ENTEROTOXIC SHOCK IN RHESUS MONKEYS: THE ROLE OF SELECTED BLOOD--ETC(U)

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Enterotoxic Shock in Rhesus Monkeys:
The Role of Selected Blood-Borne Factors

by

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Abbreviated Title: ENTEROTOXIC SHOCK IN Rhesus MONKEYS

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

The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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Staphylococcal enterotoxin B, a protein exotoxin from Staphylococcus aureus, produced progressive hypotension and shock when injected (1 mg/kg, intravenous) into rhesus monkeys. Plasma levels of factors which have been implicated in the pathogenesis of other types of shock were measured. Endotoxin-like activity (ELA) was measured by the Limulus lysate technique, fibrin degradation products (FDP) were quantified by electroimmunoassay, and activation of the complement system was assayed by measuring total hemolytic complement. Activation of the intrinsic coagulation cascade was assessed by measuring activated (cont’d)
partial thromboplastin time (APTT). Activation of the kinin system was evaluated by measuring prekallikrein activity and kininogen. Myocardial depressant factor (MDF) was measured by paper chromatography. EIA did not appear in plasma, and the complement system was not activated. The appearance of FDP and significant trend for prolongation of APTT indicated activation of fibrinolysis and the intrinsic coagulation cascade, and suggested that disseminated intravascular coagulation (DIC) was occurring. Activation of the kinin system was evidenced by a progressive and significant depletion of kininogen from 338 ± 37 to 226 ± 22 ng kallidin generated/ml, and a significant depletion of plasma prekallikrein activity from 169 ± 8 to 110 ± 15 tosylarginine methyl ester (TAME) esterase units/ml. Analysis of covariance indicated that activation of the kinin system was related to changes in blood pressure. MDF did not increase until immediately before death (increase from 1.08 ± 0.15 to 1.92 ± 0.11 paper chromatographic units per μl, n = 6. We conclude that kinins, MDF, and DIC, but not complement or endotoxin, may contribute to the pathogenesis of enterotoxic shock in rhesus monkeys.
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SUMMARY Staphylococcal enterotoxin B, a protein exotoxin from Staphylococcus aureus, produced progressive hypotension and shock when injected (1 mg/kg, intravenous) into rhesus monkeys. Plasma levels of factors which have been implicated in the pathogenesis of other types of shock were measured. Endotoxin-like activity (ELA) was measured by the Limulus lysate technique, fibrin degradation products (FDP) were quantified by electroimmunoassay, and activation of the complement system was assayed by measuring total hemolytic complement. Activation of the intrinsic coagulation cascade was assessed by measuring activated partial thromboplastin time (APTT). Activation of the kinin system was evaluated by measuring prekallikrein activity and kininogen. Myocardial depressant factor (MDF) was measured by paper chromatography. ELA did not appear in plasma, and the complement system was not activated. The appearance of FDP and significant trend for prolongation of APTT indicated activation of fibrinolysis and the intrinsic coagulation cascade, and suggested that disseminated intravascular coagulation (DIC) was occurring. Activation of the kinin system was evidenced by a progressive and significant depletion of kininogen from 338 ± 37 to 226 ± 22 ng kallidin generated/ml, and a significant depletion of plasma prekallikrein activity from 169 ± 8 to 110 ± 15 tosyl arginine methyl ester (TAMe) esterase units/ml. Analysis of covariance indicated that activation of the kinin system was related to changes in
blood pressure. MDF did not increase until immediately before death (increase from $1.08 \pm 0.15$ to $1.92 \pm 0.11$ paper chromatographic units per µl, $n = 6$). We conclude that kinins, MDF, and DIC, but not complement or endotoxin, may contribute to the pathogenesis of enterotoxic shock ir rhesus monkeys.
ENTEROTOXINS isolated from cultures of Staphylococcus aureus are protein exotoxins (28 – 29,000 daltons molecular weight) which are capable of producing acute food poisoning in humans\(^1\) and other primates.\(^2\) Intravenous (iv) injection of enterotoxins in very small doses into rabbits and monkeys produces lethargy, fever, shock, and death.\(^3\) There is evidence that during staphylococcal wound infections and purulent skin lesions, enough enterotoxin is released into the circulation of an infected patient to stimulate synthesis of antibody specific to the toxins produced.\(^4\) The enterotoxins thus released might contribute to the hypotension and shock often observed in these patients.

When Josefczyk\(^4\) measured anti-enterotoxin antibodies in normal patients and patients with staphylococcal infections, antibody to staphylococcal enterotoxin type B (SEB) was the most common type in each group. SEB has been isolated in purity greater than 99 per cent,\(^5\) and its amino acid sequence has been determined.\(^6\) In order to help to elucidate the mechanisms by which enterotoxins produce shock, we injected SEB (1 mg/kg, iv) into rhesus monkeys and measured plasma levels of various factors (complement, endotoxin, fibrin degradation products, myocardial depressant factor, and kinin system components) which have been implicated in the pathogenesis of other types of shock.

Besides the possibility that enterotoxins are released
into the circulation from staphylococcal infections, enterotoxic shock possesses unique aspects for study as a shock-model; animals have natural antibody to endotoxin, but it is possible to screen monkeys for presence or absence of antibody to enterotoxins and, thereby, study the influence of specific antibody on the progression of shock.

Rhesus monkeys were chosen for the present studies since most of the previous work on enterotoxin has been done in this species. Also, since the cardiovascular responses of endotoxemic rhesus monkeys have been shown to be similar to the responses of humans to Gram-negative sepsis, we hypothesized that the response of the rhesus monkeys to SEB might be similar to that of man.

Methods

Experimental animals were eight healthy, well-conditioned adult rhesus monkeys (Macaca mulatta) weighing 3-5 kg and were seronegative for hemagglutinating antibody to SEB. Monkeys were anesthetized intramuscularly (im) with ketamine hydrochloride (5-10 mg/kg, calculated as the base), prior to implantation of femoral or carotid artery plus femoral or jugular vein catheters (polyethylene catheters). Monkeys were restrained in Plexiglas chairs during the study. All catheters were coated with TDMAC-Heparin Complex (Polysciences Inc., Warrington, Pa.) and infused with saline (1-2 cc per hour) to inhibit clot formation and maintain patency. In each experiment
the first control blood sample was taken 1 hour before injecting SEB, and the second control sample was withdrawn at time 0, immediately before injection of the toxin. Each monkey was injected iv with 1 mg/kg of highly purified SEB (Lot 14-30, USAMRIID, Ft. Detrick, Md); this SEB was from the same lot used in other studies reported from this institute. Antigenicity and potency of the SEB were verified by Oudin immunodiffusion analysis. After injection of toxin, blood samples (8 cc per sample) were collected at various intervals from the arterial catheter; about 70% of erythrocytes removed were returned to one of the animals. In two control animals which were subjected to the same experimental procedure over an 18-hour period, but were not given SEB, this blood sampling procedure resulted in no appreciable change in hematocrit or plasma protein concentration.

Mean arterial blood pressure (MABP) was measured using a Statham (P-23)- pressure transducer coupled to an Electronics for Medicine (VR-6) recorder. Heart rate was obtained from the pressure tracings, and a water manometer was used to measure central venous pressure (CVP). CVP was measured at the phlebostatic level (right atrium) as described by Burch and Winsor.

Plasma endotoxin levels were measured by the Limulus amebocyte lysate technique (E-Toxate®, Sigma Chemical Company Inc.) which has been shown to be capable of detecting very small quantities of endotoxin in plasma. Since evidence
supporting the specificity of this test for endotoxin in plasma is not complete, positive test results are referred to as the ability of a plasma sample to cause gelation of Limulus amebocyte lysate (GLAL). The GLAL is scored (scale of 0 to +3) by the degree and rapidity of gel formation during incubation at room temperature. Under these conditions, addition of 1 µg of endotoxin (Escherichia coli, B:011 Difco) to 1 ml of freshly drawn plasma from normal monkeys resulted in the formation of a solid gel in less than 2 hours (+3); addition of 0.005 µg of endotoxin to 1 ml of plasma produced a solid gel within 18 hours (+1), and 0.001 µg of endotoxin in 1 ml of plasma gave equivocal results (i.e., a turbid solution) at 18 hours.

Myocardial depressant factor (MDF) was assayed by a modification (manuscript in preparation) of the paper chromatographic technique of Barenholz et al. Plasma samples were deproteinized, ether-extracted, and spotted (200 µl) on chromatographic paper (Whatman No. 3 MM) along with a serine standard. The paper was then placed in a tank and subjected to descending flow for 18 hours in a solvent-saturated atmosphere (n-butanol:glacial acetic acid:H₂O, 25:25:6). The papers were sprayed with 0.3% ninhydrin in ethanol and developed at 90°C for 10 minutes. Spots corresponding to Barenholz' spot G (distance traveled relative to serine = 1.6 – 1.8), and the spots developed from serine standard were eluted
from the paper using 3 ml of 1% NaHCO₃, and the absorbance at 570 nm of each eluate was measured. Under these conditions we define 1 MDF paper chromatographic unit as equal to the A₅₇₀ of 1.3 nmol of serine. Using this technique we found excellent correlation (r = 0.92) between MDF measured by bioassay versus MDF measured by paper chromatography.

Activation of the kallikrein-kinin system was assessed by measuring plasma prekallikrein and kininogen activities. Prekallikrein activity, measured according to the method of Colman et al., is based on hydrolysis of tosyl arginine methyl ester (TAMe) after kaolin activation and is expressed as mol of TAMe hydrolyzed/ml/hour. Kininogen was measured by bioassay on guinea pig ileum (in vitro) according to the technique of Rocha e Silva as modified by Pierce and Guimarães. In this procedure an excess of human urinary kallikrein (generously supplied by Dr. J. V. Pierce, National Institutes of Health, Bethesda, Md), followed by the plasma sample to be bioassayed, was added to the solution bathing the guinea pig ileum, and the magnitude of the isotonic contraction of the ileum loaded with 1 g was measured by a displacement transducer. Since human urinary kallikrein is a tissue kallikrein, the kinin generated from plasma kininogen is kallidin (lysyl-bradykinin) and results are expressed as μg/ml of kallidin generated per milliliter of sample. Each sample was assayed in duplicate and compared to samples of standard kallidin (Schwarz-Mann, Orangeburg, New York,
lot no. 2039).

Complement levels in serum were measured using the hemolytic titration of whole complement described by Rapp and Borsos. Results are expressed as the reciprocal of the dilution of serum which causes 50% hemolysis (CH50).

Fibrin degradation products (FDP) in serum were measured by electroimmunoassay through agar containing antibody against monkey fibrinogen (Chappel Laboratories, Cochranville, Pa.). Serum was obtained from blood collected in tubes containing sodium citrate (3.8 mg/ml of blood) and clotted according to the technique of Mersky et al.

Activated partial thromboplastin (APTT) in citrated plasma was measured by a one-stage assay using PlatelinR (General Diagnostics, Division of Warner-Lambert Co., Morris Plains, N. J.) reagent.

Some of the data were subjected to linear regression (least squares) analysis, and the slopes and 99% confidence limits for the slopes of the lines were determined. Student's t-test was employed for comparisons of data obtained immediately before injection of SEB and data obtained immediately before termination of the experiment. Analysis of covariance was used to compare changes in plasma kininogen and prekallikrein to changes in blood pressure.

Results

Six of eight monkeys died after SEB injection. The
two survivors were included in the study since they became moribund, and their mean blood pressure dropped below 60 mm Hg. Mean time to death or lowest blood pressure (for survivors) was 18.6 hours (range 6 – 35). Since the variability in time to death was so great, we calculated data as percent of time to final measurement for each animal. For monkeys which died, the final measurements were the last ones before death; for survivors the final measurements were those taken at the time of lowest blood pressure.

CARDIOVASCULAR DATA

Intravenous SEB resulted in a progressive and significant decrease in mean arterial blood pressure (Fig. 1). The slope of a regression line fit to the data was significantly negative (P < 0.01); a polynomial equation did not fit these data significantly better than a linear equation. Central venous pressure remained within normal limits and did not change significantly in any of the animals.

Heart rate data are presented in Figure 2. Injection of SEB was followed by a rapid increase in heart rate which was maintained until death.

KININ SYSTEM

In our laboratory, mean kininogen concentration in normal control rhesus monkeys (n = 15) is 350 ± 30 ng of kallidin generated per milliliter of plasma. After SEB, plasma kininogen
decreased significantly and progressively (Fig. 3). The slope of the regression line fit to these data was significantly negative (P < 0.01).

Plasma prekallikrein activity (Fig. 4) also underwent a significant and progressive decrease. The slope of the regression line fit to these data was significantly negative (P < 0.01).

To investigate the possibility of a causal relationship between kinin-system activation and blood pressure, we used analysis of covariance to compare blood pressure to changes in plasma kininogen and plasma prekallikrein. This test compared regression lines between prekallikrein versus blood pressure and kininogen versus blood pressure (F statistic for slopes = 2.2) and even more marked covariance between kininogen and blood pressure (F statistic for slopes = 0.643).

COMPLEMENT

As seen in Figure 5, total hemolytic complement CH50 decreased, but the change was not significant. The slope of the regression line fit to the data was not significantly negative (P > 0.05) and the value for CH50 at t = 100% was not significantly greater than the value at t = 0%.

MYOCARDIAL DEPRESSANT FACTOR

Mean MDF was significantly increased in the preterminal sample. The slope of the regression line fit to the data was significantly
positive, but MDF varied widely before the preterminal sample.

**ACTIVATED PARIAL THROMBOPLASTIN TIME AND FIBRIN DEGRADATION PRODUCTS**

APTT was measured in plasma from three monkeys given SEB and one given saline only (Figure 7). Applying linear regression analysis to the data from enterotoxic monkeys we found that there was a significant ($P < 0.01$) trend for prolonged APTT as the shock progressed; this increase did not occur in the control monkey.

Fibrin degradation products (Table 1) were undetectable in all monkeys before injection of SEB ($t = 0\%$) and were not detected at any time in a control monkey given iv saline instead of SEB. Each monkey which received SEB developed FDP at some time during the course of enterotoxemia.

**PLASMA ENDOTOXIN LEVELS**

Endotoxin as measured by quantitating the gelation of *Limulus* amebocyte lysate (lower limit of assay = 5 ng endotoxin/ml of plasma) was not detected in plasma samples from any of the monkeys at any time.

**Discussion**

The dose of SEB which we used (1 mg/kg, iv), is lethal or near-lethal for nonimmune rhesus monkeys; it consistently produces severe hypotension. The progressive hypotension which we observed after
SEB was similar to results obtained by others, but appears to be at variance with observations reported by Liu et al. Liu's group found that mean arterial blood pressure (MABP) in monkeys was relatively constant for at least 6 hours after iv SEB and then decreased, but never to values less than 90 mm Hg until immediately before death. These variant results are probably related to the fact that monkeys used by Liu's group had higher initial blood pressures (MABP = 135 ± 10 mm Hg) compared to our monkeys (MABP = 112 ± 5 mm Hg) or those used by Elsberry et al. (MABP = 118 ± 4 mm Hg); others have reported that the mean arterial blood pressure of a normal unstressed monkey is about 110 mm Hg.

The immediate and sustained increase in heart rate which we observed after SEB has been reported by others and is probably due to the increase in plasma epinephrine level which occurs soon after administration of the toxin. In later stages, hypotension would result in tachycardia through the baroreceptor mechanism.

The absence of change in central venous pressure corroborated similar results obtained by Elsberry et al. who noted only a very slight fall (decrease of 1.5 cm H₂O).

KININ SYSTEM

Progressive and significant depletion of kininogen and prekallikrein indicated that iv SEB causes activation of the
kinin system in monkeys. The sequence by which the kinin system is activated could involve damage to capillary endothelium which results in exposure of collagen to the vascular lumen and, thereby, causes activation of the kinin system through activation of Hageman factor (factor XII of intrinsic coagulation cascade). However, it was found (personal communication, Dr. John L. Middlebrook) that a very high concentration (1 mg/ml) of SEB applied to vascular endothelium in tissue culture, does not cause damage evidenced by morphologic changes (light microscopy) nor inhibition of growth. Alternatively, SEB may cause activation of the kinin system by interaction of toxin with leukocytes. Both in vivo and in vitro evidence indicates that this is the mechanism by which endotoxin causes kinin system activation in subhuman primates and in man. In this regard, it has been demonstrated that, like endotoxin, SEB binds to leukocytes, which are subsequently bound to capillary endothelium in the lung. Pulmonary edema is the most severe and consistent pathologic lesion in monkeys given SEB, and areas of endothelial cell necrosis in the lung are associated with sequestered leukocytes. Furthermore, it has been shown that iv SEB causes increased lymph flow in the thoracic duct and altered radiiodinated serum albumin kinetics, indicative of increased vascular permeability. Thus, the SEB-leukocyte complex may be involved in the increased permeability and damage to capillary endothelium.
observed in enterotoxic monkeys. Activation of the kinin system and subsequent generation of kinin in the area around these leukocytes is an attractive explanation for the increased capillary permeability observed in enterotoxic monkeys.

Kinins also cause peripheral vasodilatation. The covariance found between prekallikrein and blood pressure and the very marked covariance found between kininogen and blood pressure support but do not prove the hypothesis that there is a cause-effect relationship between kinin-system activation and the hypotension observed in enterotoxic shock. It is not surprising that covariance between kininogen and blood pressure is especially marked, because depletion of kininogen is a direct reflection of generation of kinin.

COMPLEMENT

Activation of the complement system may be involved in the early hypotension which occurs after iv endotoxin. The animals which we used in our experiments had no hemagglutinating antibody to SEb, so we would not expect SEb to activate the complement cascade via the classical pathway. In vitro experiments performed by Craig et al. indicated that SEb does not activate the complement cascade via the alternate pathway (properdin). However, we considered it possible that complement could be generated in vivo as a result of damaged capillary endothelium, and subsequent activation of Hageman factor (factor XII)
resulting in production of plasmin which, in turn, activates complement. Therefore, we measured total hemolytic complement (CH50) during the course of these experiments. Our results indicate that complement activation did not occur. However, it must be reemphasized that our monkeys did not have hemagglutinating antibody to SEB in their serum. Antibody (IgG) to SEB is common in rhesus monkeys and is often very high in humans. Thus, SEB could activate the complement system via the classical pathway, in individuals with antibody, by forming antigen-antibody complexes. Indirect evidence for occurrence of such activation is contained in work by Denniston et al. in which a sudden decrease in blood pressure was noted after giving SEB iv to immune monkeys. When the immune monkeys of Denniston et al. were skin-tested with SEB, an Arthus reaction (intermediate hypersensitivity) was elicited. Since antigen-antibody complexes and resultant activation of complement are involved in the Arthus reaction, its occurrence in monkeys immune to SEB indicates that complement is probably activated in immune monkeys challenged with SEB.

MYOCARDIAL DEPRESSANT FACTOR

After iv SEB, β-adrenergic activity increases, as evidenced by tachycardia and elevated plasma epinephrine levels. Since β-adrenergic activity should increase myocardial contractility, one might consider the possibility
that blood-borne factors which decrease myocardial contractility might have been involved in the development of hypotension. Myocardial depressant factor (MDF) is a low molecular-weight polypeptide shown to be present in the plasma of humans and animals in a variety of types of shock. Our data demonstrate that preterminal levels of MDF are significantly increased in monkeys during enterotoxic shock. In other work (unpublished observations) we found that an increase in MDF (measured by paper chromatography) equal to the mean change which we observed in our enterotoxemic monkeys would result in a 30% increase in bioassayable MDF activity. This means that the plasma from our monkeys in enterotoxic shock, when compared to pre-shock plasma, would cause a 30% depression in the contractility of an isolated papillary muscle. As in other types of shock, this increased level of MDF could be partially responsible for the demise of the animal.

FIBRIN DEGRADATION PRODUCTS AND ACTIVATED PARTIAL THROMBOPLASIN TIME

Appearance of fibrin degradation products and prolongation of activated partial thromboplastin time in our monkeys are indicative of activation of the fibrinolytic and intrinsic coagulation systems respectively; activation of fibrinolysis has come to be regarded as secondary to activation of intravascular coagulation in shock. Activation of fibrinolysis and coagulation plus the thrombocytopenia observed by others in enterotoxic animals are all laboratory criteria consistent with
the diagnosis of disseminated intravascular coagulation (DIC). Although these laboratory data indicate the presence of DIC, we could not find consistent gross or histopathologic evidence for this diagnosis in the monkeys which died; Hawley et al. obtained similar results in rhesus monkeys infected with Salmonella typhimurium in which they found laboratory evidence of DIC with no concomitant pathologic evidence.

ENDOTOXIN

In studies in rabbits we found that iv injection of SEB resulted in appearance of appreciable levels (up to 1 ug/ml) of endotoxin in plasma, presumably absorbed from the gut; death was associated with endotoxemia, and survival was associated with lack of endotoxemia. In the present study we obtained contrasting results in that endotoxin was never detectable in any of the monkeys despite measurement at repeated time intervals during enterotoxemia. Lack of enterotoxin-induced endotoxemia dispells the notion that SEB may be exerting its toxic action in monkeys via secondary release of endotoxin from the gut; such an hypothesis is attractive since there are many similarities between endotoxic and enterotoxic shock in monkeys.

As in other types of circulatory shock, a variety of factors appear to contribute to hypotension and death in enterotoxic shock. Along with pulmonary edema and alterations
in circulatory physiology, metabolic derangements might also be involved in enterotoxemia. Canonico and Van Zweiten documented mitochondrial swelling and subsequent loss of mitochondrial function in rabbits given SEB. Crawley et al. described mild hyperglycemia followed by progressively developing modest hypoglycemia in enterotoxemic monkeys; these changes were similar to those seen after endotoxin, in which case glucose and insulin initially rose then subsequently fell, and the animal eventually reached a state of hypoglycemia associated with glycogen depletion and hypoinsulinemia. The present study addresses the cardiovascular derangements present in enterotoxemia; our data indicate that activation of the kinin, fibrinolytic, and coagulation systems plus increased plasma levels of MDF may contribute to enterotoxic shock.
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FIGURE 1  Change in mean arterial blood pressure in 8 monkeys
given SEB (●) and two controls (○) given saline. Mean time
to final measurement was 18.6 hours (range 6 - 35). Solid line
is regression line for monkeys given SEB. Dashed line is regression
line for control monkeys.

FIGURE 2  Change in heart rate in 7 monkeys given SEB (●), and
in two control monkeys (○) given saline.

FIGURE 3  Change in plasma kininogen in 5 monkeys given SEB (●)
and two controls (○) given saline. Solid line is regression line
for monkeys given SEB. Dashed line is regression line for control
monkeys.

FIGURE 4  Change in plasma prekallikrein activity (TAME esterase
activity) in 7 monkeys given SEB (●) and in two controls (○)
given saline. Solid line is regression line for monkeys given
SEB. Dashed line is regression line for control monkeys.

FIGURE 5  Change in total hemolytic complement (CH50) in 7
monkeys given SEB (●) and two controls (○) given saline.
Solid line is regression line for monkeys given SEB. Dashed line
is regression line for control monkeys.
FIGURE 6  Change in plasma myocardial depressant factor in 7 monkeys given SEB (●) and one control (○) given saline. Solid line is regression line for monkeys given SEB. Dashed line is regression line for control monkeys.

FIGURE 7  Change in activated partial thromboplastin time in 3 monkeys given SEB (●) and one control monkey (○) given saline. Solid line is regression line for monkeys given SEB. Dashed line is regression line for control monkey.