Serum-mediated Depression of Neutrophil Chemiluminescence Following Blunt Trauma

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To investigate one possible mechanism responsible for decreased neutrophil bactericidal activity following trauma, the chemiluminescence response of normal neutrophils was measured following incubation in nonseptic and septic serum from 19 blunt trauma patients. Incubation of normal neutrophils in septic patients' sera (61 studies) resulted in a marked decrease in the chemiluminescence response (36 ± 26% of control), compared to incubation in nonseptic sera (92 studies, 80 ± 53% of control; $p < 0.005$). This difference between nonseptic and septic serum was apparent immediately after injury, *prior* to the development of sepsis (47 ± 4% *versus* 77 ± 12%; $p < 0.05$). The depression of the CL response was due to a suppressive factor present in septic patients' sera. This factor was nondialyzable and was present in high performance liquid chromatography (HPLC) fractions containing protein of molecular weight 50 to 100,000. Removal of albumin using Affi-gel-blue® did not remove the suppressive factor. In contrast to the suppressive effect of septic trauma serum, septic patients' neutrophils had a normal chemiluminescence response after their isolation and washing. We conclude that trauma results in the generation of a serum factor that suppresses neutrophil chemiluminescence and that is present in greater amounts in patients who eventually become septic. This factor may be responsible for the decreased bactericidal activity and depressed host defense following injury.

SEVERE BLUNT TRAUMA results in a number of acquired, reversible host-defense abnormalities,[1-6] which are hypothesized to predispose the traumatized patient to the development of sepsis.[7,8] This hypothesis remains unproven, although there is experimental data to support such a concept.[9,10] In clinical studies, it has been extremely difficult to identify the alterations responsible for the development of sepsis following blunt injury, as cause and effect cannot easily be determined. Few of these host-defense abnormalities have conclusively been shown to be of predictive value.[1,3] The complex interrelationships between these immunobiologic variables and as yet unidentified variables are undoubtedly responsible for the inability to implicate a specific abnormality responsible for the subsequent development of sepsis. Each immunobiologic variable of presumed importance should be fully analyzed before establishing the relative contribution of each in determining resistance to sepsis following injury.

Neutrophil bactericidal activity following trauma remains poorly characterized. There have been no studies that have exclusively investigated neutrophil bactericidal activity following blunt injury. Previously published studies in this area have dealt with either burn injury or a mixed population of surgical, trauma, and burn patients.[2,7] These studies, although demonstrating a loose association between cyclic alterations in neutrophil bactericidal activity and bacteremic episodes, were unable to establish a relationship between injury, depressed neutrophil bactericidal activity, and the development of sepsis. This study was undertaken to investigate the relation between blunt trauma, depressed neutrophil chemiluminescence activity, and the subsequent development of sepsis in a homogenous group of blunt trauma victims.

Chemiluminescence was used as an indirect measure of bactericidal-related oxidative metabolism,[11,12] as it is a rapid, sensitive, and noninvasive index of bactericidal activity[13,14] that may be applied to clinical screening of large numbers of samples.

Materials and Methods

Patients

Nineteen consecutive patients sustaining blunt injury and admitted to the Maryland Institute for Emergency Medical Services Systems (MIEMSS) with injuries involving at least two organ systems were studied (Table 1). Sepsis was defined as a positive blood culture, autopsy, or operative evidence of infection, or clinical,

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TABLE 1. *Clinical Characteristics of Nonseptic and Septic Patients Admitted to MIEMSS*

Nonseptic	Septic	Cause	Day of sepsis after trauma
1. 28 M; ruptured aorta; hemothorax; fractured ribs, ulnar, supra-orbital rim	48 M; L flail chest; splenic lac; fractured humerus, fibula, ribs; closed head injury	Pneumonia	3
2. 48 M; fractured pelvis, ribs, tib-fib; lung contusion; closed head injury	22 M; fractured pelvis, hip, C-1, T-6; closed head injury	Sinus infection	6
3. 25 M; liver lac.; perf. bowel; renal artery lac.; fractured knees	21 M; perf bowel; fractured humerus, radius, bilat tib-fib, pelvis	Peritonitis	5
4. 25 F; lac. spleen; retroperitoneal hematoma; fractured pelvis	34 F; fractured pelvis; lung contusion; closed head injury	Pneumonia	3
5. 20 M; liver lac; LeFort II	21 M; fractured pelvis, femur, ribs; hemothorax	Soft tissue infection	5
6. 19 M; ruptured aorta; closed head injury; fractured ribs	21 M; LeFort III; closed head injury; nasoethmoid; lung contusion	Meningitis	4
7. 18 F; fractured pelvis, femur, tibia, L-4	45 M; liver lac; fractured ribs; lung contusion	Liver abscess	4
8. 58 M; fractured tib-fib, ribs; lung contusion	70 M; compartment syndrome lower ext.	Soft tissue infection	17
9. 41 M; lung contusion; fractured T-spine, hip	28 M; brachial artery, fractured tib-fib, humerus, metatarsals	Soft tissue infection	5
10. 32 F; lung contusion, fractured femur, bilateral tib-fib			
Age 31.4 ± 4.5	34.4 ± 6.0		
Injury severity score (ISS) 26.1 ± 0.6	25.0 ± 3.6		

roentgenographic, and laboratory evidence of pneumonia. All patients were admitted to MIEMSS within 1 hour of injury and all underwent operation within 2 hours of admission. Blood samples were drawn for neutrophil isolation and serum collection within 24 hours of injury (1 day after trauma). Thereafter, blood samples were drawn daily until death, discharge, or for 3 weeks after injury. Serum was immediately frozen at −70 C until use. Anticoagulated blood (5 ml EDTA) was drawn for neutrophil isolation for use in chemiluminescence assays. Normal neutrophils were collected from healthy volunteers with type O blood. Normal serum was collected from healthy donors of different blood groups and stored at −70 C until use. Informed consent was obtained from all patients or their families in accordance with institution guidelines.

Chemiluminescence Assay

Chemiluminescence, as described by Allen,[11,15] represents light emitted by phagocytic cells, and is the end product of membrane stimulation and activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.[16] Oxidation of numerous poorly defined molecular intermediates by superoxide anion and oxygen-free radicals formed by NADPH oxidase results in visible light emission. Thus, chemiluminescence indirectly measures bactericidal-related metabolic activity.[11,12] Under proper *in vitro* conditions, such emission correlates well with directly measured bacterial killing.[13,14]

In performing this assay, 5 ml of anticoagulated (EDTA) whole blood was mixed with 2 ml of 6% dextran (T500, Pharmacia) in 0.9% NaCl, and the red cells were allowed to settle at room temperature for 45 minutes at 27 C. The leukocytes were then washed three times with Geys Balanced Salt Solution (GBSS, Gibco) and resuspended to a concentration of 2×10^6/ml. A differential count was performed in order to correct for the number of neutrophils present. Next, 1×10^6 neutrophils were added to the reaction cuvette together with 0.1 ml of test serum and incubated at 27 C for 15 minutes, followed by the addition of 0.25 ml of 1.25 mM Luminol® in GBSS, plus GBSS to a total volume of 0.9 ml. Chemiluminescence was initiated by the automatic addition of either a 0.1 ml solution of washed latex particles (0.81 μ Difco®) or 0.2 mg Zymosan A (Sigma). The Zymosan was preopsonized in either normal serum or patient serum. Monitoring of chemiluminescence was performed in a Luminometer® (1251 LKB). Output in millivolts was recorded every minute for 15 minutes. Results were reported as the peak response, the 15-minute integral response, and ratio of patient values to control. Zymosan was used to evaluate neutrophil activation by complement-mediated phagocytosis, while latex was used to evaluate nonspecific, noncomplement-mediated phagocytosis in order to more completely characterize the nature of the neutrophil dysfunction.

Opsonization of Zymosan

Zymosan A was suspended in GBSS at a concentration of 2 mg/ml and heated in a boiling water bath for 30 minutes, washed twice with GBSS, and opsonized with 20% normal or patient serum in a shaking water bath

for 30 minutes at 37 C. The opsonized Zymosan was then washed three times and resuspended in GBSS to a concentration of 2 mg/ml. In the chemiluminescence studies, 0.1 ml of this suspension was used.

Serum Fractionation

Serum was fractionated on an aqueous gel filtration column (Protein-Pack 300, Waters-Millford, MA) using high performance liquid chromatography (HPLC). The column was equilibrated with GBSS, and undiluted serum was pumped through the column at a rate of 0.5 ml/min at room temperature. Fractions were eluted according to molecular weight, with proteins of highest molecular weight eluting first.

Statistics

One-way analysis of variance was used throughout. Results were considered significant if p was less than 0.05.

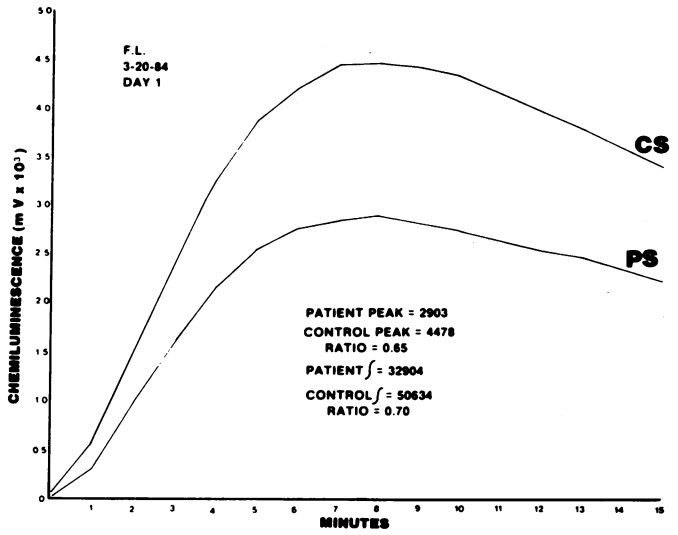


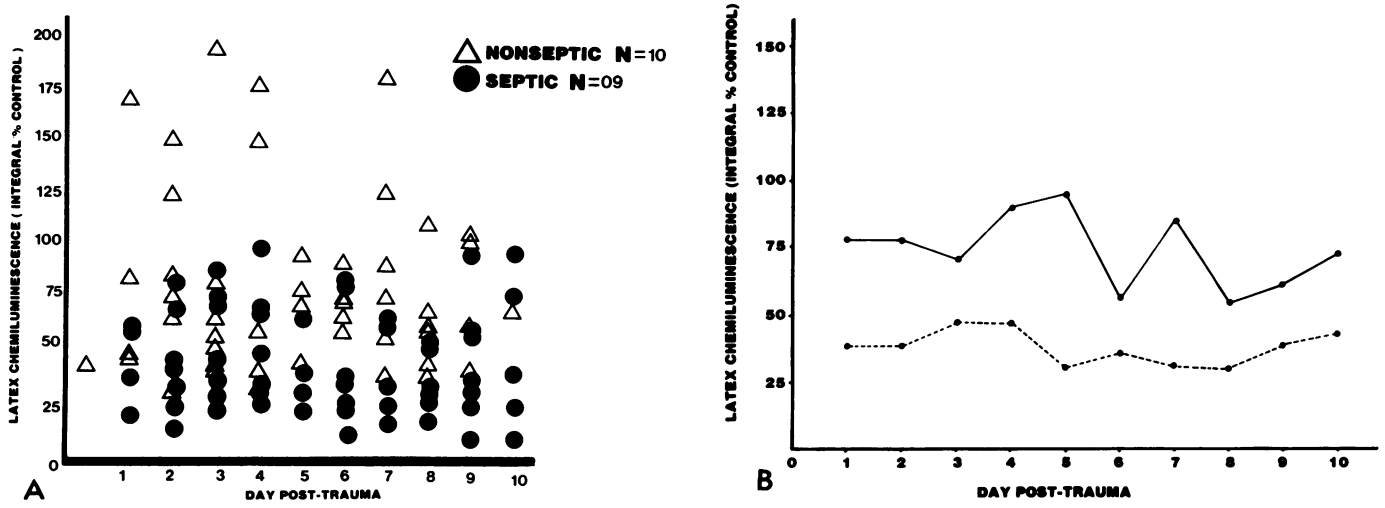
FIG. 1. Chemiluminescence response using normal neutrophils and patient's serum following blunt trauma. Normal neutrophils were incubated in either control serum (CS) or patient serum (PS) prior to testing. Chemiluminescence was initiated by the addition of 100 μl of preopsonized Zymosan.

Results

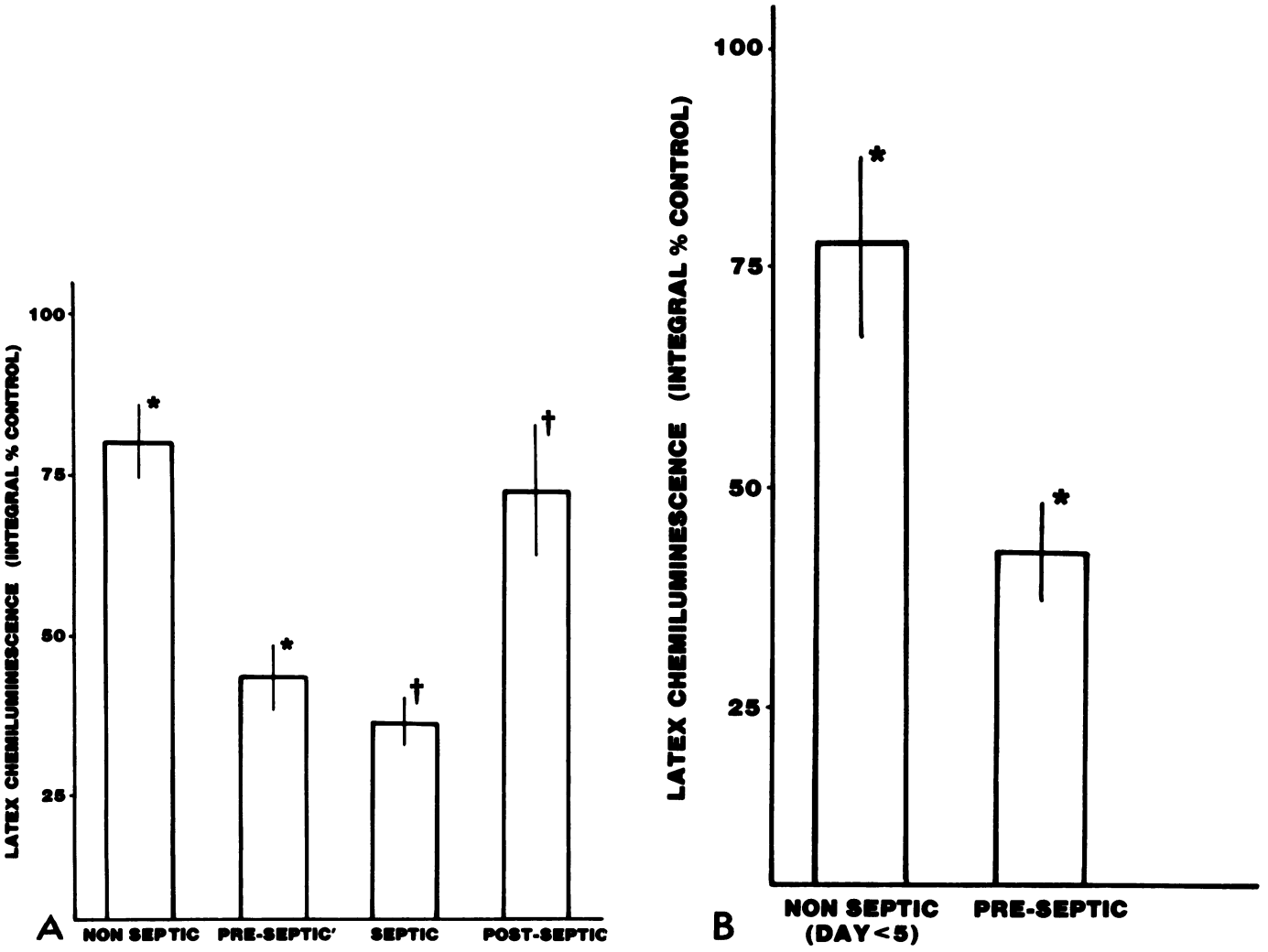
The clinical characteristics of the ten nonseptic and nine septic patients are shown in Table 1. There were no significant differences in either age or injury severity score between the groups. All patients had at least two organ systems injured, with the exception of septic patient 8, who had only an extremity crush injury. There were three intraabdominal injuries in the nonseptic group (one splenic laceration, one liver laceration, and one bowel perforation), and three intraabdominal injuries in the septic group (one splenic laceration, one liver laceration, and one bowel perforation). There were four lung contusions in the nonseptic group and three in the septic group; both cases of pneumonia occurred in patients with lung contusion. The three soft tissue infections occurred in two patients with severe grade III fractures and in one crush injury without fracture. The two intraabdominal infections occurred in patients with intraabdominal injuries (one perforated bowel and one liver laceration). Meningitis developed in a patient with a nasoethmoid and other severe facial fractures, while a sinus infection developed in a patient with a nasogastric tube in place and closed head injury. The onset of clinically detectable sepsis ranged from 3 days to 17 days after trauma, with a mean of 5.8 ± 1.3 days.

An example of the results obtained using the chemiluminescence assay is shown in Figure 1, which compares the response of normal neutrophils incubated in normal serum to that obtained using normal cells incubated in a patient's serum obtained 1 day following injury. The chemiluminescence response of the normal cells incubated in the patient's serum was depressed compared with the response using control serum. The results of all chemiluminescence assays using latex as the stimulant are shown in Figures 2A and B. Higher values were obtained using nonseptic patients' sera compared to septic patients' sera. This was true even when measured shortly after admission, prior to the onset of sepsis. Data using patients' sera are shown only until day 10, as an insufficient number of samples were available thereafter for complete analysis. Nonseptic trauma sera were less effective than control sera for approximately 1 week after injury, followed by a gradual return to control values thereafter. Septic patients' sera activity remained depressed until after the resolution of sepsis. Further analysis of these septic patients is shown in Figures 3A and 3B. The values obtained using septic patients' sera *prior to* the onset of sepsis (presepsis) were compared to either all values in nonseptic patients (Fig. 3A) or to the values obtained with nonseptic sera before the fifth day after trauma (Fig. 3B). The sera from septic patients taken early, even before sepsis intervened (presepsis), adversely effected the chemiluminescence response of normal neutrophils. The "preseptic" value was calculated as the mean obtained from all values using septic patients' sera prior to the onset of sepsis. Furthermore, the chemiluminescence response remained low during sepsis and only recovered following the resolution of the septic episode (Figure 3A).

In contrast to this serum defect, patients' neutrophils incubated in either serum-free medium or medium containing normal serum revealed no evidence of intrinsic cellular dysfunction. When tested using latex, nonseptic neutrophils had a chemiluminescence response of 100 ± 64 (% control), while septic neutrophils had a



FIGS. 2A and B. Latex chemiluminescence response using serum from nonseptic and septic patients following blunt trauma. *A.* Raw data from all patients. *B.* Daily mean values. Normal neutrophils were assayed in nonseptic or septic patients' sera $p < 0.005$; septic compared to nonseptic (ANOVA).



FIGS. 3A and B. Chemiluminescence response of normal neutrophils using nonseptic and septic serum from various periods after trauma. *A.* Nonseptic sera compared to septic sera from various periods related to sepsis. *B.* Early nonseptic period (before day 5 after trauma) compared to preseptic period. "Presepsis" includes all values prior to onset of sepsis. (*$p < 0.05$ and †$p < 0.05$)

TABLE 2. *Early and Late Chemiluminescence Responses Using Nonseptic and Septic Serum**

Assay conditions			Nonseptic serum	Septic serum			
Cells	Particle	Serum		All	Presepsis	During sepsis	After sepsis
Normal	Latex	Patient§	80 ± 53 (92)†	47 ± 38 (106)‖	43 ± 23 (20)‖	36 ± 26 (61)¶	72 ± 54 (27)¶
Normal	Z(NS)‡	Patient†	99 ± 25 (97)	85 ± 25 (106)‖	89 ± 13 (20)‖	79 ± 25 (60)¶	96 ± 27 (26)¶
Normal	Z(PS)§	Normal	106 ± 25 (50)	102 ± 13 (46)			

* Results expressed as integral (% control); mean ± SD.
† Numbers in parenthesis are number of determinations.
‡ Zymosan (Z) was opsonized with either normal serum Z(NS) or patient's serum Z(PS).
§ Denotes the variable assayed with the other two remaining constant.
‖ $p < 0.05$ compared with nonseptic.
¶ $p < 0.01$ compared with each other within row.

response of 106 ± 61 (% control). There was no difference between septic and nonseptic patients' neutrophils in this respect.

Preopsonized Zymosan was compared with latex particles as to their ability to stimulate the chemiluminescence response of normal neutrophils. Using either particle, incubation of normal cells in septic serum resulted in a diminished response compared to incubation in nonseptic serum. Using latex particles, *nonseptic* patients' sera yielded a diminished chemiluminescence response early after trauma, while these same sera yielded initial values similar to control values when preopsonized Zymosan was used (Table 2). In addition, preopsonization of Zymosan with either nonseptic or septic patients' sera yielded results similar to that of preopsonization with normal serum (Table 2). This finding indicates that serum opsonization *via* the alternative complement pathway remains intact following injury.

In order to differentiate between a deficiency of some needed serum component and the presence of an inhibitor, increasing amounts of patient serum was added to 100 μl of control serum for neutrophil incubation and testing. Addition of increasing amounts of a septic patient's serum resulted in decreasing chemiluminescence in a dose-related fashion, indicative of the presence of a serum inhibitor of neutrophil chemiluminescence contained in the septic serum (Fig. 4). Because increasing quantities of serum protein are known to quench light emission,[12] addition of a septic patient's serum was compared with the addition of normal serum. Figure 5 demonstrates the calculation of a 50% inhibition dose (Inh 50) as a reflection of the amount of inhibiting substance present in a serum sample. Addition of increasing amounts of normal serum to 100 μl of control serum resulted in some diminution of the integral chemiluminescence response in accordance with the known effect of increasing protein concentration. In contrast, addition of this septic patient's serum to 100 μl of control serum resulted in a far greater diminution of the chemiluminescence response for the same amount of serum added. This was in spite of lower total protein concentrations in the patient's serum compared with the control serum. The lower Inh 50 (82.6 μl *versus* 298.7 μl for control serum) indicated the presence of a serum inhibitor that could then be quantified using this method. The chemiluminescence response of a given sample was then correlated with that sample's Inh 50 as calculated in the inhibition assay. The results, shown in Figure 6 for ten randomly chosen samples from five septic and five nonseptic patients, demonstrate the correlation between the natural log of the Inh 50 and the chemiluminescence response. These results indicate that the decreases in chemiluminescence following trauma are due to the presence of a circulating inhibitor and

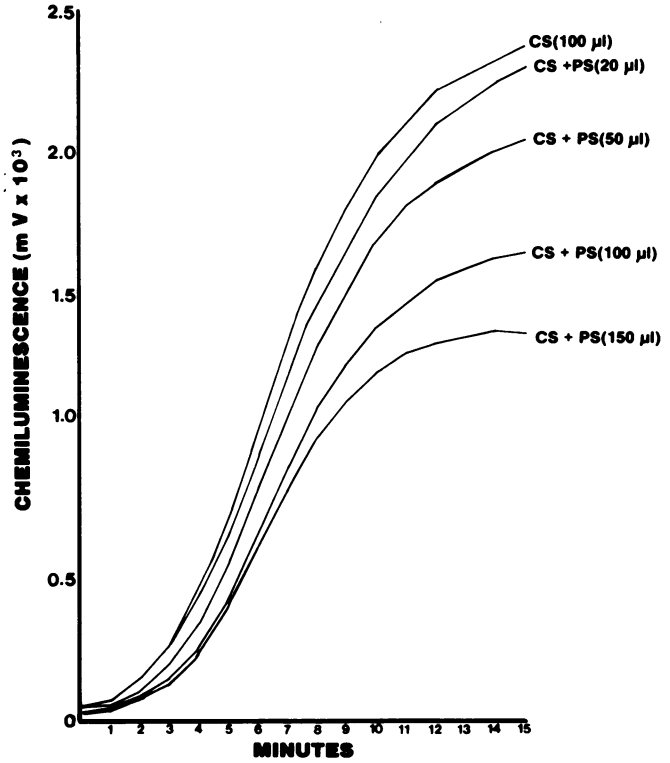


FIG. 4. Inhibition of latex chemiluminescence by addition of a septic patient's serum. CS = control serum; PS = patient serum. Addition of normal serum in corresponding amounts did not result in as marked a decrease in CL (see Fig. 5).

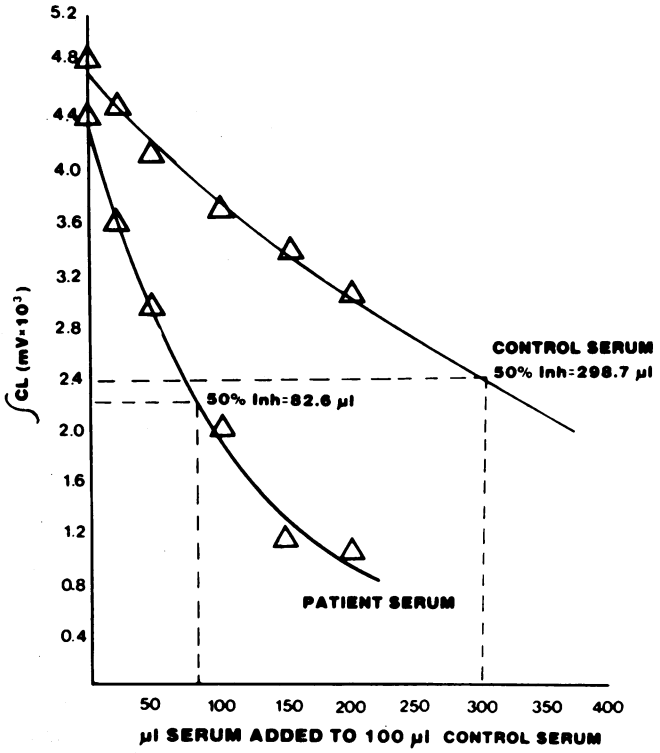


FIG. 5. Calculation of the 50% inhibition dose (Inh 50) as a semiquantitative measure of inhibitory activity in a serum sample.

not a deficiency of some sera factor. This inhibitor is present in greater quantity in those patients who eventually become septic.

Preliminary characterization of this substance has been performed. It has been found to be nondialyzable

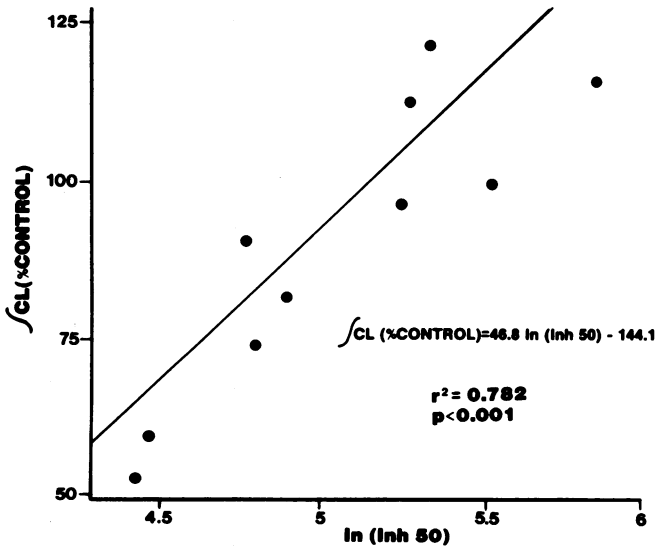


FIG. 6. Correlation between integral chemiluminescence response and Inh 50. The Inh 50 was determined for each sample as shown in Figure 5. This was correlated with the CL response of the same sample determined in the standard assay as shown in Figure 1.

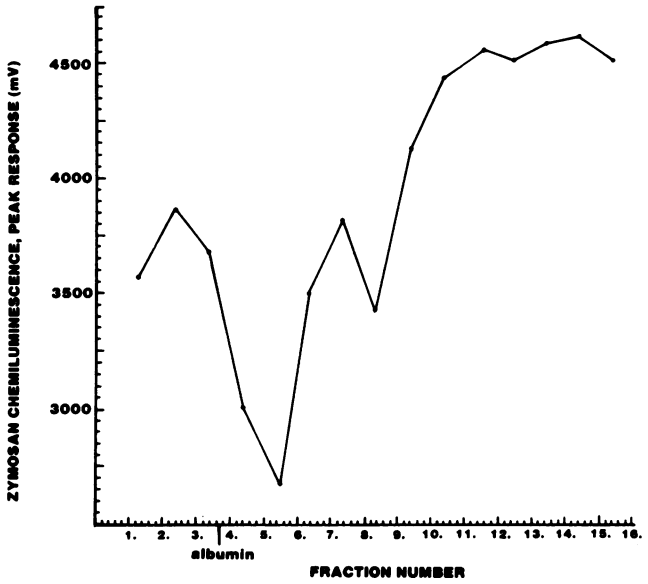


FIG. 7. Serum fractionation using HPLC. Three serum samples from a septic patient were pooled and fractionated using HPLC. Molecular weight decreases with increasing fraction number. 100 µl of each fraction was then added to 100 µl of control serum and the chemiluminescence response measured using preopsonized Zymosan and normal neutrophils.

by overnight dialysis using dialysis tubing of 12,000 molecular weight pore size. Results of HPLC are shown in Figure 7. Fractions demonstrating the lowest chemiluminescence activity contained protein with molecular weight between 50 and 100,000. Removal of albumin using Affi-gel-blue chromatography (BioRad) did not remove the inhibitor.

We attempted to remove this inhibitor from a septic patient's serum using plasma exchange in an effort to increase circulating neutrophil bactericidal activity. The following case history illustrates this preliminary attempt:

JK, a 52-year-old man, was brought to MIEMSS following a fall. He sustained a severe left-sided lung contusion and multiple rib fractures. His hospital course was characterized by a necrotizing left lung infection due to *Pseudomonas*. Abscess formation necessitated a left lower lobectomy followed later by a total left pneumonectomy for control of the infection. He developed severe systemic sepsis secondary to the pulmonary infection and empyema, with multiple organ failure characterized by severe respiratory failure, hypotension, jaundice, and coagulopathy. The chemiluminescence response of normal neutrophils tested in this patient's serum was 14% of control. In view of the patient's clinical deterioration, plasma exchange was performed using fresh frozen plasma as replacement in an attempt to remove any "inhibitors" from his plasma. Following ten units of plasma exchange, the chemiluminescence response increased to 43% of control, and after 20 units reached 58% of control. His clinical state stabilized and slowly improved over the course of the next 48 hours.

This case is reported only to confirm the existence of a circulating serum inhibitor of neutrophil chemiluminescence and the feasibility of its removal using plasma exchange.

Discussion

Neutrophil bactericidal activity has not been adequately investigated following blunt injury. Alexander et al.[2] studied neutrophil bactericidal activity in six trauma patients, together with a number of burn and transplant patients. Only one of these trauma patients was a victim of blunt injury. One of the six patients demonstrated a significant defect in neutrophil bactericidal activity associated with a penetrating injury, although no infection developed.

Using chemiluminescence as a rapid, noninvasive indicator of neutrophil bactericidal-related metabolism, we have described the formation of a trauma-derived serum inhibitor of neutrophil function. Numerous studies have demonstrated that chemiluminescence measurements correlate with *in vitro* bactericidal activity[13,14] under carefully standardized conditions. It should be pointed out that nonparticulate stimuli including formyl-methionyl-leucyl-phenylalanine (FMLP), phorbol myristate acetate, and Concanavalin A are capable of initiating the respiratory burst and chemiluminescence.[18,19] Membrane perturbation from these or other stimuli results in Ca^{++} flux,[18] superoxide production,[19] arachidonic acid metabolism,[17] and ultimately chemiluminescence production. Under rare experimental conditions such as partial heat-inactivation of neutrophils, chemiluminescence can be uncoupled from bactericidal activity.[14] Acknowledging these limitations, we feel that, under the conditions of the present study, the chemiluminescence response accurately reflects serum-mediated bactericidal activity *in vitro* and probably *in vivo* as well.

Variables influencing the chemiluminescence response include availability of divalent cations and glucose, endotoxin, erythrocytes, hemoglobin, and prostaglandins.[17,20,21] Our serum samples were not hemolyzed, and it is unlikely that significant amounts of endotoxin would be present immediately after blunt injury. The final serum concentration used was 10% in the assay, and all measurable electrolytes were within normal limits at the time of blood sampling, so that divalent cation content was presumably the same in all samples. The nonseptic and septic patients did not differ in the type or amount of vasoactive or other drugs administered, except after the onset of sepsis, when septic patients required more hemodynamic support. There is no evidence that adrenergic drugs inhibit chemiluminescence, although increased intracellular cyclic adenosine monophosphate (AMP) does inhibit chemiluminescence *in vitro*.[21] Overnight dialysis performed on a number of samples, which would remove any antibiotics, did not restore the response. Prostaglandins E_1 and E_2 are known inhibitors of chemiluminescence.[17] These species are present in elevated concentration following burn as well as traumatic injury.[22] These or other prostaglandins cannot be ruled out as inhibitors of the chemiluminescence response in our study. Prostaglandin E has other immunosuppressive properties and binds to albumin.[23] In our study, the maximum suppressive activity was contained in fractions of molecular weight similar to that of albumin. However, removal of albumin with Affi-gel-blue did not remove the inhibitor. We conclude that the inhibitor is a large molecular weight species, either a protein or abnormal cleavage product generated by trauma-induced proteolytic activity. Further identification and characterization of this inhibitor is in progress.

The exact site of action of such an inhibitor remains to be defined. The use of latex particles revealed a larger difference between early nonseptic patients and controls then did the use of preopsonized Zymosan. The mechanisms involved in latex-membrane interaction and phagocytosis are nonspecific and are poorly understood, but are not complement-mediated, in contrast to membrane binding of Zymosan. Therefore, the inhibitor does not appear to exert its effect by inhibiting complement-mediated binding, but rather by either inhibiting membrane alterations necessary for membrane-ligand interaction or intracellular oxidative metabolism. An additional result of this study was that opsonization using patients' sera was intact, indicating no important deficiency in functional alternative complement pathway activity. Decreases in immunoreactive levels of some components of the alternative pathway have been described following injury,[4] although the functional significance of such a finding remains unclear.

The early separation of nonseptic from future septic patients on the basis of their sera's chemiluminescence response is an important finding of this study. As far as we could ascertain, the two groups of patients were similar in all important aspects. It appears that different individuals respond to similar injuries differently, with some forming larger amounts of circulating inhibitors capable of compromising host-defense function to a greater degree. Chemiluminescence may, therefore, be a reliable indicator of a specific host-defense abnormality predisposing to the development of sepsis following blunt injury.

The improvement in the chemiluminescence response and the patient's clinical state following plasma exchange is consistent with the existence of such an inhibitor. This preliminary observation provides a rationale for a controlled clinical trial to test the usefulness of plasma exchange in this setting while monitoring serum inhibitory activity.

In summary, we have identified the presence of a serum inhibitor of neutrophil chemiluminescence in a number of severely injured patients. Patients destined

to become septic may react to injury by forming larger amounts of this inhibitor as compared to nonseptic patients. This inhibitor appears to be a protein or protein clearage product and can be partially removed by plasma exchange transfusion. This may provide a rational form of therapy for high-risk injured patients who can be identified using chemiluminescence measurements.

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