wash, a fifth showed neutralizing antibody rise to this agent. The sixth patient had adenovirus complement fixing antibody rise. The final patient had no evidence of adenovirus infection. The only significant abnormality of pulmonary function in the group as a whole was diffusion of carbon monoxide which was below normal in 5 cases. In these 5 the abnormality reverted to normal upon retesting several weeks later. Of interest, even the two patients (#30, #71) in whom initial DL co was within normal limits showed marked increase upon later testing suggesting a functional change had been present initially.

Table XXV

Results of Pulmonary Function Studies

Patient #	Week of Training Cycle	Virus Implicated	Carbon Monoxide Diffusion[1] Initial	Final
10	6	Adenovirus Type 4 cold agglutinin pos	73%	89%
14	3	Adenovirus Type 4	68, 52, 59, 59	80
30	5	Adenovirus Type 4	91	136
40	5	Adenovirus Type 4	61	109
71	2	Adenovirus Type 4 cold agglutinin pos	84	119
72	4	Adenovirus Type undetermined	75	152
74	3	No specific etiology	66	106

[1]% of predicted value

11. Varicella Virus In Pharyngeal Secretions.

a. Although epidemiologic observations have suggested that varicella is transmitted by the airborne route and that a patient may be infective as early as 4 days prior to appearance of the rash, isolation of the causative virus has been reported only from cutaneous lesions and cerebrospinal fluid. The present study of virus excretion in the pharynx was undertaken when serendipity provided a primary infection with chickenpox in a household in which a susceptible child was then exposed.

b. The rash in the primary case developed on Feb. 18, 1963, and was typical of varicella in a 6 year old child. Throat swabs were taken from the susceptible 3 year old brother each day from Feb. 26 to Feb. 28 when rash occurred. Exposure had been continuous from long before and after onset of rash in the first patient.

c. Throat swab extracts were inoculated into continuous human amnion cell cultures (WISH) and blind passed once for a total of 24 days in this cell culture system. Difficulty in interpreting cytopathology

in these cultures necessitated passage to WI26 cells in which CPE was observed 5 days later. Tubes inoculated with throat swabs taken 2 and 1 day prior to the eruption showed CPE of the type described for varicella. Throat swab taken the day of onset of rash did not contain the agent.

d. Serial transmission of CPE and serologic identification of the agent were unsuccessful; nevertheless, comparison of the agent under study with the behavior in cell culture of vesicle fluid from a classic case of varicella indicate similarity of the agents.

e. Thus, an agent similar to varicella was isolated from the pharynx of a child 2 days and 1 day prior to onset of the rash. This study affords laboratory confirmation of the respiratory tract as a site of virus multiplication of chicken pox and therefore of the mechanism for production of infective aerosols.

12. Changes in the Small Intestine During Acute Respiratory Infections.

a. The concept that many viral illnesses with localized symptomatology, such as respiratory disease, are truly systemic infections is now widely accepted. Since further knowledge of multiorgan involvement in acute respiratory disease might enlighten methods of prevention or treatment, a study of the small intestinal mucosal changes in this disease was undertaken. Recruits with ARD were selected from the wards of Walson Army Hospital, Ft. Dix, N.J. Intubation with the Crosby capsule was performed in the usual fashion. Following biopsy, specimens of throat washing, intestinal fluid, stool and blood were obtained for virologic survey. The biopsy itself was apportioned for gross and histologic examination, electron microscopic study and attempt at in vivo proliferation of mucosal cells.

b. Twelve patients were biopsied. Clinical and virologic diagnoses are presented in Table XXVI. Histologic examination of the mucosal biopsies is incomplete at this time, but the following observations have been made. The specimens from patients with rubella are unusually hemorrhagic but villus configuration appears normal. Most of the cases diagnosed as ARD demonstrate normal areas alternating with abnormal regions of flattened villi. Hemorrhagic tendency was much less obvious. Further study of the sections is in progress.

Table XXVI

Diagnoses of Patients with Intestinal Biopsy

Patient #	Clinical Diagnosis	Virus Recovery
51	ARD	Adeno 4 - throat Polio 2 - stool
52	ARD	None
53	ARD	Adeno 4 - stool
54	Rubella	Rubella - throat
55	Rubella	None
56	ARD	None
57	ARD	None
58	Rubella	None
60	ARD	Herpes simplex - throat Polio 2 - stool
61	ARD	Herpes simplex - throat Polio 2 - stool
62	ARD	None
63	Rubella	Rubella - throat, stool Adeno 4 - stool

13. Adenovirus Infections In the Syrian Hamster. In order to obtain an animal model for the study of adenovirus transmission, and to obtain a better bioassay for vaccine than that currently used, attempts have been made to confirm and develop the preliminary observations by Binn et al that hamsters inoculated intranasally with tissue culture infective adenovirus type 4 develop neutralizing antibody, whereas those receiving heat inactivated virus failed to do so.

a. It has been possible to confirm these findings for adenovirus type 4 and preliminary results indicate that the response to adenovirus type 7 is similar. Neutralizing antibody to adenovirus types 4 and 7 has not been found in any animals prior to inoculation, nor in controls, nor heat inactivated virus inoculated animals after inoculation. Neutralizing

antibody was first noted 14 days after inoculation. The frequency of development of antibody and the dosage necessary to elicit an antibody response has not been determined yet. It has not been possible to recover the agent from the infected animals.

b. A plaque reduction test for neutralizing antibody to adenoviruses would be extremely useful in this test system because of the small amounts of serum available and the considerably higher degree of accuracy. Such a test would also be valuable in measuring human responses to vaccine. For these reasons, attempts to produce plaques by adenovirus in grivet monkey kidney cell cultures is being made. The chief difficulty has been in maintaining a viable cell sheet under agar for the long time necessary to produce plaques. Enrichment of the media and modifications of the technique are being tried.

14. A New Murine Hemadsorbing Virus.

a. 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, par. 2), an apparently undescribed hemadsorbing virus was recovered from the pooled tissues of 2 white-footed mice (Peromyscus leucopus). A tissue suspension composed of one-half of the brains, livers and spleens of 2 white-footed mice, trapped in Virginia in March, 1962, was inoculated into the yolk sac of eight 7-day-old embryonated eggs and intraperitoneally into 2 guinea pigs. One of the inoculated chick embryos was dead on the 8th day. From the yolk sac of the dead embryo a transmissible agent was recovered which readily propagated in embryonated eggs and a variety of primary and continuous cell lines. Neither of the guinea pigs inoculated with the original mouse tissue syspension developed any signs of illness.

b. The initial cytopathic change produced by peromyscus virus in a continuous monkey heart cell line was the appearance of a few discrete syncytia which were usually visible in 48 hours depending upon the number of infectious particles in the inoculum. Within a few days, the syncytia increased in size and appeared as darkened areas against a background of refractive normal cells. Terminal changes were evident in 7 to 10 days. Serial passage of the peromyscus virus was also possible in a number of other continuous cell lines of human origin (embryonic lung, amnion, liver, HEp 2 and HeLa). In all of these lines the cytopathic effect induced by the virus was similar to that observed in infected monkey heart cells. In primary rhesus kidney cells, however, the cytopathic change was markedly different, i.e., there was ballooning and intense vacuolation. The supernatant fluid obtained from monkey heart cell cultures infected with egg propagated virus constituted the seed virus. This material had an infectious titer of $10^{-5.5}$ in tube cultures of monkey heart cells, using 0.2 ml as inoculum.

c. Presence of adsorption of guinea pig erythrocytes was first detected in infected tube cultures of primary rhesus monkey cells and continuous monkey heart cell cultures. Hemadsorption inhibition tests were conducted in monkey heart cell cultures. Titers are expressed as the highest dilution of serum completely inhibiting the hemadsorbing activity of the test virus. Complement-fixing antigens were prepared from infected monkey heart cell cultures, as well as infected chorioallantoic membranes of embryonated eggs.

d. Peromyscus virus produced fatal disease in 7 to 12 days in suckling hamsters following intracerebral or intraperitoneal inoculation, and in suckling Swiss mice and white-footed mice after intracerebral injection. Weanling animals developed no signs of overt illness in 3 weeks post inoculation. Guinea pigs inoculated either intraperitoneally or intranasally for production of antiserum failed to show any evidence of illness.

e. Approximately $10^{5.0}$ $TCID_{50}$ of peromyscus virus were completely inactivated by 20% ethyl ether after 18 hours at 4°C. The virus was relatively resistant to thermal inactivation, 1 log of virus still being detectable after 6 hours at 56°C.

f. Attempts to identify the peromyscus virus or to relate it to a number of known viruses were made by hemadsorption inhibition and complement fixation tests. The hemadsorbing property of peromyscus virus was not inhibited by hyperimmune animal sera made against any of the viruses listed in Table XXVII. Furthermore, complement was not fixed by hyperimmune guinea pig serum prepared against peromyscus virus (homologous titer, 1:256) in the presence of any of the viral antigens listed in Table XXVIII. It thus appears that peromyscus virus is serologically distinct from any of the other viruses with which it was compared.

Table XXVII

Hyperimmune Animal Sera Which Failed to Inhibit Hemadsorption in Cell Cultures Infected with Peromyscus Virus

Myxoviruses	Other Viruses
Influenza A_1, A_2, B	Simian virus 40
Parainfluenza 1, 2, 3, 4	Polyoma
Mumps	Lymphocytic choriomeningitis
Simian virus 5	
Newcastle disease	

Table XXVIII

Viral Antigens Which Failed to Fix Complement in Presence of Peromyscus Virus Hyperimmune Guinea Pig Serum*

Enteroviruses	Myxoviruses
ECHO 4, 9, 16, 20	Influenza A_1, A_2, 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	Newcastle disease

Other Viruses

Simian virus 40	K virus (Kilham)
Vaccinia	Herpes virus hominis
Mouse hepatitis (Manaker)	Adenovirus (group)
Respiratory syncytial (CCA)	Polyoma
Pneumonia virus of mice	Psittacosis
Lymphocytic choriomeningitis	

*Homologous titer: 1:256

g. In hemadsorption inhibition tests performed with peromyscus virus and 48 paired human sera obtained from patients with a variety of diagnosed diseases all failed to show antibody rises to the virus. However, sera from 11 of the 48 individuals tested had detectable hemadsorption-inhibiting antibody against the virus which ranged in titer from 1:10 to greater than 1:160. In each instance, the same level of antibody was present in both early and late serum specimens. Table XXIX presents the results of hemadsorption inhibition tests to determine the presence of peromyscus virus antibody in different age groups in man. Of the 74 sera tested, 9 of 48 persons who were 7 years or older inhibited the activity of the virus at dilutions ranging from 1:10 to 1:160, while none of the children under 7 years of age contained detectable antibody at a 1:10 dilution.

Table XXIX

Peromyscus Virus Hemadsorption-inhibiting Antibody in Persons of Different Ages

Age Group (yrs)	Number Tested	Number Positive	Reciprocal of Hemadsorption-inhibiting antibody titer
½ - 2	12	0	
3 - 6	14	0	
7 - 9	13	4	20, 10, 10, 10
10 - 14	16	3	160, 20, 20
15 - 18	7	1	160
18	12	1	20

h. Table XXX presents the results of hemadsorption inhibition tests with peromyscus virus and sera obtained from 11 species of wild animals which were trapped in Virginia and from 10 strains of laboratory mice. All sera were negative except 5 of 8 pools of white-footed mouse sera.

Table XXX

Peromyscus Virus Hemadsorption-inhibiting Antibody in Sera of Wild Animals and in Laboratory Mice

Species	Tested	Positive	Reciprocal of Hemadsorption-inhibiting Antibody Titer
Mouse, white-footed (Peromyscus leucopus) pool*	8	5	160, 40, 40, 20, 20
Deer	10	0	
Fox, gray	10	0	
Fox, red	10	0	
Marmot	4	0	
Mouse, field (Microtus) pool**	7	0	
Mouse, laboratory***	100	0	
Opossum	10	0	
Rabbit, cottontail	10	0	
Racoon	10	0	
Rat, cotton	3	0	
Squirrel	3	0	

* Each white-footed mouse pool contained sera obtained from 4-8 animals.
** Each field mouse pool contained sera obtained from 2-6 animals.
*** Serum obtained from 10 mice of each of 10 different strains (GP, CFW, Balb/c, C_3H, CAF_1, DBA, AKR).

1. Several months after the original isolation of peromyscus virus, one-half of the tissues of the original 2 white-footed mice which had been stored at -70°C. since autopsy were thawed and used to prepare 2 separate suspensions, each containing the tissues of only one mouse. From one of the suspensions, a transmissible agent was recovered which was indistinguishable from peromyscus virus in cross hemadsorption inhibition and complement fixation tests.

Summary and Conclusions:

1. Adenoviruses types 4 and 7 were implicated in the majority of overt respiratory infections in 2 companies of recruits studied in a linear fashion at Fort Dix, New Jersey, February - March, 1962. Evidence is presented to show that the carrier state for adenoviruses is established in approximately 9% of the studied population, the majority occurring in now diseased persons. Ecologic observations suggest varying patterns of dissemination of adenoviruses among recruits in different barracks. Dual sequential and/or simultaneous infections with adenoviruses occurred in 0.5% of the studied group. Neutralizing antibody to disseminated adenoviruses appears to be of low titer or absent in recruits acquiring infection, and the specificity of this antibody is of value in supporting type specific diagnosis.

2. Quantitative study of rubella virus has continued to depend upon the interference phenomenon. The interfering dose$_{50}$ (IND$_{50}$) for GMK cultures measured by challenge at 10 days was found to be an accurate indicator of infective virus. This relationship does hold true for other cell lines in which rubella virus propagation occurs; in BSC-1 cells, onset of interference is delayed by comparison with GMK, and in WI26 fails to develop altogether. Attempts to produce infection or disease in mice and guinea pigs have been unsuccessful. Rubella virus is chloroform-ether and temperature sensitive. It differs from other viruses within this broad grouping in that it does not exhibit cytopathic effect, hemadsorption or hemagglutination. No serologic relationships with any of the other known viruses associated with respiratory or exanthematous diseases group have been found. Among rubella virus strains examined, production of the interference phenomenon, maximum infectivity titers attained and cell culture host range have all been identical. In addition, serologic comparisons among 6 virus strains of different temporal and geographic origin failed to give evidence of heterotypic variation. Minor antigenic differences cannot be ruled out, however, because of the relative insensitivity of the neutralization test as it is currently performed.

3. Tests for antibody to rubella virus have been performed by a GMK tissue culture neutralization test. In developing this test a number of variables including serum-virus incubation conditions, length of incubation of cultures before ECHO II virus challenge, and test dose of virus have been evaluated for their effect on measurement of human and animal serum antibody titers. The neutralization test currently in use employs serum dilutions, 10-30 IND$_{50}$ virus doses, incubation at 37°C and 5 day ECHO II virus challenge. This technique has proven satisfactory for detection of antibodies in patient and animal sera. Virus dilution neutralization tests were found to be of less value for measuring antibody to rubella virus. No serologic evidence for infection with rubella virus has been found in rhesus monkeys, rabbits, or burros, although 1 of 3 calves possessed "neutralizing" antibody. Human gamma globulin was found to contain constant amounts of neutralizing antibody irrespective of lot.

4. Study of the epidemiology of rubella in military recruits at Fort Dix, New Jersey, during February - March 1962 established the following significant points: (1) Attack rates in training regiments ranged from 3-18/1000/8 week training cycle. Maximal rates were observed in the 7th and 8th week of basic training. (2) Approximately 15% of young adult males entering military service from the 1st Army Area have no prior experience or immunity to rubella virus. Infection rates in these susceptibles during 8 weeks of basic training is 100%, however, overt rubella is recognized in less than 20% of those infected. (3) Serologic surveys of the military and their dependents for antibody to rubella and rubeola virus have been made. Incidence of acquired immunity to both viruses is low during the 1st 2 years of life (20-40%). After the 2nd year the rate of acquired immunity for the 2 viruses varies. For rubeola 90-100% are immune by age 10-12. In contrast, for rubella comparable incidence for immunity is not reached until 20-25 years.

5. Studies were undertaken to develop plaques by rubella virus in agar-overlaid cultures of African green monkey (Cercopithicus aethiops) kidney cells. The method uses the phenomenon of interference by rubella virus with the cytopathogenic effect of enteroviruses. After innumerable failures, plaques were finally produced.

6. During laboratory study of outbreaks of Asian Influenza in the winter of 1963, a new antigenic variant of A_2 virus was identified. The antigenic analysis of this new variant is reported. Further testing of this agent and its relationship to earlier Asian strains is in progress. Studies on a new isolate of B Influenza recovered from Taiwan in 1962 are also reported. Further investigation into its antigenic analysis are in progress in anticipation of future spread from its original focus.

7. Decay of several of the common viral respiratory pathogens in aerosols were studied in a rotating chamber to determine the effect of time and relative humidity upon survival. Adenovirus types 4 and 7, rubella, influenza A_2, para-influenza type 3, Coxsackie A9, ECHO 11 and a rhinovirus were recovered up to 24 hours after aerosolization. Adenovirus type 7 survived best at very high humidities and para-influenza 3 best at very low humidities.

8. A total of 44 adults and children were studied during the course of an upper respiratory infection to detect naturally occurring infective aerosols. Seven different viruses were isolated from throat washings of 23 of the subjects but in only one instance was virus detected in the aerosol sample.

9. Antibody to 6 common viruses in nasal mucus was determined in 15 subjects and compared to serum neutralizing antibody titers. Nasal antibody is absent when serum titer is very low or absent; with some viruses nasal antibody is present only when high serum titers occur. In some patients nasal antibody was noted despite low serum titers. The antibody in nasal secretions appears to be specific.

10. Seven patients were studied with pulmonary function tests following viral pneumonia (adenovirus etiology in six) at a time when radiographic changes had resolved. Five of the total showed abnormally low values for carbon monoxide diffusion upon initial testing and all but two improved to normal or greater after one to twelve weeks.

11. Isolation of varicella virus from the pharynx was accomplished in one patient as early as two days prior to onset of the exanthem.

12. Small bowel biopsy was performed in 8 recruits with ARD and 4 recruits with rubella using the Crosby capsule. Rubella patients were found to have marked hemorrhagic changes but normal appearing villi. ARD patients showed areas of abnormal villus structure.

13. Preliminary studies indicate that hamsters develop neutralizing antibody in response to tissue culture infective adenovirus but not in response to inactivated adenovirus. Attempts are being made to adapt the plaque reduction assay to adenovirus studies.

14. A transmissible hemadsorbing virus was recovered from the tissues of a white-footed mouse (Peromyscus leucopus). Cross hemadsorption inhibition and complement fixation tests failed to identify or relate peromyscus virus with a number of myxoviruses, enteroviruses, or a variety of other viruses. Antibodies to the new virus were found in a number of human and white-footed mouse sera.

Publications:

1. Parkman, P. D., Buescher, E. L. and Artenstein, M. S. Recovery of Rubella Virus from Army Recruits; Proc. Soc. Exp. Biol. and Med. III, 225-230, October 1962.

2. Weinberger, H. L. and Buescher, E. L. Preliminary Antigenic Analysis of Newly Recovered Type A Influenza Viruses. CDC Influenza Surveillance Report #76, pp18-21, 12 April 1963.

3. Bourke, A. T. C., Parkman, P. D. and Buescher, E. L. Plaques by Rubella Virus In African Green Monkey Kidney Cultures. Virology (in press).

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 806, Military Preventive Medicine

Task No. 03, Immunization (Immunization against smallpox and other viral diseases)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Biologics Research
Department of Applied Immunology
Department of Hazardous Operations
Department of Veterinary Virology
Department of Veterinary Pathology
Divisions of Communicable Disease and Immunology and Veterinary Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Joseph P. Lowenthal, ScD
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 03 Title: Immunization (Immunization against smallpox and other viral diseases)

Reporting Installation: Walter Reed Army Institute of Research
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1. The study of the stability of the original experimental lot of freeze-dried calf lymph type smallpox vaccine, initiated in 1958, has continued. After 48 months storage in the dried state at -20°C, +4°C and +22°C, this product still exceeded the WHO minimum requirements for smallpox vaccine. Another lot of dried smallpox vaccine, prepared by the same manufacturer in rubber-stoppered bottles and obtained through regular Army supply channels, showed no evidence of deterioration one year beyond its original expiration date when stored at -20°C and 4°C. This standard issue vaccine, after rehydration with the supplied diluent, showed no loss in infectivity titer when stored for 23 weeks at -20°C, -9°C or +4°C.

2. Neutralizing antibody response to duck embryo rabies vaccine was measured in two groups of veterinarians and animal handlers. One group received 0.2 ml intradermally on days 0, 7 and 30, and the other 1.0 ml subcutaneously on days 0, 7 and 30. Preliminary data indicate that a satisfactory percentage of each group responded, and that there is no significant difference in response rate between the two groups. Studies of the response of previously immunized subjects to a booster dose are also in progress.

3. A study on the serological and protective overlap among group A arthropod-borne (arbo) viruses has been initiated in the Department of Hazardous Operations, Division of Communicable Disease and Immunology, during the current reporting period. The object of the study is to de-

velop either an attenuated live virus or a formalin-killed vaccine suitable for use in man that will provide adequate immunity against those group A arbo viruses which cause epidemics in Africa and Southeast Asia. The group A viruses being employed are African Chikungunya, Thai Chikungunya, O'nyong-nyong, Mayaro B, and attenuated Venesuelan Encephalomyelitis. The rhesus monkey is being used as the experimental animal since it more nearly approximates man and permits the obtaining of serial bleedings in adequate amounts following challenge.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 03 Title: Immunization (Immunization against smallpox and other viral diseases).

Description: This task is concerned with the evaluation of material, methods and efficacy of immunization against smallpox and other viral diseases, not elsewhere covered.

Progress:

1. Vaccination Against Smallpox - Freeze-Dried Vaccine.

a. Studies on the stability of an experimental dried calf-lymph type smallpox vaccine packaged in glass sealed ampules by Wyeth Laboratories (lot 17B1-2a), initiated in 1958, have continued. As in the past four years, periodic titrations of the viable virus content of the vaccine, as measured by chorio-allantoic membrane (CAM) pock counts in embryonated eggs, were performed on samples which have been stored at various temperatures, ranging from -20°C to +56°C, since October 1958. The results of these titrations over the entire four year period are shown in figure 1. The infectivity titers of samples stored at -20°C, +4°C and +22°C for 48 months, still exceed those of samples which gave satisfactory results in humans, and also exceed the WHO minimum requirements for smallpox vaccine ($10^{7.7}$ pock-forming units per ml). At higher storage temperatures the infectivity titers continued to decline slowly, as previously noted (annual reports 1961, 1962).

b. Because of the excellent stability of this material, dried smallpox vaccine was made available to the Military Services in 1960, packaged in 10 dose and 100 dose quantities in rubber-stoppered bottles with an 18 month expiration period. Stability studies on this standard item material have also been carried out by the Department of Biologics Research, WRAIR. The results of the CAM pock counts are shown in figure 2. Although this particular lot of vaccine (150802A) was received in our laboratory through regular Army supply channels and was placed under surveillance only four months before its expiration date, there has been no loss in infectivity, as measured by the pock count, after 16 months storage at -20°C and +4°C, and only a slight loss at +22°C. Thus, this particular lot of dried vaccine, when stored at -20°C or at +4°C, has shown no evidence of deterioration one year beyond its original expiration date.

c. The stability of the standard issue vaccine, after reconstitution with the supplied diluent, was also studied. Although this vaccine was rehydrated 15 months beyond its expiration date, the results of CAM pock counts indicate no appreciable loss in infectivity titer of the

FIGURE 1

STABILITY OF DRIED SMALLPOX VACCINE

(WYETH, LOT 17B1 – 2a)

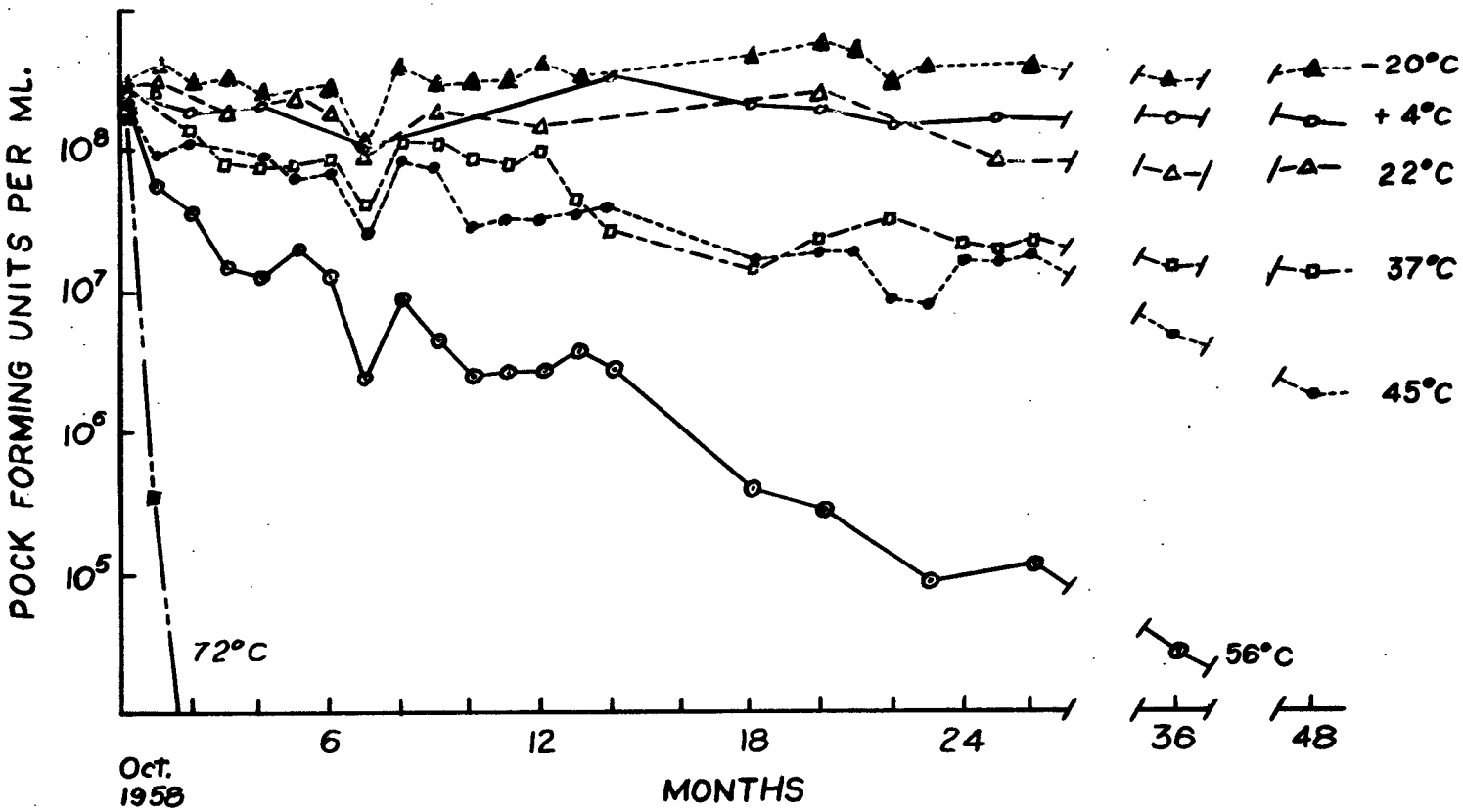
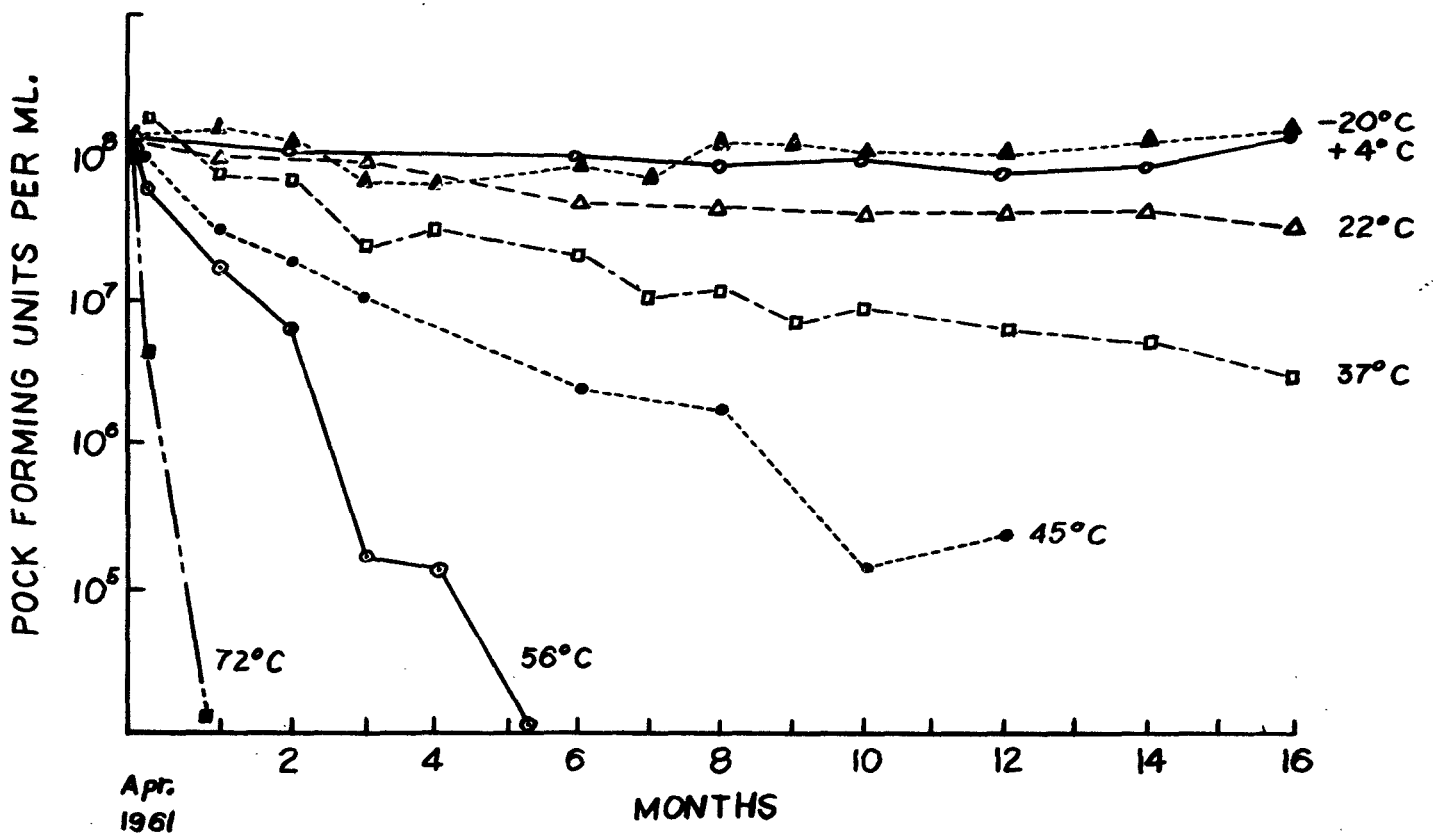


FIGURE 2

STABILITY OF DRIED SMALLPOX VACCINE
(WYETH, LOT 150802A)



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rehydrated material after storage for 23 weeks at -20°C, -9°C or +4°C. At room temperature (22°C) the titer dropped to 7.70 logs, the minimum required by WHO, after 2 weeks, and continued to decline gradually during the ensuing weeks. At 37°C there was a rapid loss in infectivity titer. These results are similar to those which were obtained earlier with the experimental lot of dried smallpox vaccine (annual report 1961).

2. Prophylactic Immunization Against Rabies.

Because of unsatisfactory results at two Army installations using duck embryo rabies vaccine given intradermally for pre-exposure immunization of high-risk personnel against rabies, a comparative study of the neutralizing antibody response following intradermal versus subcutaneous injection of this vaccine is being carried out. Subjects are approximately 60 veterinarians, animal handlers and laboratory workers who have at least potential occupational exposure to rabies virus. One group was given 0.2 ml intradermally on days 0, 7 and 30, and the second group received 1.0 ml subcutaneously on days 0, 7 and 30. Blood specimens were drawn on days 0, 30 and 60 for testing. The standard WHO screening test for neutralizing antibody (Bull. WHO, 1957, 117:915) was run on undiluted serum. Survival of 4 out of 6 test mice was considered evidence of antibody in the serum. The preliminary results are shown in the table. The fractions represent the number of specimens showing antibody out of the total number of specimens tested.

	Day Serum Sample Obtained		
Group	0	30	60
Subcutaneous	1/21	17/21	19/21
Intradermal	3/23	14/22	17/23

These preliminary data indicate that a satisfactory proportion of subjects in each group responded to the vaccine, and that there is no significant difference between the two methods evaluated. Another group of men, who had previously received some immunization against rabies, were tested for their response to a booster dose of 1.0 ml of vaccine subcutaneously. Laboratory studies on this group are still in progress.

3. Immunization Against Group A Arthropod-borne Viruses.

a. Rhesus monkeys are being employed to determine the degree of serological and cross-protection reactions encountered with the following group A arbo viruses: African Chikungunya, Thai Chikungunya, O'nyong-nyong, Mayaro B, and the attenuated strain of Venesuelan Encephalomyelitis.

b. Formalin-killed vaccines have been prepared from African green monkey kidney cell cultures and live virus vaccines from suckling mouse brains infected with the African Chikungunya virus, respectively. Both types of vaccine have shown excellent immunogenic properties when assayed in mice against the homologous virus.

c. Before considering the possibility of utilizing a tissue culture system for the propagation of African Chikungunya virus for the production of a live-attenuated or a formalin-killed vaccine, the following studies were conducted to determine the growth curve of the virus in African green monkey kidney cell cultures (MKC) followed by filtration experiments to obtain bacteriological sterility without too great a loss of viral content. The data shown in Table I and II, respectively, indicate that an excellent growth of the virus occurs in MKC and the millipore filter, type HA, may be used to obtain bacteriological sterility, with no appreciable loss in viral content.

Table I

The Growth Curve of African Chikungunya Virus (168) in Monkey Kidney Cells

Incubation 37°C	Titer Log IC LD_{50}/0.02 ml in Suckling Mice		
Time in Hours	Bottle A	Bottle B	Average Log_{10}
2	3.0	2.7	2.85
4	<2.0	<2.0	2.00
8	<3.0	<3.0	3.00
24	4.0	4.4	4.20
48	6.3	6.5>	6.40>
72	6.6	6.9	6.75

Table II

Filtration Data: Passage of Chikungunya Virus Grown in MKC Cultures Through Filters of Different Porosities with Results Determined by Log IC LD_{50}/ml in Mice

Type of Filtration	Trial A	Trial B	Average Loss (log)
None	8.6	8.4	---
Millipore HA	7.3	7.3	1.20
Sintered glass, medium	6.85	7.3	1.40
Sintered glass, fine	4.65	4.9	3.70*
Sintered glass, ultra fine	3.85	4.2	4.50**

*First through medium filter.
**First through fine filter.

d. Plaquing procedures are being currently employed in an endeavor to obtain strains of virus that may be utilized for the production of a formalin-killed or a live virus vaccine that will provide a broad spectrum protection against the group A arbo viruses under study.

e. Susceptibility of rhesus monkeys to challenge with certain group A viruses and their antibody response. Four groups of two monkeys each were inoculated with African Chikungunya (103), Thai Chikungunya (BAH 306), Mayaro B, and O'nyong-nyong (ONN) viruses, respectively. Each of

the first three groups developed a viremia except one BAH-306 monkey. All six of these animals developed complement-fixation (CF) and hemagglutination inhibiting (HI) antibodies. The two monkeys challenged with ONN virus developed neither viremia or CF and HI antibodies. Thirty days following the initial challenge, the animals were rechallenged with homologous virus and all showed a solid immunity with the exception of the ONN monkeys, who again failed to show evidence of infection. The viremia produced and the spectrum of antibody response evoked in monkeys following challenge with certain group A viruses are shown in Table III.

f. A second group of 10 rhesus monkeys first were inoculated with the attenuated strain of VEE virus; thirty days later they were divided into 5 groups of 2 monkeys each and inoculated with Chikungunya 103, Mayaro, ONN, BAH-306, and attenuated VEE virus, respectively. From available information at the time of this report, it would appear that while there is a marked serological overlap in certain instances there has been no evidence of cross protection sufficient to prevent the development of fever and viremia in the monkeys.

Summary and Conclusions:

1. The study of the stability of the original experimental lot of freeze-dried calf lymph type smallpox vaccine, initiated in 1958, has continued. After 48 months storage in the dried state at -20°C, +4°C and +22°C, this product still exceeded the WHO minimum requirements for smallpox vaccine. Another lot of dried smallpox vaccine, prepared by the same manufacturer in rubber-stoppered bottles and obtained through regular Army supply channels, showed no evidence of deterioration one year beyond its original expiration date when stored at -20°C and 4°C. This standard issue vaccine, after rehydration with the supplied diluent, showed no loss in infectivity titer when stored for 23 weeks at -20°C, -9°C or +4°C.

2. Neutralizing antibody response to duck embryo rabies vaccine was measured in two groups of veterinarians and animal handlers. One group received 0.2 ml intradermally on days 0, 7 and 30, and the other 1.0 ml subcutaneously on days 0, 7 and 30. Preliminary data indicate that a satisfactory percentage of each group responded, and that there is no significant difference in response rate between the two groups. Studies of the response of previously immunized subjects to a booster dose are also in progress.

3. The object of this study is to develop either a formalin-killed or an attenuated live virus vaccine suitable for use in man that will provide adequate immunity against those group A arthropod-borne viruses which cause epidemics in Africa and Southeast Asia. Rhesus monkeys are being used to determine the degree of serological overlap and cross protection evoked by those group A viruses endemic in Africa and Southeast Asia. A formalin-killed vaccine prepared in African green monkey kidney cell cultures infected with Chikungunya virus has shown excellent immuno-

Table III

Viremia and Antibody Response in Monkeys Following Challenge with Certain Group A Arbo Viruses

Monkey No.	Challenge Virus	Viremia	CF Antibody Against: CHIK-168	CHIK-103	Mayaro	ONN	BAH	HI Antibody Against: CHIK-168	CHIK-103	Mayaro	ONN	BAH
606	CHIK-103	+	>128	>128	16	32	>128	640	>1280	80	>1280	>1280
609	CHIK-103	+	>128	>128	16	32	>128	320	>1280	160	640	>1280
449	Mayaro B	+	4	4	64	0	4	80	40	>1280	20	40
452	Mayaro B	+	8	8	64	4	8	40	160	>1280	20	80
758	ONN-MP87	-	AC	AC	AC	AC	AC	0	0	0	10	0
769	ONN-MP87	?	0	0	0	0	0	0	0	0	0	0
619	BAH-306	+	32	>128	0	0	>128	320	>1280	40	160	>1280
621	BAH-306	-	4	8	4	0	4	20	>160	40	80	80

CHIK-103 = African Chikungunya virus
CHIK-168 = African Chikungunya virus
ONN = O'nyong-nyong virus
BAH-306 = Thai Chikungunya virus

genic properties in mice. Plaquing techniques are being used in an endeavor to select a strain of the Chikungunya virus that will be sufficiently attenuated to be used as a live virus vaccine.

List of Publications:

1. Lowenthal, J. P. Stability of freeze-dried smallpox vaccine. Presented at the meeting of the Commission on Immunization, Armed Forces Epidemiological Board, 1 April 1963.

2. Farrar, W. E. Prophylactic immunization against rabies. Presented at the meeting of the Commission on Immunization, Armed Forces Epidemiological Board, 1 April 1963.

ANNUAL PROGRESS REPORT

Project 3A O 12501 A 806, Military Preventive Medicine

Task 03, Immunization (Comparison and Evaluation of Domestic and Foreign Vaccines)

Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C. Department of Applied Immunology Division of Communicable Disease and Immunology
Period Covered by Report:	1 July 1962 through 30 June 1963
Principal Investigators:	Richard A. Finkelstein, PhD Virginia Basaca-Sevilla, MD*
Assistants:	Calvin Powell Giles C. White
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

* Bureau of Research and Laboratories, Department of Health, Manila, Republic of the Philippines.

ABSTRACT

Project 3A 0 12501 A 806	Title: Military Preventive Medicine
Task 03	Title: Immunization (Comparison and evaluation of domestic and foreign vaccines)
Reporting Installation:	Walter Reed Army Institute of Research Walter Reed Army Medical Center Washington 12, D. C.
Period Covered by Report:	1 July 1962 through 30 June 1963
Authors:	Richard A. Finkelstein, PhD Virginia Basaca-Sevilla, MD
Reports Control Symbol:	MEDDH-288
Security Classification:	UNCLASSIFIED

Our evaluation of the responses to cholera vaccines manufactured and inoculated into humans in the Philippines has continued. Sera from individuals vaccinated with three new vaccines, an El Tor vaccine, a mixed El Tor-V. cholerae vaccine and a pure cholera vaccine were tested for agglutinating antibody against Inaba and Ogawa V. cholerae strains. The results indicate that the highest titers were obtained when two doses of the El Tor vaccine were given, somewhat lower titers were obtained with the mixed vaccine, while the pure cholera vaccine elicited the lowest titers. In each case, two doses gave a somewhat greater response than one dose, but the differences were not significant in many cases in the small groups (24 subjects each) of individuals used. These vaccines had higher mouse potency than vaccines previously used in the Philippines and the human serological responses are likewise better than with the previous vaccines.

Project 3A O 12501 A 806 Title: Military Preventive Medicine

Task 03 Title: Immunization (Comparison and evaluation of domestic and foreign vaccines)

Description: Foreign and domestic vaccines, including experimental products, are evaluated in the laboratory and in the field to determine if these preparations are superior to those currently employed by the Armed Forces of the United States.

Progress: As a consequence of previous studies (WRAIR Annual Report, 1961-62), new cholera vaccines were prepared in the Philippines, starting in December, 1961, from newly opened ampules of the NIH reference cholera strains 35 (Inaba) and 41 (Ogawa). Vaccines were also prepared from Inaba and Ogawa strains of El Tor vibrios isolated during the epidemic in the Philippines and a mixed vaccine composed of El Tor and true V. cholerae strains was also manufactured in the Alabang laboratories of the Bureau of Research and Laboratories, Department of Health, Manila. These vaccines were previously (WRAIR Annual Report, 1961-62) tested and found to be of a higher mouse potency than the Philippine vaccines previously used. However, data on the human responses was lacking. Groups of subjects who received either one or two doses of the new vaccines were bled before and after immunization and the sera were tested for agglutinins against V. cholerae Inaba and Ogawa serotypes. The results are summarized in the accompanying table (Table I). In this series, the poorest responses were obtained with the pure cholera vaccine. However, in comparison with previous data (last report), even these responses were two to three times higher than were previously obtained with the old vaccines. The highest responses were obtained with the El Tor vaccine, while the El Tor-cholera mixed vaccine gave intermediate responses. Two doses of vaccine elicited somewhat greater response than a single dose, but the difference was rarely significant.

Agglutinating Antibody Response to Cholera Vaccines in Humans

(Philippines, 1962)

	El Tor Vaccine				El Tor-Cholera				Cholera Vaccine			
	1 dose		2 doses		1 dose		2 doses		1 dose		2 doses	
	In	Og	In	Og	In	Og	In	Og	In	Og	In	Og
Pre-bleeding												
Geom. Mean Titer	71	62	65	42	52	45	40	<40	67	60	60	53
95% Confidence Limits**	40-127	40-100	41-102	<40-65	<40-78	<40-66	<40-56	<40-48	48-100	<40-95	<40-105	<40-89
Post-Inoculation												
Geom. Mean Titer	360	220	601	391*	162	124	350	207*	160	113*	199*	119*
95% Confidence Limits**	236-545	166-306	425-850	338-452	103-261	78-194	249-519	129-333	103-249	80-159	122-296	77-186

* Significant difference in comparison with one or more appropriate columns to the left.

** N = 24

Summary and Conclusions: Changes in the strains used in cholera vaccine production in the Philippines are associated with increased antigenic potency in humans and increased mouse protective potency. A vaccine composed of El Tor vibrios gave the highest human agglutinin response against cholera antigens, a mixed El Tor-cholera vaccine gave intermediate responses, while a pure cholera vaccine gave the poorest responses although these were two to three times better than with previously used vaccines. Two doses gave somewhat better responses than 1 dose of the same vaccines, but the differences were rarely significant.

List of Publications: None

ANNUAL PROGRESS REPORT

Project 3A O 12501 A 806 Military Preventive Medicine

Task 03, Immunization (Responses to Immunization)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Applied Immunology
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Richard A. Finkelstein, Ph.D.
Joseph P. Lowenthal, D.Sc.
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 03 Title: Immunization (Responses to Immunization)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Authors: Richard A. Finkelstein, Ph.D.
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. A small number of individuals was inoculated with cholera vaccine produced at Forest Glen, WRAIR. Their sera were titrated in two serological procedures. The results indicate that the Forest Glen cholera vaccine is a safe product which induces a satisfactory antibody response in humans. Preliminary data indicate that it may be possible to reduce the dose of vaccine without affecting the serological response.

2. An attempt to correlate the presence and development of antibodies which inhibit the agglutination of mouse fibroblast L-929 (Earle) cells by vaccinia virus with the results of smallpox vaccination was made. Neither the presence or titre of antibody prior to vaccination could be used to predict the results of vaccination. Almost all persons had antibody present after vaccination. Studies of the results from inactivated vaccine are in progress.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 03 Title: Immunization (Responses to Immunization)

Description: This task covers the study of the reactions induced in the soldier by the administration of standard or candidate immunizing preparations, with the object of developing an immunization program with minimal adverse reactions and maximal antibody response.

Progress:

1. Responses to Immunization with Cholera Vaccine produced at Forest Glen, WRAIR. Sixteen individuals were inoculated with cholera vaccine prepared at Forest Glen for a World Health Organization field trial in India. Their sera were titrated by means of conventional agglutination tests using Ogawa and Inaba test strains and by the recently described Vibriocidal Antibody Titration. The results are summarized in Table 1. Some of the individuals had known history of previously having received cholera vaccine. In accord with our previous observations, the response to this booster dose was minimal in terms of both vibriocidal and agglutinating antibody. Subjects #7, 8 and 9, not previously immunized, received only a single dose of 0.1 ml of vaccine. It can be seen that their agglutinin response is similar to that of individuals who received a single dose of 1.0 ml. Their vibriocidal antibody rise is equivalent to that seen previously in individuals receiving 1 or 2 full doses of vaccine. No untoward reactions were observed among the individuals receiving the vaccine. It is concluded that the vaccine produced at Forest Glen is a safe product which produces an antibody response in humans similar to that which has been observed with other (commercial) cholera vaccines and cholera vaccines from other countries. The observation that a single dose of 0.1 ml in a small number of subjects produces a response similar to that with larger doses leads to the speculation whether the dose used in the routine immunization with cholera vaccine may not be reduced. These studies will be extended to a larger group of individuals who are being inoculated with 1/20 ml of vaccine.

2. Correlation of level of antibody inhibiting L cell agglutination by vaccinia virus with results of smallpox vaccination in humans. A study of the relationship of mouse "L" cell agglutination inhibiting antibody to smallpox vaccination was done in an attempt to find a faster and simpler laboratory technique for determining immunity. Vaccinia virus in high concentrations (e.g. 10^6 pfu per ml and higher) will cause agglutination of mouse fibroblast (L-929 Earle's) cells within twenty-four hours. Inhibiting antibody to this phenomenon has been reported to be related to neutralizing antibody. A study of the status of antibody levels prior to and three weeks after smallpox immunization was made.

a. Seventy-two newly commissioned officers were bled prior to and three weeks after vaccination. Following the second bleeding, all but three were revaccinated. On the initial vaccination forty had major takes, twenty-eight had minor takes and four had "no takes." On revaccination, one of the initial minor reactors and two of the initial "no takes" had major reactions.

Table 1

Response to Immunization with Forest Glen Cholera Vaccine

Subject No.	Pre- or Post-Inoculation	Antibody Titers: Agglutination Inaba	Agglutination Ogawa	Vibriocidal Inaba	Vibriocidal Ogawa
1*	Pre	320	160	10^5	10^5
	Post	320	160	10^5	10^5
2*	Pre	80	80	10^3	10^4
	Post	320	160	10^4	10^4
3*	Pre	160	160	10^4	10^4
	Post	320	160	10^4	10^4
4*	Pre	320	160	10^5	10^5
	Post	320	320	10^6	10^6
5*	Pre	320	160	10^4	10^5
	Post	640	160	10^5	10^5
6*	Pre	320	320	10^4	10^4
	Post	320	320	10^5	10^5
7**	Pre	<20	<20	$<10^1$	10^1
	Post	160	320	10^4	10^5
8**	Pre	<20	<20	10^2	10^2
	Post	320	160	10^4	10^4
9**	Pre	<20	<20	10^3	10^3
	Post	640	320	10^6	10^5
10	Pre	<20	<20		
	Post	160	160		
11	Pre	<20	20		
	Post	320	320		
12	Pre	<20	<20		
	Post	320	320		
13*	Pre	160	160		
	Post	160	160		
14*	Pre	40	20		
	Post	640	320		
15	Pre	20	<20		
	Post	160	<20		
16	Pre	<20	<20		
	Post	320	160		

*Previously immunized
**Not previously immunized: received only 0.1 ml of vaccine

b. Agglutination inhibiting antibody was present in 72.5% of the major reactors prior to vaccination and in 75% of the minor reactors. The presence of agglutination inhibiting antibody did not appear to be of value in predicting the type of response to be expected, nor did the level of antibody, since the geometric mean levels were not significantly different.

c. A four-fold or greater increase in agglutination inhibiting antibody titre was found in 85% of major reactors and 32% of minor reactors or 63% of those who had "takes." After vaccination, all persons who had major reactions had detectable antibody, 95% of those with minor reactions had detectable antibody.

d. The technical problems involved in preparing cell suspensions of over 10^6 cells/ml and the tremendous amounts of virus used as well as the limitations noted above, indicate that while this test is more practical than pock reduction tests in eggs, it is not as practical a test as the plaque reduction test in cell cultures.

Summary and Conclusions:

1. The cholera vaccine produced at Forest Glen, WRAIR, for a WHO field trial in India, is a safe product which induces a satisfactory serological response in humans. Preliminary results indicate that it may be possible to reduce the dosage of this vaccine without reducing the serological response.

2. An attempt to correlate the presence and development of antibodies which inhibit the agglutination of mouse fibroblast L-929 (Earle) cells by vaccinia virus with the results of smallpox vaccination was made. Neither the presence or titer of antibody prior to vaccination could be used to predict the results of vaccination. Almost all persons had antibody prior to vaccination. Studies of the results from inactivated vaccine are in progress.

Publications:

1. Benenson, A. S., Shively, J. N., and Vivona, S., 1963. Effect of irradiation and test system on development of tetanus antibodies. Proc. Soc. Exp. Biol. & Med., in press.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 806, Military Preventive Medicine

Task No. 03, Immunization (Development and modification of biological products).

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Biologics Research
Department of Applied Immunology
Department of Bacteriology
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Stefano Vivona, Lt Col, MC
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 03 Title: Immunization (Development and modification of biological products).

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

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Elizabeth B. Jackson, BS
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Delphine H. Clarke, MD
Jane Drzewiecki

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

Preliminary results of the WHO typhoid vaccine field trials show that the acetone-killed and dried vaccine is significantly more effective than the dried heat-phenol vaccine. A human field study of dried Q fever vaccine indicated that significant reactions can be avoided if 0.1 ml of the vaccine is given as a test dose, followed by two 1.0 ml doses if no untoward reactions occur. A phase I Q fever vaccine is being prepared, for use in comparative laboratory and human field studies with the currently used phase II vaccine. Freeze-dried Russian Spring Summer Encephalitis vaccine, after storage at 4°C for 42 months, still produced a satisfactory response in man. Field and laboratory studies on the efficacy of Eastern Equine Encephalomyelitis vaccines are continuing. Stability studies on a freeze-dried live (Strain E) typhus vaccine revealed no reduction in infectivity titer after 42 weeks storage at -20°C. A dried formalin-inactivated cholera vaccine was prepared for a WHO pilot field trial. Additional experimental freeze-drying studies to improve the potency and stability of vaccines for human use have been carried out. Studies on methods of lyophilizing, rehydrating and storing of cultures of Pasteurella tularensis have been pursued.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine.

Task No. 03 Title: Immunization (Development and modification 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 S. typhosa, Ty2 strain, seed culture, prepared in 1960 at the request of the World Health Organization for use in the production of vaccines for current and future human field studies, was continued. A summary of the results of titrations of the number of viable organisms, as determined by plate counts, on samples stored at various temperatures is as follows:

Time	4°C	22°C	37°C	45°C
6 mos.	3.50×10^9	3.93×10^8	1.46×10^6	4.67×10^4
12 mos.	1.89×10^9	1.12×10^8	$>3.00 \times 10^5$	$<10^1$
20 mos.	1.38×10^9	4.92×10^8	1.17×10^4	$<10^1$
25 mos.	1.15×10^9	4.17×10^7	2.30×10^4	$<10^1$
33 mos.	6.10×10^8	5.47×10^6	2.17×10^3	-

b. The results of the human field studies conducted by the World Health Organization on the two types of dried typhoid vaccine prepared by the Dept. of Biologics Research, WRAIR, in 1960 are still incomplete. The preliminary figures reported by the WHO from the controlled field trials in British Guiana and Yugoslavia definitely show that the acetone-killed and dried vaccine (vaccine K) is highly effective for at least two years after immunization, while the dried heat-killed phenol vaccine (vaccine L), although effective, is inferior. The results to date (May 1963) are as follows:

British Guiana

Vaccines	No. Vaccinated (Two doses)	Number of Typhoid Cases 1st Yr.	2nd Yr.	3rd Yr.*	Total
Acetone (K)	≈22,000	2	3	0	5
Phenol (L)	≈22,000	5	13	5	23
Control+	≈22,000	36	38	9	83
Total	≈66,000	43	54	14	111

*Preliminary results provided by Dr. Raymond Lewthwaite, Dept. of Technical Cooperation, London.

+Tetanus toxoid.

Yugoslavia

Vaccines	No. Vaccinated (Two doses)	Number of Typhoid Cases 1st Yr.	2nd Yr.	3rd Yr.	Total
Acetone (K)	5,028	0	15		15
Phenol (L)	5,068	0	33		33
Control+	5,039	0	67		67
Total	15,135	0	115		115

+Tetanus toxoid.

c. Approximately 1200 bottles of each of the two vaccines (K and L), of the same lot as those used in the WHO human field studies, were sent to the WHO International Laboratory for Biological Standards in Copenhagen. These have been designated by the WHO Expert Committee on Biological Standardization as the international reference preparations for typhoid vaccine. At the request of the Division of Biologics Standards, National Institutes of Health, another lot of acetone-killed and dried vaccine will be prepared in the near future by the Department of Biologics Research, WRAIR, for use by DBS as the national reference preparation for typhoid vaccine.

2. Q Fever Vaccine.

a. (1) The human field studies of dried Q fever vaccine, initiated in 1962 in cooperation with the Division of Biologics Standards, National Institutes of Health, have continued during this period. Vaccines prepared at WRAIR were distributed to a number of cooperating groups including Fort Detrick; Communicable Disease Center, Atlanta; Colorado State Department of Health, and the University of Illinois College of Veterinary Medicine. The participating groups provided, in addition to pre- and post-immunization sera from the recipients, information on residence in rural areas, previous exposure to cattle and sheep, previous contact with the Q fever vaccine or rickettsiae, results of skin tests with diluted vaccine and normal yolk sac antigen, and reactions to the three subcutaneous doses of vaccine (0.1 ml on day 0; 1.0 ml on day 4; 1.0 ml on day 11).

(2) The information received to date was analyzed to determine whether there were any consistent and practical correlations between history, results of skin tests, pre-vaccination complement fixation (CF) titers and reactions to vaccine. In Table I it is to be noted that 48 of 54 individuals (89%) with no history of exposure and 111 of 125 (89%) with exposure to cattle, sheep and/or rural areas had no detectable complement fixing antibodies in their pre-vaccination serum. The numbers in the other three groups are rather small. However, it appears that the history cannot be used reliably to predict a significant complement fixation titer and possible hypersensitivity.

Table I

Correlation of History of Exposure with Pre-Immunization CF Titer

	History of Exposure	Pre-Immunization Complement Fixation Titer						
		0	2	4	8	AC*	ND+	Totals
1	None	48	5	1	-	2	13	69
2	Cattle, Sheep and/or Rural Areas	111	7	5	2	3	19	147
3	Previous Vaccination	4		1				5
4	"2 plus 3"	11	2	2				15
5	Disease			1				1
	Totals	174	14	10	2	5	32	237

*AC = anticomplementary.
+ND = not done, sera not available.

(3) The data in Table II show that there was a very small difference in the skin test readings at 48 hours between those with no exposure and those exposed to animals and/or rural areas. There was no difference at 20 minutes. The results from the Fort Detrick group were not included in this table since they were not interpretable. (In this group all but one individual were reported to have had a positive skin test at 20 minutes.)

Table II*

Correlation of History of Exposure with Results of Skin Test

History of Exposure	Reading at 20 Mins.	No.	Reading at 48 Hours: Positive	Negative	Unknown
None (61)	Positive	1		1	
	Negative	60	3	56	1
Cattle, Sheep and/or Rural Areas (141)+	Positive	3		3	
	Negative	137	14	123	

*Ft. Detrick group excluded.
+Skin test not done on 1 individual.

(4) The data in Table III indicate that most people, regardless of history of exposure, could take 0.1 ml of vaccine or the test dose without any significant reactions. The systemic reactions reported consisted of mild headaches, feverishness from 1 to 2 days, and an occasional case with mild elevation of temperature. Thus, history cannot be used to predict the occurrence of reactions to vaccine.

Table III

Correlation of Exposure with Reactions to Vaccination

	History of Exposure	Reactions to 1st Dose	No.	Reactions to 2nd and/or 3rd Dose: Systemic	Local	None
1	None	Systemic	7	5		1
		Local	6	1	4	1
		None	51	8	22	21
2	Cattle, Sheep and/or Rural Areas	Systemic	5	3	1	
		Local	23		15	5
		None	114	14	55	44
3	Previous Vaccination	Systemic	1	1		
		Local				
		None	4	2	2	
4	"2 plus 3"	Systemic	3	2	1	
		Local	4	1	3	
		None	8	3	3	2
5	Disease	Systemic				
		Local				
		None	1		1	

(5) In Table IV an attempt was made to correlate the results of the skin test with the pre-immunization CF titer. Though there is a slight correlation between the occurrence of a positive skin test at 20 minutes and 48 hours with a significant pre-immunization CF titer, reliance upon the skin test would exclude certain persons needing vaccine and would not pick up others not needing vaccine.

Table IV

Correlation of Results of Skin Test with Pre-Immunization Titer

Results of Skin Test: 20 Mins.	48 Hrs.	Pre-Immunization Complement Fixation Titers: 0	2	4	8	AC*	ND+	Totals
Neg.	Neg.	134	11	5		5	27	182
Neg.	Pos.	12	1	1			3	17
Pos.	Neg.	22			1		2	25
Pos.	Pos.	6	2	4	1			13
Totals		174	14	10	2	5		237

*AC = anticomplementary.
+ND = not done, sera not available

(6) In Table V, where the results were tabulated in order to see what correlation there was between skin test and reactions to vaccine, it is obvious that the skin test cannot be depended upon to select

individuals for vaccination. It is seen that systemic reactions, though slight as indicated above, occurred with negative skin tests and that in other individuals with positive skin tests there were no reactions.

Table V

Correlation of Results of Skin Test with Reactions to Vaccination

Results of Skin Test		Reactions to		Reactions to 2d and/or 3d Dose Vaccine			
20 Mins.	48 Hrs.	1st Dose	No.	None	Local	Systemic	Not Given
Neg.	Neg.	None	148	61	67	20	
		Local	18	5	12		1
		Systemic	9	1	1	5	2
		Not Given	7	1			6
Neg.	Pos.	None	6	2	3	1	
		Local	9	1	5		3
		Systemic	2			2	
		Not Given					
Pos.	Neg.	None	16	3	11	2	
		Local	3		2	1	
		Systemic	2			2	
		Not Given	4				4
Pos.	Pos.	None	7		3	4	
		Local	3		2	1	
		Systemic	3		1	2	
		Not Given					

(7) In Table VI an attempt was made to correlate the pre-immunization CF titer with the reactions to vaccine. It is to be noted that some individuals with no pre-vaccination complement fixation titer had either local or mild systemic reactions and that others with a significant pre-immunization CF titer had no reaction to the vaccine. Therefore, the pre-immunization CF titer cannot be used to predict reactions to vaccine.

Table VI

Correlation of Reaction to Vaccine with Pre-Immunization CF Titer

Reactions to Vaccine Dose		Pre-Immunization Complement Fixation Titer					
1st	2nd and/or 3rd	0	2	4	8	AC*	ND*
None	None	51	4	1		2	8
	Local	74	3	3		1	9
	Systemic	16	2	3	1	1	4

Table VI cont'd

Reactions to Vaccine Dose		Pre-Immunisation Complement Fixation Titer					
1st	2nd and/or 3rd	0	2	4	8	AC*	ND#
Local	None	4					1
	Local	17	1				1
	Systemic	1		1			
	Not Given	2	1				1
Systemic	None						1
	Local	2					
	Systemic	5	2	1			3
	Not Given	1	1				

*AC = anticomplementary.
#ND = not done, sera not available.

(8) In Table VII there is a correlation presented between the pre-immunization CF titer and the post-immunization CF titer. It is to be noted that 72 out of 160 individuals with a negative pre-titer did not convert. Percentage of failure to convert was much higher among the Communicable Disease Center group that it was for the others. The history of exposure to animals and rural areas was not much different for this group, but perhaps the animals differed in that they had not been exposed to Rickettsia burneti and consequently, did not expose individuals to R. burneti.

Table VII

Correlation of Pre-Immunization with Post-Immunization CF Titers

Pre-Imm.	Post-Immunization Complement Fixation Titers									
CF Titers	0	2	4	8	16	32	64+	AC*	ND#	Totals
0	72	16	15	23	19	9	6		13	173
2		4	2	1	2	2	3			14
4				1	1	5	2		1	10
8					1				1	2
AC				2	1			2		5
ND									33	33
Totals	72	20	17	27	24	16	11	2	48	237

*AC = anticomplementary.
#ND = not done, sera not available.

(9) As indicated above the reactions to the vaccine which have been arbitrarily classified as local or systemic were mild, ordinarily. Local reactions consisted of tender and enlarged nodes, induration, local pain and tenderness at site of inoculation. The systemic reactions consisted of malaise, myalgia, fever, headache, and arthralgia. In several individuals, who had a history of previous immunization, the administration of 1.0 ml of vaccine reactivated the skin test site resulting in induration and tenderness at the site of the skin test. This occurred as long as two or three weeks after the skin test.

b. In cooperation with the Division of Biologics Standards, National Institutes of Health, production of a lot of phase I Q fever vaccine was initiated. This vaccine is being prepared by the same procedures used for the manufacturing of earlier lots of Q fever vaccine (phase II). However, the Henzerling strain of R. burneti used as seed for the current production was converted to phase I by guinea pig passage by Dr. R. Ormsbee, Rocky Mountain Laboratory, NIH. This phase I vaccine will be used in comparative laboratory and human field studies with the phase II vaccines previously prepared.

3. Russian Spring Summer Encephalitis Vaccine.

a. The study of the stability at 4°C of freeze-dried RSSE vaccine of mouse brain origin (Lot 18) was continued. The results of mouse protection tests, summarized in the following table, indicate that this particular lot of vaccine has maintained its potency when stored at 4°C for at least 38 months.

Lot No.	Period of Storage (mos.)	Protective Index (logs)
18	0	4.9
	12	4.8
	24	5.0
	38	4.6

b. Evidence was obtained by Dr. Delphine Clarke of the Rockefeller Foundation Virus Laboratories, that lot 18 dried RSSE vaccine, after storage at 4°C for 42 months, was still effective for the immunization of humans. The results of neutralization tests run at the Rockefeller Laboratories with the sera of 5 individuals receiving the full primary course of vaccine showed that 2 converted to fully protective, 1 partially protective, and 2 remained negative. Of 5 people receiving booster shots, 3 became fully protective and 2 partially protective. The 50% response level, previously observed with this vaccine, is maintained after 42 months storage.

c. Experimental studies on the preparation of RSSE vaccine by the growth of the virus in chick embryo fibroblast tissue culture were interrupted because of other commitments. However, the limited results obtained to date (annual report 1962) were sufficiently encouraging to warrant continuation of this study when time permits.

4. Eastern Equine Encephalomyelitis Vaccine.

a. An evaluation of the response of human recipients to the dried purified chick embryo Eastern Equine Encephalomyelitis (EEE) vaccine is being carried out by determination of the pre- and post-immunization serum neutralizing antibody levels employing the standard mouse neutralization test technique. The results obtained to date with several lots of the vaccine 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 Imm. Doses	Vaccine	Neutralization Index(Log_{10}) Increase* Negative	Equivocal	Positive
3 Doses	Lot 20	11	4	17
(original series)	" 21	33	3	12
	" 24	28	4	13
	25	3	1	11
		75	12	53
		(54%)	(8%)	(38%)
1 Dose	Lot 20	2		3
(Booster)	" 21	4	1	13
	" 24	6		8
	" 25	1	1	5
		13	2	29
		(30%)	(4%)	(66%)

*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 an average of 38% of the recipients of an initial series of three 0.5 ml doses of the vaccine convert from negative to positive. On the other hand, previously immunized individuals respond very well to a single booster dose, with 66% of those who are negative before the booster dose converting to positive. In addition, almost all individuals who demonstrate neutralizing antibodies before the booster show an increase in titer following the single booster dose.

b. Studies on the adaptation of the plaque-reduction technique in a tissue culture system for quantitatively measuring the neutralizing antibody levels in sera of immunized individuals have continued. Virus titrations in different lots of chick embryo fibroblast monolayers have continued to give inconsistent results, indicating the presence, in some preparations at least, of an inhibitory or an interfering agent. A systematic investigation of the plaque method is currently underway in an attempt to determine the reasons for the inconsistencies so that they can be eliminated and thus permit the use of this potentially sensitive system for evaluating the human responses to the EEE vaccines.

c. Studies on EEE vaccine produced in chick embryo fibroblast tissue culture have continued in an effort to increase the shelf life of the dried product. The addition of 2% human serum albumin to the preparation appears to protect the antigen during the freeze-drying procedures and also increases the stability of the dried vaccine.

d. Investigations are being carried out in an effort to improve the sensitivity and reliability of the guinea pig potency test for EEE vaccines. Various routes of immunization and dose levels were investi-

gated in an attempt to obtain a graded response which would permit the determination of the 50% effective dose (ED_{50}). Results obtained thus far, as shown in the following table, indicate that immunization of the guinea pigs with 0.5 ml of vaccine subcutaneously is preferable to the current procedure which utilizes intradermal doses of 0.1 ml.

Effect of Dose and Route of Immunization on Guinea Pig Response to EEE Vaccines

Vaccine	Dose* (ml)	Route	Vaccine Dilution 1/1	1/4	1/16
Tissue Culture	0.1	ID	4/10+	7/10	
	0.5	SC	10/10	9/10	
	1.0	SC	10/10	8/10	
	0.5	SC	10/10	6/10	1/10
Chick Embryo	0.1	ID	3/10	5/10	
	0.5	SC	7/10	7/10	
	1.0	SC	8/10	6/10	

*Guinea pigs immunized on days 0 and 7; challenged IC on day 21 with 100 LD_{50}.
+Survivors/Total.

5. Typhus Fever Vaccine.

a. Stability studies on lot CRD-1 of freeze-dried live (Strain E) typhus vaccine, prepared for the Commission on Rickettsial Diseases, Armed Forces Epidemiological Board (annual report 1962), were performed by Miss E. Jackson, Division of Biologics Standards, National Institutes of Health. Material stored at -20°C maintained a high embryo lethal titer and was quite stable over a 42 week period. Vaccine stored at 5°C for 31 weeks lost about one log in titer, and at 22°C for the same period a 3.5 log loss in titer was observed. At 35°C a steady drop in infectivity was noted over a 4 week period for a total loss of 2.5 logs. The results of the tests performed by Miss Jackson, in embryonated eggs and in mice, are summarized in the following table:

Stability Studies on Epidemic Typhus (Strain E) Dried Vaccine, Lot CRD-1

Storage Temperature	Storage Time	Titers Embryo LD_{50}*	Titers Toxin Mouse LD_{50}
-20°C	12 days	6.7	1:55
	8 weeks	6.6	1:55
	42 weeks	6.6	1:29
5°C	4 weeks	6.3	1:30
	31 weeks	5.9	1:30
22°C	3 weeks	6.3	1:30
	31 weeks	3.0	<1:10

Stability Studies on Epidemic Typhus (cont'd)

Storage		Titers	
Temperature	Time	Embryo LD_{50}*	Toxin Mouse LD_{50}
35°C	1 week	5.6	<1:10
	2 weeks	5.0	ND
	3 weeks	4.5	ND
	4 weeks	4.0	ND

*Embryo LD_{50} expressed as log of reciprocal of dilution at which 50% of embryos died.

Approximately 1500 bottles of Strain E typhus vaccine (lot CRD-1) are currently stored at -20°C. This material has passed all of the required tests and is available for human field studies.

b. Experimental studies on the effect of the addition of stabilizers, different methods of freezing, different rates of drying and various residual moisture levels on the stability of the live typhus vaccine were limited by other commitments. No conclusions can be drawn from the limited data obtained during this period.

6. Dried Cholera Vaccine.

a. Methods have been developed for the large scale production of a freeze-dried formalin inactivated cholera vaccine, in support of a World Health Organization project involving human field trials in India and extensive interlaboratory assay studies. Numerous small experimental lots of vaccine were prepared prior to the manufacture of vaccine for human use, to establish the methods and conditions required for the production of a large quantity of sterile, potent dried vaccine, as well as to establish methods for standardizing the final vaccine in terms of the number of bacterial cells, nitrogen content, dry weight, etc.

b. The National Institutes of Health standard cholera vaccine strains, 35A-3 (Inaba) and 41 (Ogawa), were used in these studies. Freeze-dried seed cultures of these strains, for use in the experimental studies and in the production of vaccine for human use, were prepared by harvesting the agar-grown organisms in a medium consisting of 5.2% dextran, 7.5% sucrose and 2% sodium glutamate (as described by Muggleton), freeze-drying in a chamber dryer, and sealing under vacuum with rubber closures. The dextran-sucrose-glutamate medium was selected as a result of studies of freeze-dried cholera vibrios suspended in various formulations which indicated that survival was greatest in this medium.

c. In the initial experimental studies on vaccine production, the methods which were successfully used in the production of the dried typhoid vaccines (annual report 1960) were applied to the cholera vibrio. A number of small lots of vaccine were prepared by harvesting the 18 hour growth of the cholera vibrios in Kolle flasks containing veal infusion agar. Portions of the harvest were freeze-dried after inactivation of

the organisms with 0.5% phenol or 0.1% formalin, while another portion was acetone-killed and dried. The results of mouse potency tests on these preparations indicate that the formalinized preparation gave the best results. Phenol, when present during the freeze-drying process, was deleterious. The acetone treatment also yielded a considerably less potent product.

d. Additional experimental studies demonstrated that consistently greater yields of vibrios were obtained when 3% casamino acids was employed as the growth medium in place of veal infusion.

e. The following procedure, based on the results of the experimental studies and on small scale production runs, was adopted for the production of freeze-dried cholera vaccine for human use. Kolle flasks containing 3% casamino acids in 2% agar were seeded with approximately 2 ml of a 4 hour seed culture in 3% casamino acids. After 18 hours incubation the residual seed inoculum was poured off, the growth on the surface of the agar was harvested by means of a raking device into approximately 10 ml of buffered saline, and the resulting suspension was aspirated through a gauze filter into a collecting flask. The harvests from not more than 100 Kolle flasks, all seeded with the same seed culture, were pooled as one sub-lot. After removal of a sample for the required tests and for characterization of the live harvests, sufficient neutral formalin was added to make the final concentration of formalin 0.1%. Inactivation was carried out for 4 days at room temperature and then at refrigerator temperature for at least 10 days. The two monovalent harvests were combined, just before filling and freeze-drying, in the proper proportion to yield equal quantities of Inaba and Ogawa components for the divalent vaccine. The formalin was neutralized with sodium bisulfite.

f. The equivalent of 20 liters of divalent vaccine for human use was prepared for the WHO's 1963 pilot field study. In addition, approximately 400 bottles of each of the freeze-dried monovalent components of the divalent product were sent to the WHO International Laboratory for Biological Standards, Copenhagen, for use as reference material and for the extensive laboratory studies which are planned.

g. Stability studies on the freeze-dried cholera seed material, as well as on the freeze-dried and rehydrated vaccines, are currently in progress. In addition, experimental studies are continuing in an attempt to obtain the most potent and most stable cholera vaccine for use in the large controlled field study scheduled by the WHO for 1964.

7. Freeze-Drying.

a. Additional studies have been made on methods of plug freezing and drying of Q Fever vaccine and of its suspending medium, buffered physiological saline with 2% dextrose. It was found that storage of the frozen product at -60°C for several days not only resulted in a dried product with an excellent physical appearance, but also aided in preventing

dextrose from binding with water during the drying process. Residual moisture as determined by the official NIH method was less than 0.1%. By Karl Fischer titration, which measures residual moisture and water of hydration, an average of 1.4% was found. With a product that is not stored at -60°C, the total moisture amounted to 2.6%. A 48 hour drying cycle gave a more acceptable product with regard to total moisture content than a 24 hour cycle. There was no preceptible difference in product appearance with the two cycles. No physical or chemical differences were found when the product was dried with drying stoppers in position on the bottles as opposed to drying without stoppers.

b. Most vaccines containing formalin as an inactivating agent are treated with sodium bisulfite, or some other agent, to neutralise the formalin prior to freeze-drying. The procedure is to add sufficient bisulfite to give a negative reaction in the Shryver test for aldehydes. Recent studies have indicated, however, that with certain preparations the Shryver test is not always a reliable measure of the neutralization of formalin. For example, the Shryver test gives a negative reaction with phosphate buffered physiological saline (BPS), containing 0.1% formalin, BPS containing 2% human serum albumin and 0.1% formalin, and with water containing 2% sodium glutamate and 0.1% formalin. On the other hand, quantitative formalin assays, using fuchsin-sulfurous acid as the developing agent, indicate the presence of 0.1% formalin in all cases. Neutralisation of the formalin in a given volume of vaccine can be satisfactorily accomplished by adding the calculated amount of sodium bisulfite necessary to form the addition product. After a 30 minute period (to permit the reaction to proceed), a sample of the neutralized fluid is assayed by back-titration with a standard amount of formalin.

8. The Effect of the Prefreezing Temperature, Storage Temperature and Culture Media on the Viability of Pasteurella tularensis.

a. Preservation of P. tularensis by conventional methods of lyophilization have heretofore been unsuccessful. This has necessitated maintaining the organism in a viable state by subculturing on Francis' blood-glucose-cystine agar. In order to eliminate this procedure studies were initiated on the relationship of the various physical factors involved in the freeze-drying process to survival of these organisms.

b. P. tularensis B38 was grown for 24 hours on Francis' cystine agar and the cells harvested in 10 per cent skim milk. Two-tenths ml of the cell suspension was placed in small lyophile ampules and held at room temperature until frozen. Dry ice-ethylene glycol mixtures were used to obtain the desired temperatures. The filled ampules were placed in the freezing mixtures for varying times and at various temperatures. The rate of freezing was varied between 1 and 14 minutes. Inasmuch as prefreezing for lyophilization is usually carried out at temperatures between -30°C and -60°C, temperatures of -10°C and -15°C were selected for these studies. Samples were frozen at the latter temperatures, immediately connected to the lyophile apparatus and dried overnight. Ampules were sealed under vacu-

um using a cross-fire torch. Randomly-selected ampules were opened and tested. The unopened ampules were divided into two groups; one group was stored at room temperature and the other was placed in a conventional refrigerator at 4°C. All ampules were reconstituted at 0.2 ml of glucose-cysteine-peptone broth. Two drops of the reconstituted mixture were placed on a slant or on a plate of Francis' cystine agar and the remainder was inoculated into 5.0 ml of the peptone broth medium. All cultures were incubated at 37°C and examined daily for evidence of growth. Broth media were subcultured daily to the Francis' medium. Additional ampules were opened and tested after varying storage periods.

c. Results from this small series indicated that (1) the rate of prefreezing did not appear to affect viability, (2) prefreezing within the narrow range of -10°C to -15°C resulted in recovery of viable cells, (3) the best medium for recovering rehydrated mixtures of P. tularensis B38 from the lyophilized state was the Francis cystine agar, (4) a greater number of viable cells were recovered from ampules stored at 4°C than from those held at room temperature, and (5) lyophilized cells kept at 4°C were viable for at least 5 months and cells held at room temperature remained viable for at least 3 months. Additional studies of temperature effects on survival of freeze-dried cultures are planned.

Summary and Conclusions:

1. The stability of the dried typhoid seed culture, prepared in 1960 for the World Health Organization, was followed by periodically evaluating samples stored at various temperature conditions. Preliminary figures reported by the WHO from the controlled typhoid field trials in British Guiana and Yugoslavia show that the acetone-killed and dried vaccine (K) is significantly more effective than the dried heat-killed phenol vaccine (L). Approximately 1200 bottles of each of the two typhoid vaccines (K and L) were sent to the WHO International Laboratory for Biological Standards in Copenhagen to serve as the international reference preparations for typhoid vaccine.

2. A human field trial of dried Q fever vaccine is currently being carried out in cooperation with the Division of Biologics Standards, NIH. From the data obtained thus far, it appears that neither pre-vaccination complement fixation titer or skin test, nor history of exposure to animals and rural areas will satisfactorily select those individuals who will react unfavorably to vaccination with a Q fever vaccine. A practical method to avoid significant reactions is to give 0.1 ml of the vaccine as a test dose, followed by two 1.0 ml doses if no untoward reactions occur. A phase I Q fever vaccine is currently being prepared, for use in comparative laboratory and human field studies with the currently used phase II vaccine.

3. Studies on the stability of freeze-dried Russian Spring Summer Encephalitis vaccine of mouse brain origin indicated that the product maintains its potency for mice after storage at 4°C for approximately 38 months. Results obtained by the Rockefeller Foundation Virus Laboratories demonstrate

that this vaccine, after storage at 4°C for 42 months, still produces a satisfactory response in man.

4. An evaluation of the serological response of humans to the dried purified chick embryo Eastern Equine Encephalomyelitis vaccine indicated that an average of 38% of the recipients convert from negative to positive after an initial series of the vaccine. Studies are continuing on the adaptation of the plaque-reduction technique for use in the quantitative determination of the neutralizing antibody content of immune sera. Addition of human serum albumin to an EEE tissue culture vaccine appeared to protect the antigen during the freeze-drying procedures and increased the shelf-life of the product. Preliminary studies on the guinea pig potency test for EEE vaccines indicated that immunization of animals by the subcutaneous route may be more satisfactory than by the intradermal method currently used.

5. Stability studies on a freeze-dried live (Strain E) typhus vaccine (lot CRD-1), processed at this laboratory for human use, were conducted at the National Institutes of Health. No reduction in infectivity titer was observed after 42 weeks when the vaccine was stored at -20°C.

6. The physical conditions and techniques required for the production of dried seed culture and of large quantities of sterile potent dried cholera vaccine for use by the WHO in human field trials were determined from the results obtained in experimental studies. The equivalent of 20 liters of a freeze-dried formalin inactivated divalent vaccine were prepared for the 1963 pilot field trial. In addition, freeze-dried monovalent vaccines were prepared for reference material and for extensive laboratory studies.

7. Additional experimental freeze-drying studies of Q fever vaccine and its suspending medium indicated that a dried product with an excellent physical appearance and with an optimum residual moisture content can be obtained consistently by storing the frozen product at -60°C for several days before subjecting it to a 48 hour drying cycle. Studies on the neutralization of formalin in vaccines prior to freeze-drying indicated that the Shryver test for aldehydes is not always a reliable method for determining the presence of residual formalin.

8. This study demonstrated that (1) prefreezing of P. tularensis at -10°C and -15°C was superior to prefreezing at -60°C, (2) Francis' cystine agar was better than a glucose-cysteine-peptone broth for recovery of P. tularensis from the lyophilized state, and (3) storage of lyophilized cells at 4°C was preferable to storage at room temperature.

Publications:

1. Gochenour, R. B., Lowenthal, J. P., Webster, M. E. and Berman, S. Debridement of burns on various animal species by Clostridium histolyticum proteinases. Amer. Jour. Vet. Research, 1962, 23:1089-1096.

2. Benenson, A. S., Shively, J. N. and Vivona, S. Effect of irradiation and test system on development of tetanus antibodies. Proc. Soc. Exper. Biol. & Med., 1963, 112:527-531.

3. Vivona, S. Report on the evaluation of Q fever vaccination procedures. Presented at the Annual Meeting of the Commission on Rickettsial Diseases, Armed Forces Epidemiological Board, Washington, D.C., 1 March 1963.

4. Lowenthal, J. P. Preparation of a freeze-dried cholera vaccine for WHO field studies. Presented at the Annual Meeting of the Commission on Immunization, Armed Forces Epidemiological Board, Washington, D.C., 1 April 1963.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 806, Military Preventive Medicine

Task No. 03, Immunization (Rift Valley fever - immunization of man)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Hazardous Operations
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigators: Raymond Randall, DVM*
Leonard N. Binn, PhD
Venton R. Harrison, MS

Report Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

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ABSTRACT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task No. 03 Title: Immunization (Rift Valley fever - immunization of man)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

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Reports Control Symbol: MEDDH-288

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1. The manufacture of pilot lots of Rift Valley fever virus vaccine and the determination of their immunogenic capacity in animals and man was continued during the report period. Approximately 800 doses of the vaccine were distributed to laboratories in the United States and Africa where studies on the virus are being conducted.

2. Preparations have begun toward developing commercial production scale methods and licensing of the vaccine. A lot of lyophilized vaccine has been produced that has proved to be suitable for use as the standard reference vaccine. In addition, a lyophilized lot of highly potent rabbit antiserum, prepared against the pantropic Entebbe strain of the virus, will be made available to the vaccine manufacturer as well as approved strains of the virus.

3. Several lots of the vaccine produced in this laboratory were shown to be highly antigenic for mice by the antigen extinction assay test, and the same lots when used for the immunization of human beings resulted in more than a 90% conversion to positive antibody titers of log 1.7 or greater.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Title: Immunization (Rift Valley fever - immunization of man)

Description: The object of this task is the development and production of a safe and highly immunogenic inactivated Rift Valley fever (RVF) virus vaccine for use in man.

Progress:

1. More than 1,200 persons have received either the fluid or lyophilized form of the vaccine without untoward results. More than 90% of the sera from vaccinees tested in this laboratory have shown antibody serological conversion of log 1.7 or greater.

2. Studies are being directed toward developing commercial production scale methods and finalizing specifications for procurement of the vaccine for military use. With the assistance of Dr. J. E. Smadel of the National Institutes of Health and Lt. Col. E. W. Grogan, VC, of the U. S. Army Medical Unit, Walter Reed, a tentative draft of "Minimum Requirements" for the commercial production of the vaccine is being prepared to provide a basis for the granting of a license for commercial production of the vaccine.

3. During the report period several meetings have been held with personnel of the U. S. Army Medical Unit, Walter Reed, and representatives of the National Drug Company of Swiftwater, Pa., relative to production and processing methods. This laboratory has provided vaccine for immunization of National Drug Company personnel and is prepared to furnish approved strains of the virus, a lyophilized standard reference vaccine, and an adequate supply of highly potent lyophilized rabbit antiserum free from adventitious simian agents.

4. Further studies on the immunogenic capacity of RVF vaccine in man and animals: A comparison of the antigenic potency of several lots of the vaccine both in fluid and lyophilized form was made in man and animals. The neutralizing antibody response in man was determined after a 3 dose primary series or after a single booster dose in previously vaccinated persons. In addition, simultaneous duplicate antigen extinction assays of the vaccines were carried out in mice. Two-fold dilutions of the vaccine were employed instead of the usual five-fold dilutions to increase the accuracy of the end points. The data in Table 1 shows the neutralization indices obtained in groups of individuals receiving selected lots of fluid or lyophilized vaccine and the corresponding response in mice to the antigen extinction type assay. Considering certain obvious limitations, a strong inference can be drawn between the minimal amounts of vaccine protecting 50% of the mice upon challenge with live virus and

the immunogenic response shown in the sera of man, thus permitting recognition of highly immunogenic lots of vaccine in contrast to those of lower potency.

5. Lot No. 12 lyophilized RVF vaccine was subjected to 5 replicate extinction type assays over a period of 6 months. Since a high degree of uniformity in the end points was obtained, this lot of vaccine has been selected for use as the standard reference vaccine.

Summary and Conclusions:

1. During the report period the manufacture of Rift Valley fever vaccine has been continued in order to supply current needs until the vaccine is produced commercially. Approximately 800 doses were distributed during the period for personnel working with the virus in the United States and Africa. There have been no reports of untoward reactions.

2. Further studies on the potency of Rift Valley fever virus vaccine have been conducted with human beings and animals. A strong inference can be drawn between the minimal amounts of vaccine protecting mice upon challenge and the serological immunogenic response observed in man. These data suggest that the mouse antigen extinction potency test is of value in selecting those lots of vaccine of high immunogenicity suitable for use in human beings.

3. Preparations are being made for commercial production of Rift Valley fever virus vaccine on a large scale.

List of Publications:

1. Randall, Raymond, Gibbs, C. J., Aulisio, C. G., Binn, L. N., and Harrison, V. R. The development of a formalin-killed Rift Valley fever virus vaccine for use in man. J. Immunol. 89:660-671, 1962.

2. Randall, Raymond, Binn, L. N., and Harrison, V. R. Rift Valley fever vaccine. Presented at the 11th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Atlanta, Ga., 2 November 1962. (Am. J. Trop. Med. & Hyg., in press).

3. Randall, Raymond, Harrison, V. R., and Binn, L. N. The immunological response of human beings to a formalin-inactivated Rift Valley fever virus vaccine. Presented at the 13th Annual Southwestern Conference on Diseases in Nature Transmissable to Man, San Antonio, Texas, 4-5 April 1963.

4. Binn, L. N., Randall, R., Harrison, V. R., Gibbs, C. J., Jr., and Aulisio, C. G. The serological reactions in a case of Rift Valley fever. Am. J. Trop. Med. & Hyg. 12:236-239, 1963.

5. Binn, L. N., Randall, R., Harrison, V. R., Gibbs, C. J., Jr., and Aulisio, C. G. Immunisation against Rift Valley fever: The development of vaccines from non-primate cell cultures and chick embryos. Am. J. Hyg. 77:160-168, 1963.

ANNUAL PROGRESS REPORT

Project 3A 0 12501 A 806, Military Preventive Medicine

Task 04, Ecology and Control of Disease Vectors and Reservoirs
(Arthropods of Medical Importance in Overseas Areas)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Entomology
Division of Communicable Disease and Immunology

Period Covered by Report: 1 July 1962 - 30 June 1963

Principal Investigators: Captain Bruce F. Eldridge, MSC
Captain Moufied A. Mousse, MSC
Lieutenant Eugene G. Thompson, MSC

Assistant: Pfc. Francis H. Allenza

Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

ABSTRACT

Project No. 3A 0 12501 A 806 Title: Military Preventive Medicine

Task 04 Title: Ecology and Control of Disease Vectors and Reservoirs (Arthropods of Medical Importance in Overseas Areas)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 - 30 June 1963

Authors: Captain Bruce F. Eldridge, MSC
Captain Moufied A. Moussa, MSC
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Reports Control Symbol: MEDDH-288

Security Classification: UNCLASSIFIED

1. Experiments have been conducted which indicate that ovarian development in Culex tritaeniorhynchus is influenced by the length of daily photoperiod, and that the phenomenon commonly known as "gonotrophic dissociation" exists in this species. Body fat deposition has been shown to be strongly influenced by temperature in this species, but no clear-cut evidence of photoperiodic influence has been uncovered. Mosquitoes from a colony of Culex gelidus originally collected in Malaya were found to take blood meals irrespective of the photoperiod regime to which they were exposed.

2. Laboratory studies on the effects of larval density and diet upon the growth of a colonized strain of Anopheles stephensi were initiated. It was found that maximum body weight and minimum mortality were obtained with a density level of 150 larvae per 11 x 16 inch pan. It was also found that a larval diet of dry dog food resulted in lower larval mortality and adults of greater body weight than a yeast-water mixture.

3. Laboratory studies on the bionomics of Culex gelidus were undertaken in an effort to determine criteria for estimating the physiological age of females of this species. Adults of both sexes tend to live longer under laboratory conditions when they are confined at low density levels. The relationship of female age to blood-feeding, mating, and ovarian development has been determined.

BODY OF REPORT

Project 3A 0 12501 A 806 Title: Military Preventive Medicine

Task 04 Title: Ecology and Control of Disease Vectors and Reservoirs (Arthropods of Medical Importance in Overseas Areas)

Description: This task covers a variety of studies on the ecology and bionomics of arthropods in relation to several groups of pathogenic agents and to a variety of vertebrates involved in infectious disease ecology. Current investigations are being conducted into the behavioral and physiological responses of laboratory strains of Asiatic mosquitoes to environmental factors.

Progress:

1. Influence of Daily Photoperiodicity on the Biology of Culicine Mosquitoes.

a. A question of significance in the epidemiology of Japanese encephalitis is whether or not females of the mosquito species Culex tritaeniorhynchus exhibit the phenomenon of gonotrophic dissociation (in pre-hibernating individuals, the use of ingested blood for fat synthesis rather than egg production). Although it has been shown that under the influence of autumn-length photoperiods, most C. tritaeniorhynchus females do not take blood meals, there are always a small percentage of such individuals which do. If these individuals in nature ingest virus-containing blood and subsequently hibernate, they could then serve as overwinter carriers of the virus. Gonotrophic dissociation is a phenomenon which has been thought to be limited to anopheline species, although several Russian workers claim its existance in Culex pipiens. Furthermore, these workers have found gonotrophic dissociation to be under the direct influence of daily photoperiod. Our experiments have shown that a blood meal in C. tritaeniorhynchus does not always result in ovarian development, and that a correlation does exist between the number of blood-engorged females failing to develop ovaries and exposure to short photoperiod. No quantitative determinations have as yet been made to verify the synthesis of fat from the ingested blood, but visual observations clearly show that in blood-engorged females failing to develop ovaries, the fat body is greatly enlarged. Among long-photoperiod females (17 hours of illumination daily), nearly all individuals which take a blood meal experience normal ovarian development. Out of 190 such individuals tested, 187 developed a full batch of eggs. Among 83 short-photoperiod females (11 hours of illumination daily) tested, only 63 developed eggs. These results are complicated, however, by a number of factors. Among a given number of short-photoperiod females offered a blood-meal by placing a chick in their cage overnight, only a few will take blood. We theorized that this small number of fed females could be divided into two groups: (1) those females which have been altered physiologically by exposure to short-photoperiod, and which will take blood only after many hours of exposure to the test chick, and (2) those females which for some reason are insensitive to the effects of

the short-photoperiod, remain quite aggressive, and take a blood-meal within a very short time. To test this hypothesis, we offered a chick overnight to a cage of short-photoperiod females. In the morning, those females containing bright red blood (we assumed these to be recent feeders and referable to the first group described above) were segregated from those containing dark red blood (assumed early feeders, referable to second group). These mosquitoes were held for three days and then dissected. There were only eight females which took a blood meal. Three showed dark red blood and five bright red blood. Each of the former females experienced normal ovarian development, but the ovaries of each of the latter group remained undeveloped. Although the number of individuals involved in this experiment was very small, the results tend to support our hypothesis.

b. In order to obtain larger numbers of blood-fed short-photoperiod females, groups of C. tritaeniorhynchus females were provided with a 50% defibrinated rabbit blood, 5% sucrose in water mixture. Although no attempt was made to determine the amount of blood reaching the mid-gut of individual mosquitoes, it can be assumed that any mosquito surviving more than 48 hours ingested some of the mixture, since it was the only source of moisture available to them. Furthermore, both short-photoperiod females and long-photoperiod controls were observed to have red-colored, distended abdomens. In the short-photoperiod group, of 54 mosquitoes examined after one week of this regime, only one had fully developed ovaries. Among 37 long photoperiod controls examined, 30 had ovaries with some follicles fully developed, and 6 more had partially developed ovaries. Among these control mosquitoes, however, the usual case was to find 8-10 mature follicles and the remainder of the follicles at stage 1 (resting stage). This probably reflects the low nutritional value of the blood-sucrose mixture for ovarian development.

c. Experiments are now underway to determine the effect of photoperiod and temperature on fat deposition in non-blood fed C. tritaeniorhynchus. Preliminary results show that temperature affects rate of deposition and also the amount deposited. Photoperiod has been shown to exert some influence on fat deposition, but so far no clear-cut relationship has been demonstrated.

d. A strain of Culex gelidus originally collected in Malaya was tested for the effect of daily photoperiod on blood-feedings response. It was found that females of this species took blood independently of daily photoperiod length. Since Culex gelidus does not hibernate in Malaya, and since there is very little fluctuation in daily photoperiod length, this result was anticipated.

2. Laboratory Studies on the Biology of Anopheles stephensi.

Laboratory studies on the biology of Anopheles stephensi were initiated in order to establish standard rearing techniques and to facilitate the use of this mosquito in chemotherapy studies. Larval density studies were conducted to determine the effects of crowding on mosquito growth. Three replications were run with larvae reared at four density levels - 75, 150, 300 and 600 per 11 x 16 inch pan, respectively. The average period required for all larvae to pupate at the 75 and 150 density levels was 6 and 5 days,

respectively, while larvae at the 150 and 300 levels required 9 and 8 days, respectively. Larval mortality was approximately the same at the 75, 150 and 300 levels (3-6%), while mortality at the 600 level was 29%. Fifteen adult females from each of the four levels were weighed. The mean weights of these females were 1.49 mg. for those from the 75 density level, 1.52 mg. for the 150 level, 1.31 mg. for the 300 level and 1.13 mg. for the 600 level group. A t-test indicated there was a significant difference in weight between the 150 and 300 density level groups and between the 300 and 600 level groups. The difference in weight between the 75 and 150 level groups was not significant. Studies to determine the effect of diet upon larval mortality were undertaken. Two diets are widely used for the rearing of mosquito larvae: a yeast and distilled water mixture and dry dog food. These diets, plus a combination of both, were compared. Larval mortality was highest in the yeast-water mixture series with a mean mortality of 45%. The larval mortality in the dog food series and the combination series was 18% and 24% respectively. Mean weights of females reared in these tests were 1.29 mg. for the yeast-water mixture series, 1.51 mg. for the dog food series and 1.38 mg. for the combination series. There was no significant difference between the dog food series and the combination series or between the yeast-water series and the combination series. Various degrees of pupal crowding (75-300 pupae/pint container) and variations in depth of water in the pupal emergence container did not affect the pupal or adult mortality.

3. Bionomics of Culex gelidus.

Laboratory studies were conducted on the bionomics of a laboratory strain of Culex gelidus with special emphasis on the adult stage. Results thus far indicated that, under insectary conditions, survival potentials of the adults appear to increase when they are confined at low density level (55 pairs in a 10" round-cage) and when offered a combination of 5% sugar solution, apple-slices and blood for food. Mating in cages did not occur before the third day after adult emergence. The rate of insemination rose steadily until the tenth day at which time 70% of the females had been inseminated. Blood-feeding activity began on the third day after emergence though occasionally some females fed on blood when one day old. Females took from 83 to 410 seconds to become fully engorged on human blood imbibing from 1.189 to 1.769 milligrams of blood. Blood-feeding was a prerequisite for egg maturation in this mosquito. The first sign of ovarian development appeared 5 hours after blood ingestion, when the egg follicle became oval, with the yolk granules occupying up to half the follicle. The eggs reached maturation 144 hours following a blood-meal (human-blood) at which time the nurse cells had disappeared and the micropile had formed.

In an attempt to determine the physiological age of females it was observed that considerable variation existed in the length of the first gonotrophic cycle especially in relation to age of female at time of blood-feeding and the kind of blood offered. Follicular relics in the ovarioles are easily recognizable although the number of successive gonotrophic cycles has not yet been established. Further studies are planned to determine whether any external characters can be utilized in age-grading females of this species.

Summary and Conclusions:

1. Experimental evidence indicates that females of the species Culex tritaeniorhynchus exhibit the phenomenon of gonotrophic dissociation. Temperature has a much greater effect upon fat deposition in this species than does daily photoperiod length. Blood-feeding response in the Malayan mosquito Culex gelidus is not affected by daily photoperiod length.

2. A larval diet of dry dog food and a density level of 150 larvae per 11 x 16 inch pan result in maximum body weight and minimum larval mortality in a colonized strain of Anopheles stephensi.

3. Results of laboratory experiments indicate that density of adults in cages influences longevity of adult life. The relationship of female age to blood-feeding, mating, and ovarian development has been determined. These data will contribute to age grouping techniques for this species.

Publications:

1. Barnes, W. W., Eldridge, B. F., Greenberg, J. H., and S. Vivona. A field evaluation of malathion dust for the control of body lice. Jour. Econ. Ent. 55: 591-594, 1962.

2. Eldridge, B. F. The influence of daily photoperiod on blood-feeding activity of Culex tritaeniorhynchus Giles. Amer. Jour. of Hyg. 77: 49-53, 1963.

ANNUAL PROGRESS REPORT

Project No. 3A 0 12501 A 806, MILITARY PREVENTIVE MEDICINE

Task No. 04, Ecology and Control of Disease Vectors and Reservoirs
(Diagnosis of canine filariasis)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Department of Veterinary Microbiology
Division of Veterinary Medicine

Period Covered by Report: 1 July 1962 through 30 June 1963

Principal Investigator: John Rigg, D.V.M.

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

ABSTRACT

Project No. 3A 0 12501 A 806 Title: MILITARY PREVENTIVE MEDICINE

Task No. 04 Title: Ecology and Control of Disease Vectors and Reservoirs (Diagnosis of canine filariasis)

Reporting Installation: Walter Reed Army Institute of Research
Walter Reed Army Medical Center
Washington 12, D. C.

Period Covered by Report: 1 July 1962 through 30 June 1963

Author: John Rigg, D.V.M.

Reports Control Symbol: MEDDH-288

Security Classification: Unclassified

Surveys of canine microfilariasis in sentry dogs located in Pacific Air Force Commands were completed. The prevalence of positive findings ranged from 1.4 to 21.6%, which was within the range of findings obtained in various sections of the U. S. The percentages of infected dogs in bases in Japan and Korea (5.5 and 1.4%) were remarkably low, although 30% of the same animals had been found to be infected previously. The reduction in prevalence reflects the efficacy of chemotherapeutic measures that were applied in that area.

BODY OF REPORT

Project No. 3A 0 12501 A 806 Title: MILITARY PREVENTIVE MEDICINE

Task No. 04 Title: Ecology and Control of Disease Vectors and Reservoirs (Diagnosis of canine filariasis)

Description:

A survey of Air Force sentry dogs was conducted to determine the relative incidence of canine filariasis in various geographical regions to provide guidelines on the efficacy of current control measures.

Progress:

A survey of sentry dogs in various Air Force installations in the Pacific area was completed employing the acridine orange staining technic for blood examination. The distribution of positive dogs by country is shown in the following table:

Country	No. of Dogs Examined	Positive Dogs No.	%
Japan	135	10	5.5
Korea	73	1	1.4
Okinawa	113	11	9.7
Philippine Islands	51	11	21.6

The range of positive findings, 1.4 to 21.6% was within that found in the United States. The highest prevalence of filariasis was found in dogs from the Philippine Islands. A remarkably low percentage of dogs in Korea and Japan had filariasis. From information provided from the Commands, in Japan and Korea, filariasis had been found previously in 30% of the same dogs. The remarkable reduction in positive findings reflect the efficacy of treatment measures being applied in these areas. Most of the bases in this area reported treatment of dogs either with "Filicide" or "Caparsolate" given intravenously at dosage of 1 mg per pound body weight daily for 2 to 3 days, followed by oral administration of either Diathiazamine (10 mg/lb/for 10 days) or "Coracide" (10 mg/lb/for 3 days).

Screening of European Air Force sentry dogs is now in progress. To date, no positives were found in 159 blood samples submitted from Germany.

Summary and Conclusions:

Surveys of canine microfilariasis in sentry dogs located in Pacific Air Force Commands were completed. The prevalence of positive findings ranged from 1.4 to 21.6%, which was within the range of findings obtained in various sections of the U. S. The percentages of infected dogs in bases in Japan and Korea (5.5 and 1.4%) were remarkably low, although 30% of the same animals had been found to be infected previously. The reduction in prevalences reflects the efficacy of chemotherapeutic measures that were applied in that area.