NEW LIMITATION CHANGE

TO
Approved for public release, distribution unlimited

FROM
Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; MAR 1966. Other requests shall be referred to U.S. Army Medical Unit, Fort Detrick, MD 21701.

AUTHORITY
USAMRIID ltr, 9 Jul 1971
U. S. ARMY MEDICAL UNIT
FORT DETRICK, FREDERICK, MARYLAND  21701

SPECIAL REPORT
TO THE
COMMISSION ON EPIDEMIOLOGICAL SURVEY
OF THE
ARMED FORCES EPIDEMIOLOGICAL BOARD

MARCH 1966

Project No. 1B533001D164 03

COMPILED BY

HARRY G. DANGERFIELD, MAJOR, MC
ABSTRACT

A review of the research program directed toward determining the biological effect of staphylococcal enterotoxin B is presented.
FOREWORD

The papers presented here were given at the Executive Session of the Commission on Epidemiological Survey on 10 September 1965. Other unclassified reports were presented 9 September and were published separately February 1966.

In conducting the research described the investigators adhered to the principles of laboratory animal care as established by the National Society for Medical Research.

The technical assistance of Mrs. Phebe W. Summers in preparing the illustrations and editing the report is gratefully acknowledged.

DAN CROZIER
Colonel, MC
Commanding
# TABLE OF CONTENTS

| Abstract | iii |
| Foreword | iv |
| Pyrogenic Effects of Staphylococcal Enterotoxin B, Frank A. Carozza, Jr., Captain, MC | 1 |
| Clearance and Localization Kinetics of Radioactive Labeled Staphylococcal Enterotoxin B | |
| Part I, Morton I. Rapoport, Captain, MC | 14 |
| Part II, Leland F. Hodoval, Captain, VC | 29 |
| Part III, Earl L. Morris, Captain, VC | 35 |
| Effects of Staphylococcal Enterotoxin B on the Coagulation Mechanism and Leukocytic Response in Beagle Dogs --- A Preliminary Study, Charles F. Gilbert, Captain, MC | 42 |
| Serological Methods for Detection of Staphylococcal Enterotoxin B Antibody, Martha K. Ward, Captain, USPHS | 49 |
| Detection and Measurement of Immunological Responses to Staphylococcal Enterotoxin B, Virginia G. McGann, Ph.D. | 56 |
| Distribution List | 68 |
| DD Form 1473 | 69 |
Recent evidence has indicated that staphylococcal enterotoxin B (SEB) exhibits a pyrogenic effect when administered parenterally to laboratory animals. Laboratory workers accidentally exposed to this material have also become markedly febrile. The present investigations were carried out in an attempt to define more closely the febrile response to enterotoxin in one species, the rabbit, and describe its mechanism of action.

Because fever and other physiologic changes, induced in experimental animals by SEB are similar to those induced by bacterial endotoxin, it was necessary to demonstrate first that the pyrogenicity of enterotoxin was not due to contaminating endotoxin. At the spring meeting of the Commission, evidence obtained in preliminary studies was presented indicating that pyrogenicity was an intrinsic property of enterotoxin. These data are reviewed briefly. The possibility of endotoxin contamination seemed unlikely since rigorous analysis of the SEB prepared and used at Fort Detrick had revealed it to be an essentially pure substance. Nevertheless bacterial endotoxins are ubiquitous and elusive substances; contamination of other materials with them is difficult to control. In fact, certain pharmacologic effects of endotoxin such as fever may be caused by trace amounts, hundredths of a microgram. To rule out the possibility that the pyrogenicity of SEB was due to contamination by trace amounts of endotoxin, a comparison was made of the febrile responses of 2-kg albino rabbits to each of these substances.

In Figure 1 the solid line shows the composite febrile response of 6 animals each given 10.0 μg/kg of SEB intravenously (IV). The interrupted line represents the composite response of 5 normal rabbits to a single IV injection of 1.0 μg/kg of Escherichia coli endotoxin. The area beneath each curve is called the fever index and is a measure of pyrogenic activity. The fever indices for each group are almost identical over a 5-hr period; the shapes of the curves, however, are markedly different. The pyrogenic response to endotoxin was significantly higher at 1 hr than that produced by enterotoxin. At approximately 1 to 1½ hr the response of endotoxin recipients leveled off to form the initial part of the typical biphasic response. At 2 hr, temperatures of endotoxin-challenged animals began to rise rapidly. A peak of 3.3°F above baseline temperature was reached by 3 hr; over the following 2 hr the temperature rapidly fell to near normal. In contrast the temperature of enterotoxin recipients rose gradually and by 5 hr reached a significantly higher mean temperature increment than the endotoxin group. In fact, peak increments did not occur until 6-7 hr following challenge. The difference in the shapes of these two curves is significant and suggested that the fever produced by enterotoxin was not due to endotoxin contamination.

* Medical Division.
FIGURE 1. COMPOSITE FEBRILE RESPONSE OF RABBITS TO A SINGLE IV INJECTION OF STAPHYLOCOCCAL ENTFROTOXIN B (SEB) OR ENDOTOXIN (ET).
If pyrogenic effects of enterotoxin had been due to endotoxin contamination it should have been possible to render rabbits tolerant by repeated injections; in addition after repeated injections of enterotoxin, rabbits should have been tolerant to a single injection of bacterial endotoxin. Figure 2 shows that this was not the case. A group of normal albino rabbits was injected IV on day 0 with 10.0 μg/kg of enterotoxin. The mean 5-hr fever index was 1062. This was not significantly different from the response of 5 normal rabbits given 1 μg/kg of E. coli endotoxin, where the 5-hr index was 1106. When the enterotoxin group was rechallenged with the same dose of enterotoxin on days 3, 9 and 14, there was some suggestion of a decrease in mean fever index but this was not significant. On day 15, after 4 injections of enterotoxin, these animals were given 1.0 μg/kg of endotoxin. The response of these enterotoxin pretreated animals was approximately that observed in normal animals receiving the same endotoxin dose.

The converse of this experiment was also performed. If the pyrogenic effects of SEB were due to endotoxin contamination, animals rendered tolerant to endotoxin should have been refractory to the pyrogenic effects of enterotoxin. Examination of Figure 3 reveals that tolerance to a large amount of bacterial endotoxin did not confer tolerance to the pyrogenic effects of enterotoxin. Rabbits were rendered tolerant by 5 IV injections of 10 μg/kg of E. coli endotoxin during a 9 day period. The mean fever index on day 9 was 802. This reflected a high degree of tolerance since 5 normal rabbits given half the dose of the same endotoxin had a mean fever index of 1532. In spite of the high degree of endotoxin tolerance, rabbits challenged with 10.0 μg/kg of SEB on day 12 reacted with as much fever as normal animals receiving the same amount of enterotoxin. Thus endotoxin-tolerant animals were not refractory to the pyrogenic effects of staphylococcal enterotoxin. The results of these studies indicated that pyrogenicity was an intrinsic property of SEB and was not due to endotoxin contamination.

Since these data were obtained, a further study has confirmed that pyrogenicity of SEB is not due to trace amounts of endotoxin. Although 4 injections of enterotoxin over a 2-week period had failed to induce significant diminution of the febrile response, rabbits were rendered pyrogenically refractory to staphylococcal enterotoxin by single daily injections (Figure 4). The bar on the left shows the mean pyrogenic response of 5 normal animals to 10 μg/kg of SEB; the mean fever index is 988 and not significantly different from the response of normal rabbits to 1.0 μg/kg endotoxin shown at the extreme right. Enterotoxin was then administered by single daily IV injections. On day 4 the response to the fifth injection of 10 μg/kg of enterotoxin was reduced significantly to 187. On the following day these animals, pyrogenically refractory to enterotoxin, were challenged with 1.0 μg/kg endotoxin and responded as normals receiving the same dose. Therefore it is apparent that pyrogenic tolerance to endotoxin could not be induced with repeated challenges of enterotoxin even though after 4 daily enterotoxin injections animals became markedly refractory to its pyrogenic effects.
FIGURE 2. FEBRILE RESPONSE OF RABBITS, PRE-TREATED WITH STAPHYLOCOCCAL ENTEROTOXIN B (SEB), TO E. COLI ENDOTOXIN (ET).
FIGURE 3. FEBRILE RESPONSE OF RABBITS, MADE TOLERANT TO E. COLI ENDOTOXIN (ET), TO STAPHYLOCOCCAL ENTEROTOXIN B (SEB).
FIGURE 4. FEBRILE RESPONSE OF 5 RABBITS TO E. COLI ENDOTOXIN (ET) AFTER 5 DAILY INJECTIONS OF STAPHYLOCOCCAL ENTEROTOXIN B (SEB).
Having established that enterotoxin was intrinsically pyrogenic, studies were performed to determine the mechanism of this effect. Because of the latent period of \(\frac{1}{2}\) - 1 hr from the time of injection until an appreciable rise in the temperature of the rabbit, it seemed likely that an intermediate pyrogenic substance might be activated in vivo by the toxin. Furthermore, since enterotoxin affects leukocytes in vivo similarly to endotoxin, \(\frac{1}{2}\) it seemed likely that this intermediate substance might be an endogenous pyrogen similar to that which mediates fever after endotoxin administration. Figure 5 shows that after enterotoxin administration an endogenous pyrogen is released. Six normal rabbits were given 10 \(\mu\)g/kg of SEB and the resultant composite temperature curve is shown at the left. Four hr postinjection animals were exsanguinated; the plasma was separated and frozen at -20 \(^\circ\)C. Because peak temperatures occurred 6-7 hr after enterotoxin administration, 4 hr was felt to be a time when endogenous pyrogen would be maximal. Two days later this plasma was assayed for endogenous pyrogen in rabbits rendered refractory to SEB by 8 daily injections. Recipient animals were rendered refractory in order to negate the possibility that any pyrogenic substance in the donor plasma was merely enterotoxin. The broken curve on the right shows the composite response of the 6 recipients to 10 \(\mu\)g/kg SEB on the day prior to the endogenous pyrogen assay and reflects a high degree of refractoriness. When these animals received 15 ml/kg of donor plasma IV, a sharp increment in temperature occurred as shown by the solid line on the right. This fever occurred promptly, peaked to about 1.5 \(^\circ\)F above baseline and disappeared by 3 hr. Such a response is typical of that caused by the endogenous pyrogen released by bacterial endotoxins. \(\frac{1}{3}\) Enterotoxin-refractory rabbits who received normal donor plasma showed no response.

A similar study further confirmed that the release of endogenous pyrogen by enterotoxin is not due to endotoxin contamination. Donor animals were pretreated with daily injections of E. coli endotoxin for 1 week prior to enterotoxin administration as shown at the left in Figure 6. The monophasic temperature curve represents the endotoxin-tolerant state. These animals presumably had no circulating endogenous pyrogen 4 hr after endotoxin injection. The remainder of this study was carried out as described previously. Four hours after enterotoxin administration animals were bled; plasma was assayed in enterotoxin refractory recipients for endogenous pyrogen. A prompt monophasic fever similar to that seen before ensued. Thus staphylococcal enterotoxin does induce an endogenous pyrogen in recipient animals, which probably mediates the febrile response.

It was stated earlier that after 4 single daily IV injections of SEB rabbits become refractory to its pyrogenic effects. The remainder of this report is concerned with a further examination of this state and a preliminary investigation of mechanisms responsible for it.

Figure 7 shows the response of 4 normal animals to 10 \(\mu\)g/kg enterotoxin, the curve labeled day 0. The mean 6-hr fever index of 1,000 is shown on the right. On the next day when rechallenged with the same dose there was a slight, but not significant, decrease in the mean fever index. Single daily
NORMAL DONORS (6)  
SEB REFRACTORY RECIPIENTS (6)

\[ \text{SEB} 10.0 \, \mu g/kg \, \text{IV} \]

\[ \text{SEB} 10.0 \, \mu g/kg \, \text{IV} \]

**FIGURE 5.** TRANSFER OF ENDogenous PYROGEN INDUCED BY SEB IN NORMAL ANIMALS.
ENDOTOXIN TOLERANT DONORS (7)

SEB REFRACTORY RECIPIENTS (4)

E. COLI ENDOTOXIN 1.0 µg/kg IV

SEB 10.0 µg/kg IV

BLEED

PLASMA (E.P.)

18 ml/kg IV

FIGURE 6. TRANSFER OF ENDOGENOUS PYROGEN INDUCED BY SEB IN ENDOTOXIN-TOLERANT ANIMALS.
FIGURE 7. PYROGENIC RESPONSES OF 4 RABBITS TO REPEATED SEB ADMINISTRATION FOR 5 DAYS AND FOLLOWING 2 DAYS REST.
Injections were continued and by day 4, the 5th challenge, animals were pyrogenically refractory. Animals were not challenged with enterotoxin on the subsequent 2 days. However, after this period of rest rabbits challenged with the same dose exhibited an almost normal febrile response to enterotoxin with a fever index approaching that observed after the 1st injection. Thus the pyrogenic refractory state induced by 4 injections of enterotoxin was transient and almost completely abolished by 2 days rest.

Studies were then initiated to determine the mechanism of the enterotoxin pyrogenic refractory state. Its transient nature suggested that the animals might be nonspecifically refractory due to loss of sensitivity of the thermoregulatory centers to pyrogenic stimuli or inability to further mobilize endogenous pyrogen. Because refractory animals responded normally to passive transfer of endogenous pyrogen and to bacterial endotoxin, however, this possibility seemed unlikely.

Another possibility was that refractory rabbits might be protected from the pyrogenic effect of enterotoxin by a circulating humoral factor or antibody. No antibody, however, could be detected in control or refractory state sera employing the agar diffusion test or tanned sheep cell hemagglutination test.

Nevertheless, the possibility of a circulating protective factor, not measured by the serologic tests employed, remained. Therefore an attempt was made to demonstrate such a factor by means of passive transfer of the refractory state. Figure 8 shows that this could not be done. Eight rabbits were rendered pyrogenically refractory to SEB by 4 daily injections. The pyrogenic reaction to the 5th challenge is shown on the left. On the following day animals were bled; plasma was separated and frozen. Normal rabbit plasma was also collected and similarly treated. Two weeks later 6 normal rabbits were given IV 15 ml/kg of refractory state plasma and 6, 15 ml/kg normal plasma. One hour postinjection all animals were challenged with 10 μg/kg IV of SEB. Results are shown to the right. The mean 6-hr fever index did not differ significantly between the 2 groups receiving donor plasma. Moreover, the mean fever index for each group was the same as normal animals receiving the same dose. Thus, no circulating protective factor could be demonstrated in animals pyrogenically refractory to SEB.

Other mechanisms, which may mediate this state such as hyperactivity of the reticuloendothelial system and specific desensitization to enterotoxin are presently under investigation.

SUMMARY

Pyrogenicity is an intrinsic property of staphylococcal enterotoxin B.

Upon intravenous administration in the rabbit, enterotoxin releases an endogenous pyrogen.
COMPOSITE FEBRILE RESPONSE OF 8 PYROGENIC REFRACTORY DONORS TO SEB.

SEB 10.0 µg/kg IV

NORMAL PRETREATMENT

NORMAL REFRACTORY DONOR

PLASMA

PLASMA

MEAN 6- HR. FEVER INDEX X10^2 (±S.E.)

FIGURE 8. PYROGENIC EFFECT OF SEB ON RABBITS PRE-TREATED WITH PLASMA FROM PYROGENIC REFRACTORY DONORS.
Rabbits become pyrogenically refractory to enterotoxin after 4 single daily intravenous injections. This state is transitory and disappears within 2 days if animals are not repeatedly challenged. The refractory state is probably specific for enterotoxin since refractory animals react normally to endotoxin and endogenous pyrogen. The refractory state is not mediated by a demonstrable circulating humoral protective factor.

LITERATURE CITED


Clearance and Localization Kinetics of Radioactive Labeled Staphylococcal Enterotoxin B

Part I

Morton I. Rapoport, Captain, MC

Detailed physiological data obtained as a result of investigations into the mechanism of action of staphylococcal enterotoxin B (SEB) have been presented in a previous session of this Commission. Despite the chemical dissimilarity of SEB and bacterial endotoxin, they do share some important physiological responses. In addition to fever, both are capable of producing hypotension, anemia, leukopenia and alterations of glucose metabolism and catecholamine response. In contrast to these similarities, the author presented data at the last session of the Commission that suggested that SEB was reversibly bound to tissues and that this phenomenon was important in defining its mechanism of action.

This binding concept became apparent with the use of the highly specific equine origin antibody, staphylococcal enterotoxin B antitoxin Lot 1, prepared by Lt Colonel Grogan.* We have continued to use this antiserum as a means of learning more regarding the mechanisms by which SEB is lethal. As a result, we have gathered significant additional information by a variety of approaches all of which support the concept of reversible tissue binding as a factor in toxemia and provide a valid approach toward therapy. Data presented in April 1965 are reviewed. Results of recent studies on the interaction of SEB with the reticuloendothelial system (RES) and specific binding sites in tissues is presented. This work permits the binding concept to be considered in a much more detailed fashion.

The interaction of toxin and tissue sites first became apparent as a result of clearance studies. SEB can be tagged easily with radiiodine (I131); subsequent work indicated this tag was relatively stable. The isotope appeared to have no demonstrable physiological or immunological effect on the toxin.

Figure 1 shows the clearance from plasma of SEB-I131 in a group of monkeys given 1,000 µg/kg (approximately 30 LD50) of body weight. The solid line indicates total plasma radioactivity and the broken line, toxin activity. The latter was determined by trichloroacetic acid (TCA) precipitation of protein bound radioactivity. Throughout this discussion protein bound radioactivity will be equated with toxin radioactivity since spontaneous adherence of free I131 to serum proteins is comparatively minimal. Clearance is plotted as per cent of initial counts per minute (CPM).

* Physical Sciences Division.
# Assistant to the Commanding Officer.
Disappearance of toxin from plasma was exceedingly rapid, with a half-disappearance time ($t_{1/2}$) of 7 min. During the initial 60 min, total plasma radioactivity appeared to reflect accurately toxin activity. Thereafter, delodination, indicated by the separation of the curves, progressed at a rate sufficiently rapid so as to render a measurement of total plasma radioactivity misleading. The slight rise in total plasma radioactivity after 3 hr represents the recirculation of free $1^{131}I$.

In contrast are the results shown in Figure 2 from a group of monkeys given type-specific antitoxin prophylactically 30 min prior to challenge; a marked delay in clearance of SEB-$I^{131}$ was evident, the $t_{1/2}$ being $>5$ hr rather than the previously observed 7 min. The rate of delodination of toxin was also inhibited as demonstrated by the minimal separation of the 2 curves. The toxin was held in the circulation and was metabolized, presumably very gradually, or altered in a manner which prevented it from causing death.

In view of earlier work indicating a reversal of toxemia with delayed administration of antitoxin, experiments were designed to test the effect, if any, on clearance of SEB-$I^{131}$ when antitoxin was administered therapeutically.

Figure 3 shows the effect of different quantities of antitoxin administered 2 hr after SEB-$I^{131}$ challenge. The open circles indicate a group of 4 animals given antitoxin, 6 ml/kg of body weight, and the closed circles 4 animals given 3 ml/kg. Toxin removal from the circulation was not only halted but there was a rather pronounced return of radioactivity to the plasma. While the differences were not statistically significant due to the relatively small number of animals studied it appeared that the higher dose of antitoxin returned a larger amount of radioactivity to the circulation; returned radioactivity was almost exclusively toxin radioactivity as indicated by the broken lines. In addition, the return of toxin to the circulation was even more pronounced when animals were given antitoxin 30 min after challenge.

It was clear that despite rapid removal of SEB from the circulation this did not imply detoxification since animals given type-specific antibody prophylactically, were protected but, paradoxically, cleared SEB at a much slower rate. One might conclude that animals not receiving antitoxin cleared the material rapidly with the result that the toxin established itself upon or within vital sites, whereas, toxin did not reach those vital sites in animals receiving prophylactic antitoxin. Furthermore, delayed administration of antibody reversed the clearance pattern and returned a portion of the toxin to the circulation. This finding suggested that SEB was bound or attached to certain sites from which it could be removed and concomitantly, toxicity reversed.
FIGURE 1. DISAPPEARANCE OF SEB-1\(^{131}\) IN 6 MONKEYS.

FIGURE 2. DISAPPEARANCE OF SEB-1\(^{131}\) IN 4 MONKEYS GIVEN ANTITOXIN PRIOR TO CHALLENGE.

FIGURE 3. REAPPEARANCE OF SEB-1\(^{131}\) AFTER ADMINISTRATION OF ANTITOXIN AT TWO DOSAGE SCHEDULES.
The concept is presented in equation form in Figure 4. The very rapid removal of SEB from the circulation implies that the number of potential tissue binding sites far exceeds the number of toxin molecules administered.

Figure 5 shows that the addition of type-specific antibody reverses the equilibrium; so that less SEB is bound to tissue sites. More simply stated, the binding affinity of SEB for antibody seemed to exceed the binding affinity of SEB for tissue sites. This concept seemed most attractive and additional studies designed to test this hypothesis are reported.

In an attempt to gain knowledge regarding interaction of SEB and a specific tissue site, and in addition, to measure a response to physiological stress, we selected adrenal cortical function as a study model. Bacterial endotoxin has been investigated extensively in regard to adrenal physiology and found to induce an adrenal glucocorticoid response. This observation has been a source of valuable information both from a mechanistic as well as therapeutic aspect.

Values of plasma Porter Silber chromogens in monkeys under several experimental conditions are shown in Figure 6. Each curve represents a group of monkeys bled serially during a 24-hr period (number of animals studied are shown in parentheses). To avoid iatrogenic hypovolemia during the study each bleeding was immediately followed by replacement of an equal volume of banked whole monkey blood. Challenge materials were administered immediately after the 0 time bleeding.

The broken line represents values from a group of control monkeys given saline intravenously (IV). Plasma corticoid concentrations showed only the slight fluctuation to be expected in a normal diurnal pattern: this implied that the handling and bleeding procedures were not in themselves sufficiently stressful to evoke an adrenal response. The dotted line indicates a group of monkeys each of which received 4 units/kg of adrenocorticotropin (ACTH) in single IV injections. The maximum stimulation occurred at 4 hr with the mean value being 81 µg/100 ml; thereafter, plasma hydroxycorticoids returned to control concentrations. The 3rd group of animals received IV 100 µg/kg of SEB; the values are indicated by the solid line. Following challenge there was a progressive rise reaching a peak of 127 µg/100 ml at 4 hr. It is apparent that SEB in the dose administered was significantly more potent in stimulating adrenal corticoid secretion than an acute single injection of 4 units of ACTH, a dose in the physiologic range.

Additional experimental variables were studied in the same fashion, as shown in Figure 7. The solid line indicates 17-OHCS values in blood from a group of animals given SEB. The broken line represents 6 monkeys given antitoxin prophylactically 30 min prior to challenge with SEB. There is only a modest rise in cortisol values in this group. In contrast, another group of 6 monkeys were given antitoxin therapeutically 30 min after SEB; the rise in 17-OHCS was intermediate between groups treated prophylactically and those remaining untreated.
**Figure 4.** Equilibrium of SEB with tissue binding sites.

\[ \text{SEB} + \text{TiSi} \underset{K}{\overset{K}{\rightleftharpoons}} \text{SEB} - \text{TiSi} \]

**Figure 5.** Effect of type specific antibody on equilibrium of SEB with tissue binding sites.

\[ \text{SEB} + \text{TiSi} \underset{K}{\overset{K}{\rightleftharpoons}} \text{SEB} - \text{TiSi} \]

\[ 1^\bot K \]

\[ \text{SEB} - \text{Ab} \]
FIGURE 6. MONKEY PLASMA 17-OHCS.

FIGURE 7. MONKEY PLASMA 17-OHCS.
The experimental design did not permit recognition of any exact site, such as the cerebral cortex, hypothalamus or pituitary, where the toxin might initiate adrenal stimulation. Previous work had indicated a pronounced catecholamine release following SEB challenge; epinephrine is a well known stimulator of ACTH. However, SEB is a considerably larger protein than the various octopeptides, known to release ACTH. The implication from this study is that antitoxin lessens adherence of toxin, or removes it from, the sites that influence adrenal secretion.

It became important to define the role of the RES in host defense against SEB intoxication with respect to tissue binding. It is doubtful that a substance initially phagocytized by the RES would remain immunologically active and capable of being released by addition of antibody.

Figure 8 taken from an article by Iio and Wagner shows clearance rates of radiolodinated aggregated albumin from plasma of dogs. The solid line represents total radioactivity and the broken line, protein bound activity. It may be seen that clearance is rapid; within 12 min a reappearance of radioactivity occurs. This secondary rise is a function of the release or dissociation of $^{1131}$ from the aggregated albumin. Protein bound $^{1131}$ continues to leave the plasma in a progressive fashion.

Results of similar work conducted in the Medical Unit in a group of monkeys are presented in Figure 9. Radiolodinated aggregated albumin was supplied by Squibb Laboratories. It is apparent that the clearance of albumin given in a dose of 3 mg/kg is relatively rapid as is the rate of deiodination. In contrast, a group of monkeys given Thorotrast, 3 ml/kg, 3 hr prior to administration of aggregated albumin, is shown on the right. The clearance rate is markedly inhibited and the rate of deiodination is delayed. This procedure then, defined a change after Thorotrast in uptake and deiodination of a substance known to be phagocytized by the RES.

A similar study performed in monkeys receiving SEB-$^{131}$ is shown in Figure 10. The disappearance of SEB in the control situation is shown on the left. The clearance of SEB-$^{131}$ in a group of monkeys given Thorotrast 3 hr prior to challenge is shown on the right. It is apparent that the rate of clearance and the rate of deiodination are unaffected by this manipulation. The implication is that the RES must play only a minor role in the clearance of this material. This conclusion is also supported by the observation that type-specific antibody actually delays removal of SEB rather than facilitating it, as is the case with bacterial endotoxin.

The fact that the RES does not appear to be a major factor in SEB intoxication is in keeping with our observation that delayed antitoxin therapy seemed to remove toxin from unidentified sites and return it to the circulation. Immunoactive or unaltered toxin would not be expected to return from intracellular sites within the RES.
Colloidal Albumin Disappearance Rate Constant
29.6 ± 4.2%/min

Colloidal Albumin Degradation Rate Constant
10.1 ± 0.8%/min

FIGURE 8. BLOOD CLEARANCE OF AGGREGATED ALBUMIN IN NORMAL DOGS.
— TOTAL PLASMA RADIOACTIVITY.
— PLASMA RADIOACTIVITY, AFTER REMOVAL OF FREE IODIDE.
Figure 10. Disappearance of SEBi\(^{131I}\) in monkeys.
The observation that the RES is not a major removal organ for SEB-\(^{131}\) leaves the obvious question: what are the sites of importance in the removal of SEB? Dr. Morris' work (presented later in this report) indicates that a large amount of SEB radioactivity localized in the kidneys. This finding was in agreement with earlier work done by Lt Colonel Dirks (Pathology Division, Medical Unit) using an entirely different experimental approach, fluorescent antibody staining of kidney sections. Figure 11 is a black and white reproduction of one of his slides. A pronounced localization of SEB within the proximal tubular epithelial cells of the kidney was noted. The light area fluoresced apple green and denoted presence of SEB.

It was observed further that antibody prophylaxis drastically altered localization in the kidney so that only small amounts of activity accumulated. These findings, in conjunction with previous work indicating a marked reduction in the rate of deiodination as a result of antitoxin prophylaxis, prompted further investigation of the renal aspect of the problem.

The clearance of SEB-\(^{131}\) in a group of monkeys which had bilateral ligations of the renal artery and vein 1 hr prior to challenge, is shown in Figure 12. There was an obvious delay in disappearance of toxin, the \(t_1\) approximated 30 min rather than the expected 7 min. Deiodination was markedly inhibited; there was no evidence of the secondary rise of radioactivity attributed to the appearance of free or disassociated \(^{131}\). The study indicated that the kidneys were not only a major site of localization but also a primary site of deiodination and perhaps other metabolic alterations. Furthermore, the prophylactic administration of specific antitoxin prevented localization of toxin in the kidney and perhaps, as a result, inhibited its deiodination.

It was apparent that despite numerous metabolic similarities with bacterial endotoxin, SEB exhibited very obvious differences in localization sites and clearance kinetics: the RES did not appear to play a major role in the removal of SEB from the circulation and work indicated that the kidneys were clearly important organs of metabolism. Whether the oliguria and azotemia associated with SEB intoxication were due to direct effect of the toxin or merely secondary to shock was less evident.

The reversible binding concept remains the most attractive explanation for the sequential changes observed.

Figure 13 illustrates in a slightly more detailed manner than that shown in Figure 5, our present concept of the distribution kinetics of SEB. The primary organs of localization are the liver and kidneys. The latter are the major sites of deiodination of labeled SEB and perhaps the major site of metabolic alteration. It was further shown by Dirks by means of the fluorescent antibody technique that SEB accumulated in the brain after a delay of 2-4 hr. Adherence of SEB to white blood cells in vitro has been observed but the significance has not been established. The RES is apparently a minor site of localization and metabolism.
FIGURE 11. LOCALIZATION OF SEB IN RENAL TUBULAR EPITHELIAL CELLS DEMONSTRATED BY FLUORESCENT ANTIBODY TECHNIQUE.
Figure 2: Disappearance of Se-75 in monkeys.

% INITIAL CPM

0 30 60 180 240 360 420 480 600 800 1000

30 60 120 180 240 300 360 420

TIME (MINUTES)

CONTROL

TOTAL PLASMA RADIOACTIVITY

PLASMA TCA PRECIPITATE

RENA L ARTERY LIGATION
FIGURE 13 & 14. DEMONSTRATION OF EQUILIBRIUM REVERSAL RESULTING FROM ANTIBODY ADMINISTRATION.
Figure 14 indicates the sequence of events occurring after antibody administration. The reversible binding or equilibrium relationship was most prominent in the kidneys from which a large amount of SEB apparently was returned to the circulation. This was explained by the binding affinity of SEB for antibody exceeding its affinity for the kidney. To a lesser extent this sequence occurred also in liver and muscle. Curiously enough, SEB was increased in the lung following antibody administration. The explanation for this observation was not apparent although this most probably represented accumulation of the toxin-antibody complex rather than toxin alone.

SUMMARY

There is a suggestion that following SEB challenge, toxin rapidly binds to certain vital tissues and in this manner manifests its toxicity. The fact that this binding can be reversed to a degree serves as a basis for the effectiveness of delayed therapy.

Considerable insight has been gained into the mechanisms by which SEB acts. Productive leads are being pursued with emphasis on the metabolic and physiologic interactions of toxin and host.
The theory of a reversible binding phenomenon between staphylococcal enterotoxin B (SEB), its specific antitoxin, and various undetermined tissue sites is expanded by description of a radiobioassay and its implications.

Evidence was presented by Captain Rapoport that antitoxin injected into the circulation 30 min after the administration of radio-iodinated SEB (SEB-I\(^{131}\)) caused a reappearance of labeled toxin in peripheral blood. In passively immunized monkeys the disappearance rate was prolonged from a normal of 7 min to approximately 5 hr.

Following these observations, it became evident that the binding between the toxin, its specific antitoxin, and undetermined tissue sites resembled the reversible binding phenomenon characteristic of several hormones and their specific carrier proteins.

It was theorized that retention within the blood stream (half-disappearance time or \(t_1\)) of a given dose of toxin would be increased if the quantity of circulating specific antitoxin were increased. Thus, it seemed reasonable that SEB protective antibody might be quantitated by its ability to prolong the disappearance rate of the SEB-I\(^{131}\) in an experimental animal. This report describes the development of such a bioassay using albino rats, and demonstrates its potential usefulness in the study of serum from a variety of animal species including humans with hemagglutinating (HA) antibodies against SEB.

White male rats weighing 250-280 gm were used throughout. Since preliminary studies failed to reveal evidence of any influence of rat weight within this range, a single uniform dose of 360 \(\mu\)g of SEB-I\(^{131}\) was used.

Radioactive toxin and specific antitoxin were identical to those previously described. For purposes of this assay, the standard, undiluted hyperimmune equine antiserum with an HA antibody titer of 1:1,600,000 was assigned arbitrarily a value of 16.0 units/ml.

Figure 1 illustrates schematically the bioassay protocol. Following anesthetization of a rat by an intraperitoneal injection of 0.6 ml of 5% sodium pentothal containing 120 units of heparin, exactly 0.5 ml of the standard antiserum in appropriate dilution, or unknown serum to be assayed, was injected into the dorsal penile vein. One minute after injection of the test sample, 360 \(\mu\)g of SEB-I\(^{131}\) was injected into the same vein.

* Physical Sciences Division.
Blood samples for determination of radioactivity were obtained from the tail artery; the tail was cut with large scissors and clamped. Samples were taken at precisely 4, 6, 8, 10, and 14 min after toxin injection. Droplets of blood were collected on a small piece of parafilm and the tail stump was reclamped. The first sample was obtained at mid-tail and subsequent samples, immediately proximal to the clamp. Following the last collection the rat was sacrificed.

The whole blood was aspirated immediately from the parafilm with a 0.1-ml micropipette and blown into the bottom of a plastic tube for counting. Two ml of distilled water were flushed through the pipette and into the counting tube to remove any residual blood. Radioactivity was then measured in a scintillation well detector. The counts obtained were plotted against time on semilogarithmic paper and the $t_d$ was determined. Figure 2 shows examples of such plots in individual animals. As can be seen, essentially linear disappearance curves were obtained.

Figure 2A shows an example of a control rat given 0.3 ml saline as a test sample; $t_d$ was 3.8 min. The average $t_d$ of 10 such control animals was 3.7 ± 0.2 min. Figures 2B, 2C, and 2D show that rats passively immunized with increasing amounts of antitoxin demonstrated a progressive prolongation of $t_d$. In Figure 2B, 2.0 units of antitoxin were administered; a $t_d$ of 10.8 min resulted. Figure 2C shows a $t_d$ of 23.7 min with 4 units and Figure 2D, 34.0 min with 8 units of antitoxin.

When the half-disappearance time in individual animals given an identical amount of toxin was plotted against the logarithm of the units of antitoxin a sigmoid dose response curve was obtained (Figure 3). Control animals and 8 concentrations of antitoxin were assayed. It can be observed that the range of values for an individual dose of antitoxin became widest with the highest concentrations. This could be attributed perhaps to the flattened disappearance curve in individual rats which tended to decrease the accuracy of $t_d$ estimation; it could also be related to increased inherent variability in the region of flattening of the assay curve itself.

It seemed appropriate to determine if further increases in antitoxin concentration would influence the bioassay curve. In order to avoid increasing the test sample volume, purified and concentrated hyperimmune equine SEB antitoxin (The National Drug Co., Lot 19575) was employed. With undiluted samples of this, the $t_d$ was increased to 45 min. The results of assays of serial dilutions of this product suggested that it was 4 times more potent than the antitoxin used for the standard. From these observations it was concluded that the dose response curve would flatten progressively with the administration of antitoxin in doses above 8.0 units/0.5 ml.

The portion of the dose response curve which showed the sharpest linear increase fell between 3 and 5 units. Therefore, this portion of the curve was studied in greater detail (Figure 4). Thirty-five animals
FIGURE 1. SEB ANTITOXIN BIOASSAY PROTOCOL.

FIGURE 2. SEB-I\(^{131}\) DISAPPEARANCE IN RAT.

FIGURE 3. SEBA ASSAY—ENTIRE CURVE.
FIGURE 4. SEBA ASSAY – LINEAR PORTION.

FIGURE 5. SEBA ASSAY – HUMAN SERA.
were employed using dilutions of the standard in the linear portion of the curve. The best-fit line, as shown, was obtained by the least squares method and exhibited a correlation coefficient of 0.99. A linear relationship was confirmed by an analysis of variance and was significant at < 0.01% level. The lambda value (criterion of likelihood) for use of this portion of the curve for assay purpose was 0.132. It should be noted that the absolute location of this curve varied slightly with different lots of SEB-1 and, accordingly, unknown sera should always be compared with antitoxin standards. In addition, unknown sera with prolonged half-disappearance times would have to be diluted as necessary to permit quantitation within the linear portion of the assay curve.

To test the assay, sera were obtained from normal and immunized horses, burros, and monkeys. The normal sera from these species all showed values similar to those obtained with normal saline controls. Assays of sera from the immunized animals showed a prolongation of the disappearance time enabling an estimation of their potency to be made.

Sera from 25 normal human subjects were studied. Although none of this group gave a history of known exposure to SEB, 19 had HA antibody titers ranging from 1:1,000-1:32,000. The average t½ of duplicate samples of these sera is plotted against HA titer in Figure 5. None was found different from that of saline controls. An interesting observation on human gamma globulin obtained commercially was that it had an HA titer of 1:82,000 and caused a prolongation of t½ equivalent to 2 units of antitoxin (note the asterisk on Figure 5).

By interpolation, one antitoxin unit was equated with a 1:100,000 HA titer. As can be seen, the lower limit of sensitivity of the bioassay system appeared to be in keeping with an HA titer of about 1:50,000.

If our interpretation of the available data approaches a true interrelationship, it implies that the rat bioassay is much less sensitive than HA titers for detecting evidence of an immune response to SEB. But it does imply that very high HA titers must exist in serum before the t½ of SEB is lengthened.

The theory upon which our assay was based would imply that similar results could be obtained in other species. To test this hypothesis, the t½ of radioiodinated toxin was determined in guinea pigs and found to be approximately 7 min; this was lengthened progressively in individual animals by the prior administration of antitoxin in increasing concentrations indicating that a similar pattern existed in other species.

The demonstration that a reproducible dose-response relationship existed between antitoxin and the rate of toxin clearance gave support to our basic theory concerning such an interaction. The findings observed in rats and guinea pigs, together with those in monkeys, implied that such a relationship...
was a general one and not species-specific. That such a dose-response relationship could be predicted added value to our supposition that binding phenomena could explain the interaction between SEB and its specific antitoxin.

The specificity of this assay appears to depend entirely upon the presence of antitoxin against SEB in the material tested. The concept that the antitoxin-toxin combination is retained within the circulation and therefore is prevented from reaching sites within the tissues to exert toxic action is based upon a single mechanism. This concept implies that protective activity and half-life-lengthening activity are one and the same property of the antitoxin. Whether the antitoxin molecules which afford protection are identical to those which hold the toxin within the blood stream cannot be determined from the information at hand.

Less well defined is the relationship between HA activity and the ability of the antitoxin to protect with or without lengthening of the t½ of the toxin.

SUMMARY

A rat radiobioassay for SEB antitoxin provided reproducible results of high precision. This system has given support to our concept concerning the toxin-antitoxin interaction.
CLEARANCE AND LOCALIZATION KINETICS OF RADIOACTIVELY LABELED STAPHYLOCOCCAL ENTEROTOXIN B
PART III

Earl L. Morris, Captain, VC*

Fundamental to obtaining an understanding of the pathophysiology of staphylococcal enterotoxin B (SEB) intoxication is the location of the site or sites of localization of the toxin. Early studies of toxin distribution were conducted in monkeys 2, 6, 12 and 24 hr postchallenge with radiodiode-labeled SEB (SEB-I^131). However, it was subsequently determined that over one-half of the SEB-I^131 was deiodinated within 4-6 hr postchallenge.

In the present study, Macaca mulatta without known prior experience while under sodium pentothal anesthesia, were challenged with 1,000 μg/kg of SEB-I^131, a dose expected to produce lethality in 80-90% of monkeys. Ten minutes and ½, 2 and 6 hr later they were sacrificed, autopsied, and organs to be counted were placed in plastic cups; the carcass was retained for counting. Radioactivity was measured in a total-animal counter which maintained a constant counting geometry in order to quantitate and localize the activity and account for 100% of the injected radioactivity. An attempt was made to determine if there was a variation in tissue distribution due to route of administration and to show the influence of antitoxin on the distribution of SEB.

Table I lists the routes of challenge, number of animals employed, and the intervals at which they were sacrificed. The antitoxin employed was hyperimmune equine serum (SERA) and was administered intravenously (IV) ½ hr prior to challenge. Following antitoxin prophylaxis virtually all of the SEB remained in the blood stream 10 min postchallenge; therefore, this time period was eliminated in the animals so treated.

The ability to study localization of toxin following oral challenge depended upon the stability of the SEB-I^131 complex following absorption through the intestinal wall. An initial study was directed toward this problem. Two monkeys were given I^131-labeled toxin intragastrically and 2 were given free I^131 by the same route. All were bled at frequent intervals over a 6 hr period. Each blood sample was centrifuged and 1 ml of plasma and the precipitate obtained from treating 1 ml of plasma with trichloroacetic acid were counted. The results are shown in Figure 1.

In the group given SEB-I^131 the majority of radioactivity was not bound to protein. Essentially the same quantity of protein-bound radioactivity was found in both controls and challenged animals. This showed

* Physical Sciences Division.
TABLE I. DISTRIBUTION OF MONKEYS IN EXPERIMENTAL PROTOCOLS

<table>
<thead>
<tr>
<th>CHALLENGE ROUTE</th>
<th>SEB-\textsuperscript{131} VARIABLES</th>
<th>NO. OF ANIMALS BY TIME (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Intra-venous</td>
<td>Inferior vena cava</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Portal vein</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>SEBA prophylaxis</td>
<td>0</td>
</tr>
<tr>
<td>Oral (intra-</td>
<td>SEBA \textsuperscript{131}</td>
<td>0</td>
</tr>
<tr>
<td>gastric)</td>
<td>Free \textsuperscript{131}</td>
<td>0</td>
</tr>
</tbody>
</table>

that the complex was not actively absorbed and the protein-bound radioactivity was due primarily to combination of \textsuperscript{131}I following absorption with nonspecific proteins in the blood. Thus, it seemed doubtful that meaningful organ localization of SEB could be obtained following oral challenge of the iodinated toxin.

The initial IV challenge studies were reported to the Commission last spring. The results of these studies will be combined with recently acquired data; however a different format will be employed.

Percentage of total radioactivity injected is plotted against time in hours for the various body parts measured (Figure 2). Various organs are presented individually in a form that clearly demonstrates the changes that occurred.

1. Carcass and blood.

Blood values were calculated on the basis of blood volume being equivalent to 5% of the animals live weight. Carcass values include radioactivity of blood trapped in the carcass. Similar patterns were seen following IV and portal challenges: there was an early increase of approximately 40% of administered radioactivity in the carcass mainly due to the high percentage in the blood. This was followed by a prompt fall from the early peak in both carcass and blood. At 30 min the carcass values began returning toward the amount of the early peak and slightly surpassed this value at 6 hr. This was attributed to tissue absorption and not solely to the amount in the blood. The blood follows a disappearance curve in complete agreement with the data reported by Captain Rapoport with the slight late increase due to circulating free \textsuperscript{131}I.

With antitoxin prophylaxis an entirely different picture was seen. Throughout the 6-hr period, carcass levels were between 75-80% of administered radioactivity. The blood level was slightly higher than the
FIGURE 1. BLOOD ACTIVITY FOLLOWING ORAL ADMINISTRATION OF SEB-1[31].
FIGURE 2. DISTRIBUTION OF SEB-[^{131}I] FOLLOWING IV INOCULATION BY THE INFERIOR VENA CAVA (IV) AND PORTAL VEIN AND ANTITOXIN PROPHYLAXIS.
carcass level until 4 hr. This was due to radioactivity of blood in various organs not counted with the carcass. The blood level showed a very gradual decline in contrast to that observed in the monkeys challenged by IV or portal routes.

2. Lungs.

Again, values for the IV and portal routes were not significantly different and the antitoxin data were in marked contrast. A peak value of 8.9% was attained at 2 hr, more than twice as much as the IV value and 2 times greater than the portal. At 6 hr the level was still more than twice that of the other two. The reason for this difference was not readily apparent. Retained blood with its high level of radioactivity after antitoxin might have accounted for part of this difference. Perhaps binding of toxin to white blood cells (WBC) and trapping of damaged WBC's in the lung (a concept presented by Captain Crawley at an earlier meeting) might have been accentuated in some manner by the presence of antitoxin. Another explanation may be that the toxin-antitoxin complex is taken up by pulmonary WBC's or macrophages. It is possible that late pooling of blood in the lung, in association with pulmonary edema, could have accounted for our findings.

3. Liver.

After IV challenge there was a slightly higher percentage of activity than following portal challenge until 6 hr when the values of both were essentially the same. In contrast to the picture in the lungs, the passively immunized animals showed a notable decrease in activity. At 30 min both IV- and portal-challenged animals had a value of approximately 20%, as compared to 10% for immunized animals. However, the IV and portal values fell steadily to a value of 6.5-6.7% at 6 hr; the antitoxin value fell to approximately 7% at 2 hr, leveled off, and at 6 hr was similar to the others. The absence of a larger accumulation of radioactivity in the liver after IV challenge with SBB-1131 seemed to increase its importance as the major organ of SBB removal from blood.

4. Kidneys and urine.

Kidney values revealed a very interesting picture. With antitoxin, very little activity was found at any time throughout the 6 hr. Although both the portal and IV values were maximal at 30 min and steadily declined to < 10% at 6 hr, there were statistically significant differences between them at both the 30- and 120-min levels.

The urine findings indicated the rate of deiodination of SEB-1131 and excretion of the label in the urine. It should be noted that in neither the IV or the portal challenged groups did the urine and kidney values at 6 hr come close to equalling the kidney values at the 30-min peak. This implied that the initially high concentrations of radioactivity had left the kidney via the bloodstream rather than via the urine.
5. Stomach and intestines.

The stomach and intestines were grouped together since the activity found here was due primarily to free $^{131}$I that had been excreted into the gastrointestinal tract. This gut localization of free iodide is well known. Note that prophylactic antitoxin which markedly lessened deiodination of the toxin was associated with a much lower gut accumulation of $^{131}$I.

6. Heart, thyroid, spleen and brain.

Radioactivity was very low in these organs. Values for heart, spleen and thyroid were similar. In the brain the greater rise in the antitoxin group was due to the large amount of activity present in the blood that bathes the brain. The values were low and probably of little significance. Since the spleen is an important site of reticuloendothelial activity, the low percentages found here have helped in eliminating the reticuloendothelial system as important in the handling of SEB. The increase seen in the thyroid represented uptake of free $^{131}$I.

From this data and that of the previous presentations by Drs. Rapoport and Hodoval, it is evident that the antitoxin is very effective in preventing the clearance of SEB from the bloodstream, i.e., greatly affecting its distribution among the various organs. The antitoxin-SEB-$^{131}$I aggregate is shown to be fairly stable. By preventing the uptake of SEB by the organs, the toxic effect of SEB is combated completely or greatly delayed.

From these distribution studies, the kidneys primarily and the liver secondarily appear to be the major organs engaged in actively localizing, deiodinating, and possibly detoxifying the SEB. The fact that antitoxin reduces the amount of SEB found in these organs, especially in the kidney, supports this contention. Another thought is that the kidney is used as a storage site for SEB to be released later and as a deiodination point with the free $^{131}$I being returned into the bloodstream. The return of free $^{131}$I is evident by the low quantities in the urine and the increase in the gastrointestinal tract.

The increase in lung radioactivity following antitoxin prophylaxis is difficult to explain.

Further work is planned, aimed primarily at the kidney, to distinguish the role of this organ in the handling of SEB.

**SUMMARY**

Oral, IV and portal routes of administration of SEB-$^{131}$I were studied. The oral route is of no value because of destruction of the complex upon absorption through the intestinal wall. The IV and portal routes are of value but there is no real difference between them.
Antitoxin is effective in preventing localization of toxin.

The major organs concerned with the handling of SEB, at least in the early stages during its very rapid disappearance from the circulating blood, are the kidneys and secondly the liver.

LITERATURE CITED

EFFECTS OF STAPHYLOCOCCAL ENTEROTOXIN B ON THE COAGULATION MECHANISM AND LEUKOCYTIC RESPONSE IN BEAGLE DOGS --- A PRELIMINARY REPORT

Charles F. Gilbert, Captain, MC*

Much of the reported work with staphylococcal enterotoxin has been in areas of investigation other than that of blood coagulation. The only reports seen relating to clotting were those of Sugiyama and co-workers in 19641 and Bernheimer and Schwartz2 earlier this year. Sugiyama and his group were mainly concerned with fibrinogen and platelet changes after enterotoxin administration in rabbits and monkeys. Bernheimer and Schwartz demonstrated the capacity of enterotoxin to injure or destroy rabbit platelets in vitro.

The finding of hemorrhages in dogs receiving staphylococcal enterotoxin B (SEB) suggested that a defect might be present in the hemostatic mechanism and indicated the need for investigation of this area. The bleeding present in dogs was especially prominent in gut, liver, and lymphoid tissue, including the lymph nodes, spleen, thymus and Peyer's patches. In fact, enterocolitis and hemorrhagic manifestations were the most striking findings at autopsy.

Ten adult purebred beagle dogs were used; 9 received an intravenous injection of 100 µg/kg of highly purified SEB, and one, 50 µg/kg. The tests employed in this study included whole blood clotting time, clot retraction and lysis, prothrombin time and partial thromboplastin time. Total and differential white blood cells counts were performed.

Blood (8 ml per sample) was obtained using plastic syringes from the external jugular vein. One sample was taken from each dog before toxin administration; after challenge, 10 specimens were collected over a 51-day period.

For the coagulation studies, 9 parts of blood were treated with 1 part of 3.2% sodium citrate. The plasma was separated by centrifugation and was either tested immediately or frozen at -20 C for subsequent testing. All coagulation studies were performed in non-coated glass. Blood for the total and differential leukocyte counts were processed by standard techniques.

The whole blood clotting time was performed by a modification of the Lee-White technique;3 the prothrombin time by the method of Quick;4 and the partial thromboplastin time by the method of Langdell, Wagner, and Brinkhous.5

* Pathology Division.
The second tube of the clotting time test was observed for clot retraction 30 min after the blood was drawn and at hourly intervals thereafter. The degree of retraction was graded from 3+ to 0. All observations were made by one individual. After retraction, the tubes were held at room temperature and observed for 72 hr for evidence of clot lysis.

The range and mean of the prothrombin time and partial thromboplastin time were established for the controls and the 18 postchallenge bleeding periods.

All dogs became ill within 3 hr after toxin administration, as evidenced by emesis and, sometimes, diarrhea. Two dogs died, one at 13 hr and one at 4 days postchallenge.

The prechallenge clotting time for the 10 dogs ranged from 6-10½ min with a mean of 8½. Figure 1 illustrates the average clotting time for each bleeding period and shows the prolongation immediately after challenge and to 22 min on the 28th day. The clotting time returned to the upper normal range by the 51st day. The alterations in the clotting times suggest there may be a deficiency of one or more of the plasma clotting factors.

All clots retracted normally (3+) before challenge and 1 hr after challenge (Figure 2). Beginning at 3 hr, however, there was diminished retraction and this continued until the 2nd postchallenge day when only a slight degree of retraction was present in 2 of the samples. Thereafter, clot retraction gradually improved and was normal on the 23rd day. These findings suggest that there may be a deficiency of fibrinogen or of platelets or possibly a defect in platelet function.

There was no evidence of fibrinolysis in any of the samples during a 72-hr observation period.

The range of the prothrombin time in the prechallenge samples was 8.3-10.5 sec, the mean, 9.5 (Figure 3). Prothrombin time decreased slightly 1 and 3 hr after challenge. At 6 hr, there was a prolongation to about 12.5 sec. Thereafter, the prothrombin time returned to the normal range, except on the 30th day when the values were slightly below the established range. The abnormality in the 6-hr samplings also suggested a deficiency of one or more of the plasma factors; this is supported by the data on whole blood clotting time.

Prolongation of the partial thromboplastin time occurred at 6 hr; it persisted until about the 8th postchallenge day when the values returned to the established normal range (Figure 4). This too, in conjunction with the clotting time and prothrombin time, is additional evidence for a deficiency of one or more of the plasma factors.
One of the more dramatic changes associated with the toxin was the rapidly developing leukopenia. As seen in Figure 5, the average control leukocyte count was 16,000/mm. One hour after toxin administration, the white count decreased to 1,800; this low level persisted in the 3-hr sampling; thereafter the count increased to about 20,000 on the 4th day. The leukocytosis, though slight, continued until the 12th or 14th day. These findings are similar to the response that may occur in an overwhelming bacterial infection in which there is an initial leukopenia followed by leukocytosis. The increased count on the last day remains unexplained.

The changes in the differential counts were also most marked immediately after challenge (Figure 6). Before toxin administration there was an average of 70% neutrophils and about 30% lymphocytes. By 1 hr after challenge these cells were present in almost equal numbers; subsequently the neutrophils gradually increased and the lymphocytes decreased. During the 1st day and continuing through the 3rd day, there was a slight "shift to the left" with the appearance of 7-8% early forms. After the 4th day the cells were present in normal numbers.

Immediately after toxin administration, there was an average of 9 normoblasts among 100 leukocytes counted. During the remainder of the 1st day, the normoblasts decreased and were absent during the remainder of the experiment.

The appearance of early forms of each series, i.e., normoblasts and bands, the leukocytosis following the leukopenia, indicate that the marrow was not impaired by the toxin and was capable of responding. Furthermore, the marrow was histologically normal in the 2 animals which died.

As mentioned earlier, one of the animals died in 13 hr and another in 4 days. Complete autopsies were performed shortly after death. There were losses of about 20% of the body weight. In addition to severe enterocolitis, there was bleeding into the lymphoid tissue and liver. There was no evidence of intravascular coagulation or infarction. Both deaths were attributed to dehydration and hemorrhage with severe pneumonia in the animal dying on day 4.

SUMMARY

Ten dogs were given staphylococcal enterotoxin B intravenously and were observed and bled over a 51-day period. The whole blood clotting time, clot retraction, clot lysis, prothrombin time, partial thromboplastin time, and total and differential white blood counts were performed on each sample. An abnormality was indicated by each test at some point during the testing period with the exception of clot lysis. The most frequent and more severe abnormalities occurred within the first 4 days after challenge. The results indicate that alterations of the clotting mechanism occur after administration
Fig. 5: Total Leukocyte Count

Fig. 6: Differential Leukocyte Count
of staphylococcal enterotoxin B and suggest that the plasma factors are primarily involved. Also indicated by these studies is the need for fibrinogen determinations and platelet counts. Changes in the leukocytic response appear to be similar to those which can occur during an overwhelming bacterial infection.

LITERATURE CITED


When the Serology Section of the Bacteriology Division began its operation, the task assigned highest priority was that of the establishment of a routine serological service for determination of staphylococcal enterotoxin B (SEB) antibody. Most of the work reported here was done by Major Johnnie Runnels.**

Several serological procedures which were used originally for the titration of toxin have been adapted by other workers for use in tests for enterotoxin antibody. Among these were the hemagglutination (HA), complement fixation (CF), and several agar-gel diffusion (AD) techniques.

Since a variety of serum specimens of interest to investigators had been examined in other laboratories by a HA technique, this procedure was selected for initial studies. The protocol used was identical with that employed in other laboratories at Fort Debrick. Sensitization of red blood cells (RBC) for the test was accomplished by coupling toxin to sheep erythrocytes with Bis-diazotized Benzidine (BDB). It should be emphasized that each step of this procedure, from the original preparation of the BDB compound through the final washing of the sensitized cells, was extremely difficult to control and apparently very sensitive to minor variations in temperature and timing and perhaps other unknown factors.

It soon became apparent that while the BDB HA test appeared to be a highly sensitive method for detection of antibody, wide variations in titers were observed when the same serum was examined repeatedly. For example, in repeated tests on a large number of serum samples the variations in end points observed were as much as 8 tubes in a 2-fold dilution scheme in a number of cases. The mean variation in titers of one group of 20 sera repeatedly run together in the same protocols was more than 4 tubes or 16-fold. Only 2 of these varied as little as 2 tubes from day to day. In addition, about 5% of sera which were negative in one test would be positive on another day with titers occasionally as high as 1:640.

In view of these results, a rather intensive examination of the test itself was begun. Recognizing that our personnel were largely inexperienced, we thoroughly checked and carefully controlled all of the obvious factors which could affect results of any serological determinations, i.e., accuracy of dilutions, timing and temperature in preparation of all reagents, as well as during incubation of the test, the reproducibility of reading end points, etc. Possible variations in sheep RBC as related to time of storage, bleedings from different animals and repeated bleedings from the same animal were

** Bacteriology Division.

** Presently at the Department of Veterinary Science, University of Wisconsin, Madison, Wisconsin.
also considered. The results of these rather extensive efforts to control and standardize all factors involved in the BDB HA test all led to the same conclusion: the major variable contributing to lack of reproducibility of the test was the procedure involving the BDB coupling of antigen to the RBC. If one can judge from other agglutination procedures, it is reasonable to assume that both the number of cells coupled properly with antigen and the concentration of antigen per cell could affect the end point of the test. Since, as has been pointed out by other workers, BDB is a highly active compound capable of reacting with a variety of chemical groupings in a totally unpredictable fashion, a theoretical consideration of the most difficult part of the test procedure to control also pointed to the coupling procedure.

Originally it was not known that other laboratories using this test had had similar experiences. We only knew that "certain difficulties" had been encountered. Upon further, more searching, questioning it was discovered that our experience was not unique, wherever the day to day reproducibility of the test had been examined. For example, Dr. Silverman of the Biological Laboratories (in whose laboratory most of the early work with the BDB test at Fort Detrick had been done) had found variations in titer of > 10-fold when a single serum was examined with different antigen preparations. Both Dr. Silverman and personnel of the Bacteriology Division arrived at the conclusion that in its present state of development the BDB HA test is of limited usefulness for quantitative studies of antibody to SEB, despite its great sensitivity and its, at least assumed, specificity.

There was no alternative but to look elsewhere for a good serological method or combination of tests which could be used for detection of antibody and which also could be quantitated accurately for studies of the immune response to antigen and immune status of particular individuals. Silverman's group has begun studies on a CF test and is preparing to initiate work with several other techniques. Dr. McGann of the Division has been working for some time on an AD test (see the following paper); the Serology Section has done some preliminary work on a HA technique employing antigen adsorbed on tannic acid-treated erythrocytes. It is our hope that with close cooperation and collaboration among these several laboratories the basic problem will soon be resolved.

Limited data comparing the results of 2 or more of these tests on the same serum specimens are available and have been summarized in several tables. Table I shows titers obtained in repeated tests on hyperimmune horse serum with the 2 HA techniques. RBC from different bleedings of the same sheep were used for antigen preparations after varying periods of storage. It is readily apparent that the tanned cell technique is markedly less sensitive than the BDB test as judged by end point titer. However, in these trials there is no significant variation from preparation to preparation with the tanned cell test while the variation in titer with the BDB test is from 1,300,000 to 1:20,000,000, or 6 tubes in a 2-fold dilution scheme, on days when the 2 tests were run in parallel.
TABLE I. VARIATION IN HEMAGGLUTINATION TITERS USING RBC FROM DIFFERENT BLEEDINGS OF SAME SHEEP

<table>
<thead>
<tr>
<th>DATE BLOOD DRAWN</th>
<th>DATE BLOOD USED</th>
<th>RECIPROCAL HA TITERS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BDRE</td>
</tr>
<tr>
<td>27 June</td>
<td>7 July</td>
<td>5,000,000</td>
</tr>
<tr>
<td></td>
<td>9 July</td>
<td>5,000,000</td>
</tr>
<tr>
<td></td>
<td>12 July</td>
<td>600,000</td>
</tr>
<tr>
<td>7 July</td>
<td>12 July</td>
<td>20,000,000</td>
</tr>
<tr>
<td></td>
<td>13 July</td>
<td>600,000</td>
</tr>
<tr>
<td></td>
<td>14 July</td>
<td>300,000</td>
</tr>
<tr>
<td>14 July</td>
<td>20 July</td>
<td>160,000</td>
</tr>
<tr>
<td>21 July</td>
<td>26 July</td>
<td>300,000</td>
</tr>
<tr>
<td></td>
<td>27 July</td>
<td>2,600,000</td>
</tr>
<tr>
<td></td>
<td>30 July</td>
<td>10,000</td>
</tr>
</tbody>
</table>

a. 100 ml of blood drawn from sheep No. 4270 on dates shown above.

b. Hyperimmune horse serum. Reciprocal of last dilution giving agglutination.

c. BDRE equals hemagglutination with Bis-diazotized Benzidine coupled antigen.

d. TS equals hemagglutination with antigen absorbed on tanned sheep RBC.

In Table II results of the 2 HA tests and the AD test on serum samples selected at random from 200 individuals are summarized.

The upper portion of the table shows the number and percent of samples positive by each of these techniques. The lower portion compares the results of the AD test with the 2 HA techniques. There is little correlation between detectable precipitating antibody and BDRE HA titers under 1:512, or tanned cell HA titers under 1:20. Much better correlation is
### TABLE II. SUMMARY OF RESULTS OF 3 SEROLOGICAL TESTS ON SAMPLES FROM INDIVIDUALS SELECTED AT RANDOM

<table>
<thead>
<tr>
<th>TEST</th>
<th>NO. POSITIVE</th>
<th>% POSITIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDB⁴/</td>
<td>153</td>
<td>77</td>
</tr>
<tr>
<td>TS⁵/</td>
<td>65</td>
<td>33</td>
</tr>
<tr>
<td>Agar diffusion</td>
<td>55</td>
<td>28</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TITER⁶/ NO. SAMPLES</th>
<th>AGAR DIFFUSION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Positive</td>
</tr>
<tr>
<td></td>
<td>% Positive</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BDB</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>47</td>
<td>4</td>
</tr>
<tr>
<td>&lt; 512</td>
<td>102</td>
<td>15</td>
</tr>
<tr>
<td>512</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td>&gt; 512</td>
<td>28</td>
<td>22</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>124</td>
<td>16</td>
</tr>
<tr>
<td>10 - 20</td>
<td>37</td>
<td>15</td>
</tr>
<tr>
<td>40 - 160</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>320 - 1280</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

a. BDB equals hemagglutination with Bis-diazotized Benzidine coupled antigen. 200 samples tested.
b. TS equals hemagglutination with antigen absorbed on tanned sheep RBC. 190 samples tested.
c. Reciprocal.

seen at higher titers in the HA tests. In Table III results of the CF tests, performed by Dr. Silverman's laboratory, as well as those obtained by the 3 techniques used in the Medical Unit laboratories, are tabulated, for serum samples taken from 15 individuals with known exposure to SEB. The samples examined were taken 16 weeks after exposure in all cases. Two points of interest are apparent from the table: (1) there is a close correlation between the results of the AD and CF tests and (2) there is a rather consistent relationship between the results of the tanned cell HA test and those of the AD test, the titers of the former being 10- to 20-fold higher.
TABLE III. RESULTS OF 4 SEROLOGICAL TESTS* ON SAMPLES FROM 15 INDIVIDUALS WITH KNOWN EXPOSURE TO ENTEROTOXIN

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>RECIPROCAL TITER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BDB</td>
</tr>
<tr>
<td>1</td>
<td>800,000</td>
</tr>
<tr>
<td>2</td>
<td>200,000</td>
</tr>
<tr>
<td>3</td>
<td>400,000</td>
</tr>
<tr>
<td>4</td>
<td>40,000</td>
</tr>
<tr>
<td>5</td>
<td>16,000</td>
</tr>
<tr>
<td>6</td>
<td>20,000</td>
</tr>
<tr>
<td>7</td>
<td>40,000</td>
</tr>
<tr>
<td>8</td>
<td>10,000</td>
</tr>
<tr>
<td>9</td>
<td>1,000</td>
</tr>
<tr>
<td>10</td>
<td>8,000</td>
</tr>
<tr>
<td>11</td>
<td>10,000</td>
</tr>
<tr>
<td>12</td>
<td>16,000</td>
</tr>
<tr>
<td>13</td>
<td>16,000</td>
</tr>
<tr>
<td>14</td>
<td>4,000</td>
</tr>
<tr>
<td>15</td>
<td>2,000</td>
</tr>
</tbody>
</table>

a. BDB equals hemagglutination with Bis-diazotized Benzidine coupled antigen.

CF equals complement fixation (tests performed by Dr. Silverman's laboratory).

AD equals agar diffusion.

TS equals hemagglutination with antigen absorbed on tanned sheep RBC.

in every case. On the other hand, there is a much less constant relationship between the titers of the BDB HA tests and those obtained by the other methods.

The final evaluation of any serological method must take into consideration the question of whether or not the results of the test provide any useful information related to previous experience with the antigen, and the immune status (or immune response to antigen) of the individual whose serum is being examined. Definitive information on this point for any of these methods must await further work carefully planned for this particular purpose.

At present only suggestive information is available. If we assume that the 200 individuals, referred to earlier, constitute a typical sampling, it appears that from 28 to 77% of the population have had some experience with enterotoxin antigen, the number depending upon the test used.
The question of whether any of the available in vitro methods to evaluate potential protection against challenge provided by detectable antibody can be used is impossible to answer at present. There has been no opportunity to go through all of the records available on the large number of monkeys which have been used in a variety of studies with enterotoxin. However, data have been examined carefully on 184 monkeys challenged by the intravenous route with known doses of SEB varying from 0.2-300 µg/kg of body weight. Unfortunately, for purposes of analysis, prechallenge antibody levels were determined after the fact, and the chance distribution of animals with antibody was such that most of them were in the lower dose groups. Of 40 animals challenged with 300 µg/kg none had antibody detectable by the AD technique and only 4 had prechallenge antibody detectable by the BDB HA test. The remaining 30 animals with HA antibody and the 16 having precipitating antibody were distributed more or less equally among the 100, 30, 10 and 0.2 µg/kg-dose groups.

Upon further examination of the data, differences between death and survival for animals with and without antibody were calculated for animals challenged with 100 and 30 µg/kg as a single group since in these 2 cases more than 50% of all animals succumbed to challenge. Similarly, differences between illness and no illness for animals with and without antibody were calculated for all animals in the 10 and 0.2 µg/kg-dose groups, since few animals died, but more than 50% became ill after challenge with these doses.

Although the number of animals with antibody was too small to draw any far-reaching conclusions, the results of this analysis showed that there was a significant difference between the responses of animals with and without antibody in both cases. The level of significance was much higher in the calculations made for animals having precipitating antibody than for those having HA antibody. It is noteworthy that none of 8 animals with precipitating antibody succumbed to the 100 or 30 µg doses while 5 of 15 animals with HA antibody and 4 of 65 without antibody detectable by either method died as a result of challenge. Similarly, only 1 of 8 animals having precipitating antibody became ill after challenge with 10 or 0.2 µg/kg. The illness rate among those having HA antibody was 6 of 15 and that among monkeys without antibody detectable by either method was 36 of 46.

Despite the small numbers involved, these data suggest that the presence of precipitating or HA antibodies affords some protection against the lethal effect of SEB at relatively high doses, and may also afford some protection against illness, at doses of toxin well below the median lethal dose but in the range of the illness dose50.
The problem of aerological tests for the detection of SRB antibodies is still unresolved. While the widely used BDB HA test appears to be more highly sensitive than any others employed thus far, it is so difficult to control that attempts to quantitate it for routine work appear doomed to failure. The less sensitive tanned cell HA, CF, and AD methods appear to be infinitely more reproducible, reliable and amenable to quantitation. It is impossible to say at the moment which, or whether, any of these techniques will prove useful in estimating protection against challenge. It appears that antibody detectable by agar gel diffusion is more closely related to protective antibody than that demonstrated by the BDB HA test.
During the initial stages of the staphylococcal enterotoxin B (SEB) program, serologic methods were developed by other investigators for monitoring production and purification of toxin. The methods, however, were not entirely satisfactory for quantitation or analysis of antibody. Consequently, studies were initiated to investigate in vitro methods for detection, measurement and analysis of the antibody response to vaccination and challenge. This report summarizes our approach to these problems.

Exploratory experiments with hyperimmune horse and rabbit sera suggested the feasibility of using Ouchterlony agar diffusion techniques. With hyperimmune sera, reproducible titers were obtained by testing increasing dilutions of serum against a constant amount of antigen, and the titers seemed to reflect antibody potency. Several problems appeared when procedures developed for hyperimmune sera were used to examine sera from animals with less enterotoxin experience. The major difficulty was the inhibitory effect of excess antigen or antibody on immune precipitation. Excess antigen completely inhibited formation of immune precipitates with sera from all species, and excess antibody in sera of man, monkey, chimpanzee and horse markedly inhibited precipitation. The optimal amount of toxin for titrations of strong sera was 5 μg, whereas for many human sera it was 0.2 μg. Consequently, it was necessary to test unknown sera against several concentrations of toxin, a procedure that made difficult a meaningful estimate of antibody potency.

To avoid this difficulty, a method was developed for demonstrating the zone of antigen-antibody equivalence by Ouchterlony techniques (Figure 1). The procedure consisted of mixing increasing concentrations of toxin with constant amounts of serum in a series of tubes. After incubation the mixtures were added in sequence to the center row of wells. Reference toxin was added to each well in the top row and reference antiserum to each well in the bottom row. After 1-3 days, plates were examined for location of immune precipitation. If a mixture contained excess antibody, precipitate formed with the reference toxin; if excess toxin, precipitate formed with reference antiserum. The neutralization zone was the area in which neither component was in excess. End-points of the zone were affected by incubation time and concentration of reference material. The mid-point, called the equivalence point, was quite insensitive to these influences; by conversion to μg toxin/ml serum it could be used to compare potency of a wide range of sera. For a hyperimmune horse serum, this method gave essentially the same equivalence value as did quantitative precipitation. When this serum was

* Bacteriology Division.
FIGURE 1. AGAR DIFFUSION TECHNIQUE FOR EQUIVALENCE DETERMINATIONS. (TOP ROW: REFERENCE TOXIN; MIDDLE ROW: SERUM-TOXIN MIXTURES; BOTTOM ROW: REFERENCE ANTISERUM.)
absorbed with toxin at equivalence, no free toxin or antibody could be demonstrated in the supernatant fluid, and tests of it for prophylactic efficacy in monkeys indicated removal of protective antibody.

Serum samples from several species of animals that had received a variety of experimental treatment were tested. Equivalence values ranged from 1-2800 μg toxin/ml serum. The relationship between equivalence point and agar diffusion titer, as measured with 1 μg toxin, was examined to determine whether titers could be used as a measure of antibody content when there was insufficient serum for equivalence determination (Figure 2). If the log₁₀ equivalence point was plotted against the log₁₀ reciprocal titer, a fairly good straight-line relationship was obtained. The reason for the difference in slope for horse sera, as compared with monkey and human sera, is not known. It may reflect the type of exposure to enterotoxin or the influence of species-specific, non-antibody serum factors. Another possibility that will be discussed later is the nature of the antigen-antibody reaction.

Limited data suggest a relationship between equivalence point and protective activity. Studies with monkeys challenged by the intravenous route indicated that with high challenge doses (300 μg/kg) the equivalence point may measure protection against lethal effects of enterotoxin, but not against illness. Several sera and serum concentrates used to treat monkeys after challenge with enterotoxin were available for study. Table I summarizes data on the fate of challenged monkeys after treatment with antiserum at, or near, the equivalence point. "Survival" indicates that all animals in the test group survived challenge; "death," that at least one succumbed. For example, with the 0.00067 ml/μg dose of Lot 19575, 1 of 6 died, whereas 6 of 6 controls died. There was good correspondence between equivalence value and the dose of antitoxin that prevented lethality.

In other studies 3 groups of 6 monkeys each were immunized with a toxin-antitoxin mixture at equivalence, or with the precipitate or supernatant fluid from the mixture. At time of challenge, all animals had circulating antibody with equivalence values that ranged from < 1-60. After challenge with 300 μg/kg, 17 of 18 animals became ill, but all survived. Median time to illness for control monkeys and for animals with equivalence values < 10 was approximately 1 hr; for those with values of > 10, almost 3 hr. The animal that did not become ill was in the latter group. When sera from monkeys that had no known prior experience with enterotoxin were examined, approximately 9% had detectable levels of precipitating antibody, ranging from amounts too weak to quantitate to titers of 1:8, all representing an equivalence of < 10. After challenge with varying doses of toxin, all animals with prechallenge antibody survived, whereas only 50% of those without detectable antibody survived.

Serial bleedings were obtained from 25 individuals known to have been exposed to type B enterotoxin. Twelve had participated in an ingestion study, and approximately 1 year later 2 of the 12 were among a group of
Figure 2. Relationship of agar diffusion titer to equivalence point for serum samples from horse, monkey, and human.
TABLE 1. COMPARISON OF NEUTRALIZATION ACTIVITY OF SERA WITH EFFICACY FOR PASSIVE PROTECTION OF MONKEYS

<table>
<thead>
<tr>
<th>SERUM SAMPLE</th>
<th>EQUIVALENCE POINT (ml serum/μg toxin)</th>
<th>SERUM DOSE (ml/μg toxin)</th>
<th>MONKEY RESPONSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEBA-1a/b</td>
<td>0.002</td>
<td>0.003</td>
<td>Survival</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.001</td>
<td>Death</td>
</tr>
<tr>
<td>SAEB Lot 1957b/c</td>
<td>0.0004</td>
<td>0.002</td>
<td>Survival</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.00067</td>
<td>Death</td>
</tr>
<tr>
<td>Human γ-globulin</td>
<td>0.01</td>
<td>0.02</td>
<td>Survival</td>
</tr>
<tr>
<td>Human serum</td>
<td>0.1</td>
<td>0.1</td>
<td>Death</td>
</tr>
<tr>
<td>Monkey pool</td>
<td>0.04</td>
<td>0.01</td>
<td>Death</td>
</tr>
</tbody>
</table>


15 individuals involved in an accidental aerosol exposure. In the ingestion experiment, subjects were fed purified toxin. Seven individuals had no detectable antibody before exposure; 6 of 7 responded to toxin, 5 with overt illness and 1 with a low level of antibody. Five individuals with pre-exposure titers showed no toxic symptoms; 2 of the 5 had high preexposure titers that remained unchanged; the other individuals had barely detectable preexposure antibody that increased significantly within 2 weeks after exposure. Two of the group with preexposure titers were subsequently involved in the accidental aerosol exposure to an unknown concentration of enterotoxin. They were ALH, whose antibody had disappeared from circulation, and EWG, whose antibody level remained unchanged. Figure 3 shows the antibody responses of the 15 subjects involved in the incident. No antibody could be detected in sera from admission bleedings of the hospitalized individuals, but REN, who was ill and not hospitalized, had a low antibody titer. Except for JFF, all who became ill had an antibody response with the maximum at 4-10 weeks and no significant change between 4 and 9 months after exposure. Of 5 individuals who did not become ill, 2 had significant levels of antibody that remained unchanged; 2 showed a weak response, and one never developed antibody.
Figure 3. In vitro neutralization of SEB by sera of 9 hospitalized and 6 nonhospitalized men.
A recent survey of 200 people selected at random indicated that 55 individuals, or 28% of the population, had precipitating antibody to SEB; 29 samples had sufficient antibody for quantitation. An unexpected observation was the sporadic appearance of antibody in sera from serial bleedings of both man and monkey. When 4 bleedings of 10 individuals with no known experience with toxin were performed at weekly intervals, antibody was detected in the 2nd and 4th bleedings of one individual, and the 1st, 3rd and 4th bleedings of another. In bleedings from one monkey, antibody was present in one serum sample, but not in a sample taken 2 days later. This pattern of response occurred only with sera that were too weak for quantitation and may represent exposure to minimal antigenic stimulation. For example, with oral challenge, one individual, who showed no overt response, had weak antibody 2 weeks postchallenge but none at 4 weeks.

Throughout these studies there has been the suggestion that more than one antigen-antibody system was involved. With hyperimmune sera clear-cut evidence was difficult to obtain, but with human and monkey sera 2 antigen-antibody systems frequently were suspected from examination of detection tests and titrations. In the simple detection test 0.1 ml of unknown serum was tested against several concentrations of toxin. Sera too weak for quantitation formed only one line of precipitate, but many stronger sera formed 2 distinct lines, as shown in Figure 4. When sera taken before and after challenge were compared (Figure 5), a 2nd antibody could be demonstrated in the postexposure sample. With the postexposure serum a double-line reaction occurred, and one of the lines crossed the line common to the pre- and postchallenge samples. In neutralization titrations 2 antibodies might be present, one requiring more toxin for neutralization than the other.

Studies using disc electrophoresis are helping to interpret some of these findings. Purified toxin was run in a large-pore gel in a cationic system. The column was sectioned lengthwise; one-half was stained for protein and the other was tested by immunodiffusion methods. Figure 6 is a diagrammatic representation, comparing the stained section with the immunodiffusion reaction against hyperimmune horse antiserum and against postexposure serum from an individual involved in the aerosol incident. Shaded areas indicate diffuse reaction. A major and a minor protein appeared in the stained section. At least 2 other minor fractions were demonstrated by immunodiffusion. The rapidly-moving material that reacted with horse serum appeared with a sample of 10 μg. Both components that corresponded to the stained areas reacted with horse serum at ≥ 5 μg of toxin, but only one reacted with 1 μg. The slow-moving material was not demonstrated with horse serum but was the only component that reacted with human serum at the 10-μg level, and the reaction occurred close to the serum slit. With 5 μg, the reaction with human serum appeared as is shown here. The slow-moving component forming a heavy, distinct line and the stained-area component forming a weak, diffuse precipitate. With 1 μg, only the stained-area material showed a reaction forming a heavy line of precipitate about half-way between the column and the antibody slit.
FIGURE 4. DETECTION TEST FOR ANTIBODY. (T=TOXIN; S=SERUM)
FIGURE 5. ANTIBODY IN PRE- AND POST-EXPOSURE SERUMS.
(T=TOXIN; C=CONTROL SERUM; S-1=PRE-EXPOSURE SERUM;
S-2=POST-EXPOSURE SERUM.)
HORSE ANTISERUM

STAINED DISC.
REACTION: SEB

HUMAN ANTISERUM (COHEN)

RUNNING pH: 4.3
RUNNING TIME: 30 MIN.
CURRENT: 5 mA

FIGURE 6. IMMUNODIFFUSION AFTER DISC ELECTROPHORESIS
OF SEB. (COMPOSITE DIAGRAM: SEB SAMPLE = 1-10 µg)
This component identified with 1 of the 2 horse serum reactions but because of concentration effects we are not yet certain which one. In many human serum samples the major antibody appeared to be directed against the slow-moving component. It is hoped that studies now in progress may clarify the significance of these results with regard to protection. The results, however, are compatible with work reported several years ago by other investigators. At that time 2 major and 1-2 minor components were demonstrated in purified toxin by starch electrophoresis and fluorescein paper. Ouchterlony tests with hyperimmune rabbit sera indicated that although 3 components could be detected, one constituted over 99% of the toxin.

Paralleling the preceding experiments, investigations have been in progress on the nature and purification of antibody. Immune electrophoretic studies demonstrated that the antibody in human, monkey and rabbit sera was a \( \gamma_2 \)-globulin; in contrast in hyperimmune horse sera the major antibody was present in the T-globulin region, and only small amounts were in the \( \gamma_2 \)-fraction. During the course of immunizing the horses, there was little difference in titer of sera obtained 7 days after cumulative doses of either 78 mg or 9 gm toxin, but during this interval the major antibody-containing fraction changed from the \( \gamma_2 \) to the T-globulin region, and with 2 of 4 animals \( \gamma_2 \)-antibody disappeared completely. Concentrates of this pooled hyperimmune plasma, prepared by The National Drug Co. from a 28-50% \((\text{NH}_4)_2\text{SO}_4\) cut, showed approximately 5-fold purification, in terms of equivalence values of \( \mu \)g serum protein/\( \mu \)g toxin, but they contained 8-9 serum factors in addition to antibody. After pepsin digestion only 5 nonantibody fractions could be detected.

Investigations in progress have been directed toward increasing the purity of plasma concentrates. Pilot studies suggested that salting-in fractionation resulted in a product with essentially the same neutralizing activity as The National Drug Co. product, but with fewer nonantibody components. Further purification could be effected by passage through DEAE-Sephadex or hydroxyapatite.

**SUMMARY**

Agar diffusion techniques have been of considerable value for studies on detection, measurement and analysis of the antibody response to immunization and challenge with staphylococcal enterotoxin B. Methods were developed for measuring antigen-antibody equivalence and for estimating equivalence values from simple titrations. Equivalence values appeared to correlate with protection against lethal effects of enterotoxin in monkeys and with protection against illness in man. Immunoelectrophoretic analysis indicated the presence of at least 2 minor antigens in the purified enterotoxin preparation. The major antibody reaction of some human and monkey sera appeared to be directed against one of the minor antigens. Consequently, it will be necessary to isolate the antigenic components in order to determine the contribution of individual antibodies to serum titer and to protection.
LITERATURE CITED

A review of the research program directed toward determining the biological effects of staphylococcal enterotoxin B is presented.
Enterotoxin  
Biological effects  
Radiotopes  
Immunology  
Serology  
Pyrogens  
Therapy

1. ORIGINATING ACTIVITY: Enter the name and address of the contractor, subcontractor, grantee, Department of Defense activity or other organization (corporate author) issuing the report.

2. REPORT SECURITY CLASSIFICATION: Enter the overall security classification of the report. Indicate whether "Restricted Data" is included. Marking is to be in accordance with appropriate security regulations.

3. GROUP: Automatic downgrading is specified in DoD Directive 5200.10 and Armed Forces Industrial Manual. Enter the group number. Also, when applicable, show that optional markings have been used for Group 3 and Group 4 as authorized.

4. REPORT TITLE: Enter the complete report title in all capital letters. Titles in all cases should be unclassified. If a meaningful title cannot be selected without classification, show title classification in all capitals in parenthesis immediately following the title.

5. DESCRIPTIVE NOTEs: If appropriate, enter type of report, e.g., interim, progress, summary, annual, or final. Give the inclusive dates when a specific reporting period is covered.

6. AUTHORS: Enter the name(s) of author(s) as shown on or in the report. Enter last name, first name, middle initial. Military, show rank and branch of service. The name of the principal author is an absolute minimum requirement.

7. REPORT DATE: Enter the date of the report as day, month, year, or month, year. If more than one date appears on the report, use date of publication.

8. TOTAL NUMBER OF PAGES: The total page count should follow normal pagination procedures, i.e., enter the number of pages containing information.

9. NUMBER OF REFERENCES: Enter the total number of references cited in the report.

10. CONTRACT OR GRANT NUMBER: If appropriate, enter applicable number of the contract or grant under which the report was written.

11. PROJECT NUMBER: Enter the appropriate military department identification, such as project number, subproject number, system numbers, task number, etc.

12. ORIGINATOR'S REPORT NUMBER(S): Enter the official report number by which the document will be identified and controlled by the originating activity. This number must he unique to this report.

13. OTHER REPORT NUMBER(S): If the report has been assigned any other report numbers (either by the originator or by the sponsor), also enter these numbers.

14. KEY WORDS: Key words are technically meaningful terms or short phrases that characterize a report and may be used as index entries for cataloging the report. Key words must be selected so that no security classification is required. Identify such as equipment model designation, trade name, military project code name, geographic location, may be used as key words but will be followed by an indication of technical control. The assignment of links, rules, and weights is optional.

UNCLASSIFIED
Security Classification