Chemiluminescence response of human phagocytes in septic patients: Priming effects by endotoxin and inflammatory cytokines

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The production of reactive oxygen species by whole blood and polymorphonuclear leukocytes (PMNs) in the peripheral blood of 20 patients with gram-negative septicemia was studied by using the method of luminol-dependent chemiluminescence (CL) stimulated with non-opsonized zymosan or phorbol myristate acetate (PMA). The CL responses of 10-fold diluted whole blood and PMNs in these patients were significantly increased when compared with those of healthy persons. The number of granulocytes in the blood of patients was increased in many cases. To clarify the reason why the CL response of phagocytes in septic patients is augumented, I studied the priming effect of inflammatory cytokines including tumor necrosis factor -α (TNF-α), interleukin-1β (IL-1β), IL-6 and lipopolysaccharide (LPS: endotoxin) on the CL response of normal human phagocytes. These cytokines and LPS are often detectable in the serum of patients with severe gram-negative sepsis. Whole blood and PMNs were incubated for 10 or 60 min at 37°C with various concentrations of each cytokine or LPS, and the integrated CL response induced by zymosan or PMA was measured for 20 min, permitting comparisons of the effects of cytokines or LPS. Preincubation with TNF-α or LPS resulted in an increase in the CL response of PMNs at concentrations of more than 1 U/ml and 100 ng/ml, respectively. Similar results were obtained in diluted whole blood samples. However, no significant priming effect was observed when PMNs were incubated for 10 and 60 min with various concentrations (1-100 ng/ml) of IL-1β or IL-6. The priming effect of TNF-α was significantly prevented when TNF-α was preincubated with a murine anti-TNF-α monoclonal antibody (TNF-α MAb) or a TNF-binding protein-II (TNF-BPII) for 10 min at 37°C prior to the exposure to whole blood sample. These inhibitory effects were dose-dependent. The present study showed that TNF-α MAb and TNF-BPII have the ability to prevent the enhanced CL response in human whole blood samples primed with TNF-α. These findings may be useful for supportive therapy in patients with gram-negative septicemia and septic shock, especially in cases involving serious PMN-mediated organ damage caused by excessive release of toxic products such as reactive oxygen species.

Key words: sepsis, chemiluminescence, cytokines, lipopolysaccharide, anti-TNF-α MAb, TNF-binding protein

Introduction

Improvements in medical care have given longer life spans to the elderly and patients with severe underlying diseases. One major cause of death continues to be their exceptional vulnerability to infection. The highest mortality occurs in patients with gram-negative infections who develop bacteremia, with reported mortality figures ranging from 10%—20% in relatively healthy individuals to greater than 50% in immuno-compromised patients[1]. Recent studies have demonstrated that a cascade of mediators is stimulated by lipopolysaccharide (LPS or endotoxin), a complex glycolipid which is the major component of the outer membrane of gram-negative bacteria[2]. The clinical syndrome of gram-negative bacterial septicemia appears to result primarily from excessive stimulation of the host immune system by LPS[2,3]. LPS induction of cytokine release, particularly that of tumor necrosis factor-α (TNF-α), is probably the central event in the pathophysiology of gram-negative septicemia, although many additional immunological events accompany cytokine release[2~5] LPS and cytokines, such as TNF-α, prime polymorphonuclear leukocytes (PMNs) for subsequent oxygen radical release, phagocytosis and bactericidal capacity, induce the surface expression of integrins, and promote the adherence of PMNs to endothelium[2,6~8]. This activation of PMNs at the sites of gram-negative infection potentiates host defense against invading bacteria. However, the high levels of circulating LPS and/or TNF-α which occur during severe sepsis may also cause unwanted activation and recruitment of PMNs into uninfected remote organs, resulting in serious PMN-mediated injury consequent to the release of toxic products such as oxygen free radicals[9,10]. Some investigators have also reported that interleukin-1 (IL-1) and IL-6, which are detectable in septic patients' sera, promote PMN activation and accumulation[3,8,11~13]. In this study, I measured the production of reactive oxygen species from PMNs in patients with gram-negative bacteremia by using a luminol-dependent chemiluminescence (CL) method, and compared the priming effect of inflammatory cytokines such as TNF-α, IL-1β and IL-6 on the CL response of PMNs obtained from healthy volunteers with that of LPS. These studies demonstrated that TNF-α and LPS potentiate PMN function directly, whereas neither IL-1β nor IL-6 has this effect. These priming effects by TNF-α and LPS were also observed in whole blood samples. Furthermore, this paper examines whether the priming by TNF-α could be blocked by preincubating TNF-α with either a murine monoclonal antibody (MAb) directed against TNF-α or a TNF-binding protein (TNF-BPII).

Materials and Methods

Patients

The present study included 20 patients with sepsis (11 males, 9 females), aged 32 to 90 years (average±SD, 64±19 years). Eighteen patients survived and two died. Controls were normal persons (18 males, 12 females), aged 18 to 38 years (30±6 years). Sepsis was confirmed by clinical signs and blood cultures. Criteria for a diagnosis of sepsis were as follows[14]: fever or hypothermia (>38.3 or<35.6°C); tachycardia (>90 beats/min in the absence of β-blockade) and tachypnea (respiration>20/min or need for mechanical ventilation); and either hypotension (systolic blood pressure≦90 mm Hg or a sustained drop in systolic pressure≧40 mm Hg in the presence of an adequate fluid challenge and in the absence of antihypertensive agents) or two of the following six signs of systemic toxicity or peripheral hypotension-unexplained metabolic acidosis (pH≦7.3, base deficit>5 mmol/l. or an elevated plasma lactate level); arterial hypoxia (PO_2≦75 mm Hg or PaO_2/FIO_2<250); acute renal failure (urinary output <0.5 ml/kg/h); elevated proth-rombin or partial thromboplastin time or reduction of the platelet count to less than half the baseline value or <100,000 platelets/mm^3; sudden decrease in mental acuity; and cardiac index of>4 l/m^2 with systemic vascular resistance of<800 dyn s/cm^5.

The causative micro-organisms were *Escherichia*

coli (10 cases), *Klebsiella pneumoniae* (3 cases), *Acinetobacter calcoaceticus* (3 cases), *Pseudomonas aeruginosa* (2 cases), *Serratia marcescens* (1 case) and *Salmonella typhi* (1 case). Primary infection sites were the urinary tract (9 cases), catheter-related (5 cases), decubitus ulcer (1 case), pulmonary tract (1 case), colon (1 case) and unknown (2 cases). A shock state evaluated by Bone's definition[3] was confirmed in 6 patients, and the endotoxin serum levels were positive (>10 pg/ml) in 5 of the 10 patients tested by Endospecy® assay (Seikagaku Kogyo Co., Tokyo, Japan). Blood samples for CL assay were obtained from patients within 48 hours after positive blood cultures.

Chemicals and media

Dulbecco's modified Eagle's medium (MEM, Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 25 mM HEPES buffer and L-glutamin 0.3 g/ml, pH 7.4, was used to dilute blood samples. Luminol, purchased from Tokyo Kasei Kougyo Co. (Tokyo, Japan), was dissolved in phosphate buffered saline (PBS) at 20 μg/ml, and zymosan particles (Sigma Chemical Co., St. Louis, MO., USA) were suspended in PBS at 25 mg/ml. Phorbol myristate acetate (PMA) (Sigma Chemical Co.) was dissolved in dimethyl sulfoxide (DMSO) at 100 μg/ml. Highly purified recombinant human TNF-α (specific activity 2.55×10^6 JRU/mg; 392 pg/U) was kindly provided by Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Highly purified recombinant human IL-1β (specific activity 2×10^7 half-maximal U/mg) and IL-6 (specific activity 5×10^6 U/mg) were kindly provided by Outsuka Pharmaceutical Co., Ltd. (Tokushima, Japan) and Ajinomoto Co., Ltd. (Yokohama, Japan), respectively. A murine anti-human TNF-α MAb (IgG_1 class antibody, 1.0 mg/ml) was kindly provided by Outsuka Pharmaceutical Co., Ltd., and recombinant human TNF-BPII (5.0 μg/8.3 μl) was purchased from R & D Systems Inc. (Minneapolis, MN, USA). None of these reagents contained endotoxins as judged by Limulus amebocyte lysate assay (Seikagaku Kogyo Co.; minimum detection level 0.3 ng/ml). LPS derived from *Escherichia coli* O111: B4 strain was purchased from Sigma Chemical Co.

Isolation of PMNs

Ten ml of freshly drawn heparinized blood (10 U/ml) from healthy human donors or septic patients was mixed in a sterile plastic tube containing 4.5% dextran solution and left to stand for 40 min. The leukocyte-rich plasma was centrifuged at 400×g on a Ficoll-Hypaque gradient for 30 min. The erythrocytes were lysed with hypotonic saline (0.2%), and the osmolarity was then restored by adding hypertonic saline (1.6%). The PMNs were washed twice with MEM and adjusted to a final concentration of 1×10^7 cells/ml in MEM.

Measurement of chemiluminescence

Luminol-dependent CL assays were performed using either ten-fold diluted whole blood or isolated PMNs. The reaction mixture contained 1 ml of PMNs in suspension (1×10^6 cells) or 1 ml of diluted whole blood (0.1 ml of heparinized blood diluted with 0.9 ml of MEM), and 20 μl of 11.3 mM luminol solution. After preincubation at 37°C for 10 min, 20 μl (500 μg) of zymosan solution or 5 μl (0.5 μg) of PMA solution was added to the mixture, and CL was continuously measured for 20 min with a six-channel Biolumat LB 9505 device (Berthold Co., Germany).

Priming effect of cytokines and LPS on CL response of human phagocytes

Heparinized blood was collected from healthy volunteers and PMNs were isolated using the method described above. The reaction mixture contained 0.1 ml of PMN suspension (5×10^5 cells), 0.1ml of solution containing various concentrations of TNF-α (0.1 U—1,000 U), IL-1β (1—100 ng), IL-6 (1—100 ng), LPS (10 ng—1 μg) or 0.1 ml of pyrogen-free 0.9% saline for control, and 20 μl of luminol solution. The final volume of each mixture was adjusted to 1 ml with MEM. In the same way, whole blood which was diluted ten-fold was used for phagocytes and pretreated with each cytokine or LPS. After preincubation with various concentrations of each cytokine, LPS or pyrogen-

free 0.9% saline for 10 min (however, isolated PMNs were preincubated with LPS for 60 min) at 37°C, 20 μl of zymosan or 5 μl of PMA solution was added to the mixture and CL was measured for 20 min. The CL-index was calculated by dividing the integrated CL of drug-exposed phagocytes by the integrated CL of untreated phagocytes (control).

Inhibitory effect of anti-TNF-α MAb and TNF-BPII

Solution containing 100 μl of TNF-α solution (100 U, 10 U, respectively) or pyrogen-free 0.9% saline (100 μl) were initially incubated with various concentrations of anti-TNF-α MAb (100—1000 ng) or TNF-BPII (10—100 ng) in a total volume of 200 μl of MEM for 10 min at 37°C, and subsequently, diluted blood samples were primed with this mixture for 10 min and CL activity was measured in the same way after the addition of PMA. The inhibitory effect was also expressed as CL-index.

Statistical analysis

Data are shown as means±SD for all assays. All assays were performed in duplicate and analyzed statistically by Student's *t*-test.

Results

1) CL response of whole blood and isolated PMNs in patients with gram-negative septicemia

Fig. 1 shows that the number of white blood cells and granulocytes in most blood specimens from septic patients increased significantly in comparison with those of healthy subjects. Fig. 2 shows the integrated counts for 20-min measurements of CL for ten fold-diluted whole blood samples from septic patients and healthy sub jects induced by non-opsonized zymosan and

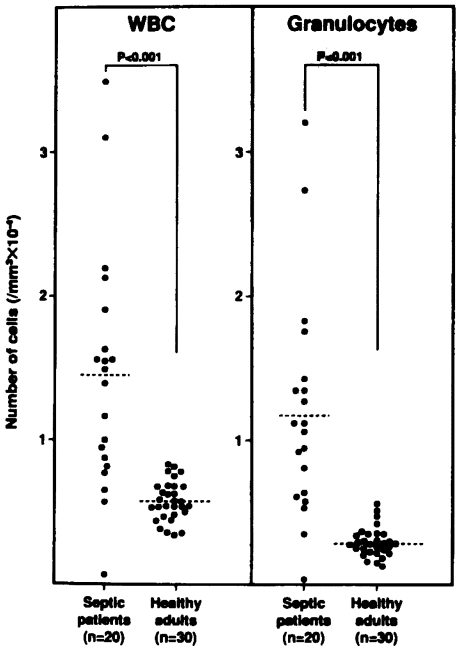

Fig. 1. Number of white blood cells and granulocytes in healthy adults and patients with gram-negative sepsis. Significantly different ($p<0.001$) from the values in healthy adults.

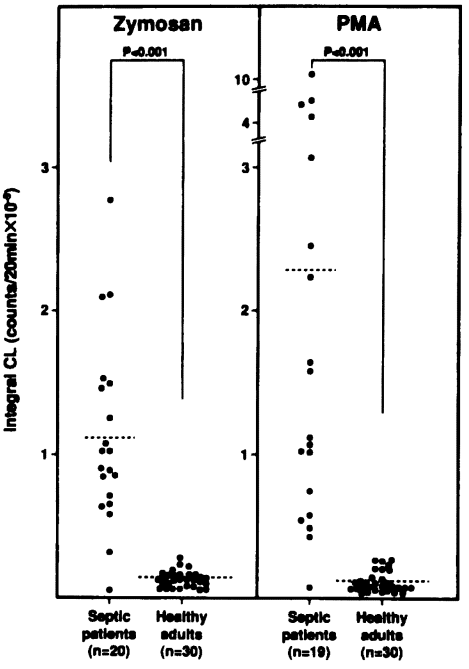

Fig. 2. Chemiluminescence (CL) values of whole blood stimulated with non-opsonized zymosan or PMA in patients with gram-negative sepsis and healthy adults. Data are shown as 20-min integrated CL counts. Significantly different ($p<0.001$) from the values in healthy adults.

PMA. The responses induced by the stimuli were significantly higher in most specimens from septic patients. Fig. 3 shows that the CL responses of isolated PMNs induced by non-opsonized zymosan or PMA were higher in septic patients than in healthy subjects. These results indicate that circulating PMNs are activated in a majority of patients with gram-negative septicemia.

2) Influence of LPS on the CL response of whole blood and PMNs

LPS alone did not induce the CL response of whole blood and PMNs at the various concentrations tested. A 10-min incubation of LPS at a concentration of more than 100 ng/ml induced a 1.2- to 1.4-fold increase in integrated CL for whole blood following stimulation with zymosan or PMA (priming effect) (Table 1). On the other hand, the CL response of isolated PMNs after incubation with more than 100 ng/ml of LPS for 60 min was significantly increased by stimulation with zymosan or PMA (Fig. 4).

3) Influence of TNF-α on the CL response of whole blood and PMNs

The priming effect of TNF-α on whole blood CL is shown in Table 1. When diluted whole blood was incubated with various concentrations of TNF-α for 10 min at 37°C, the CL response of whole blood was significantly enhanced following stimulation with non-opsonized zymosan or PMA when compared with untreated cells. A similar priming effect by TNF-α was observed in the CL response of PMNs (Fig. 5).

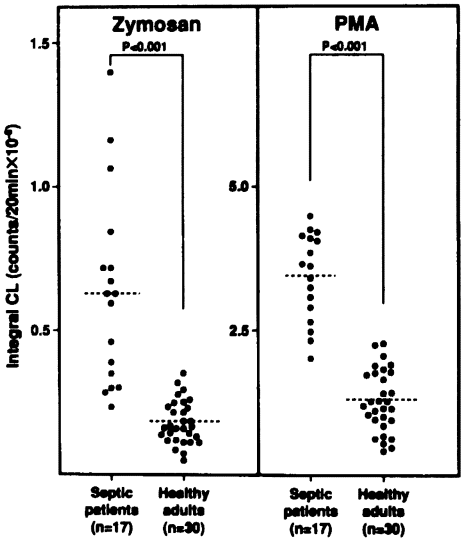

Fig. 3. Chemiluminescence (CL) values of polymorphonuclear leukocytes (PMNs) in patients with gram-negative sepsis and healthy adults in response to non-opsonized zymosan or PMA. Data are shown as 20-min integrated CL counts. Significantly different ($p<0.001$) from the values in healthy adults.

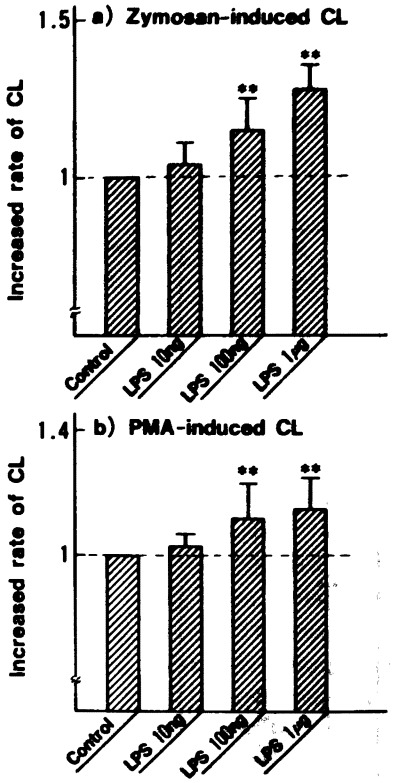

Fig. 4. Priming effect of lipopolysaccharide (LPS) of *Escherichia coli* O111: B4 on the chemiluminescence (CL) response of polymorphonuclear leukocytes (PMNs). CL-index was determined as described in Materials and Methods. Data are shown as means±SD (n=8). Significantly different (**$p<0.001$) from control without LPS.

Table 1. Priming effect of lipopolysaccharide (LPS) from *Escherichia coli* O111: B 4 and tumor necrosis factor-α (TNF-α) on the chemiluminescence (CL) response of whole blood

	Zymosan-induced CL	PMA-induced CL
whole blood+saline	1.00	1.00
whole blood+LPS (10 ng)	1.03±0.05 NS	1.04±0.07 NS
whole blood+LPS (100 ng)	1.18±0.10 p<0.01	1.26±0.09 p<0.001
whole blood+LPS (1 μg)	1.28±0.12 p<0.001	1.37±0.10 p<0.001
whole blood+saline	1.00	1.00
whole blood+TNF-α (0.1 U)	1.02±0.08 NS	1.03±0.10 NS
whole blood+TNF-α (1 U)	1.12±0.15 p<0.01	1.16±0.11 p<0.01
whole blood+TNF-α (10 U)	1.21±0.10 p<0.001	1.31±0.14 p<0.001
whole blood+TNF-α (100 U)	1.32±0.15 p<0.001	1.53±0.21 p<0.001
whole blood+TNF-α (1,000 U)	1.33±0.17 p<0.001	1.55±0.17 p<0.001

CL-index was determined as described in Materials and Methods. Data are shown as means±SD (n=7). Significantly different (p<0.01, p<0.001) from control without LPS or TNF-α.

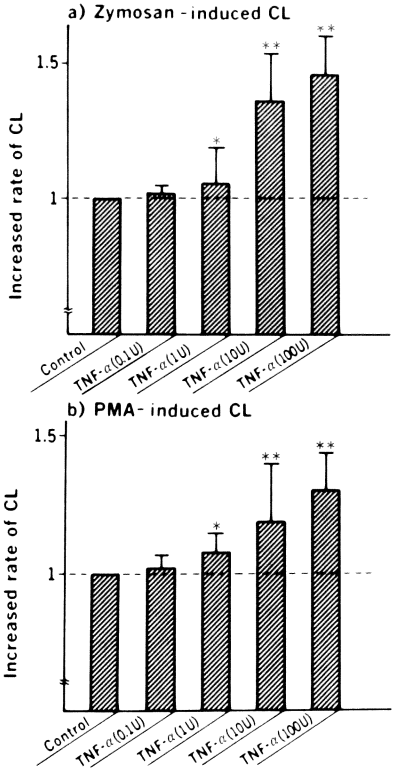

Fig. 5. Priming effect of tumor necrosis factor-α (TNF-α) on the chemiluminescence (CL) response of polymorphonuclear leukocytes (PMNs). CL-index was determined as described in Materials and Methods. Data are shown as means±SD (n=8−12). Significantly different (*p<0.01, **p<0.001) from control without TNF-α.

TNF-α alone showed slight induction of the CL response of PMNs (data not shown), with a 1.1- to 1.6-fold increase in the CL activity of whole blood and PMNs achieved following stimulation with each stimulus after 10-min incubation at the various concentrations of TNF-α. This priming effect of TNF-α was dose-dependent, and was statistically significant at concentrations above 1 U/ml. TNF-α showed a stronger effect than LPS at all levels studied.

4) Influence of IL-1 and IL-6 on the CL response of PMNs

No significant priming effect was observed when PMNs were incubated for 10 min with various concentrations (1—100 ng) of IL-1β or IL-6 (Table 2). Similar results were observed in diluted whole blood samples, and the 60-min incubation had no significant influence on the CL response (data not shown).

5) Inhibition of enhanced CL by anti-TNF MAb and TNF-BPII

As shown Fig. 6, incubation of anti-TNF-α MAb with TNF-α for 10 min during the priming period completely suppressed the enhanced CL response to PMA in blood samples primed with TNF-α (100 U/ml). This inhibitory effect of anti-TNF-α MAb was dose-dependent, and reached statistical significance at concentrations higher than 300 ng/ml. Similar suppression by TNF-BPII of the enhanced CL response primed with TNF-α (10 U/ml) was observed (Fig. 7).

Table 2. Priming effect of interleukin-1β (IL-1β) and IL-6 on the chemiluminescence (CL) response of polymorphonuclear leukocytes (PMNs)

	Zymosan-induced CL	PMA-induced CL
PMNs+saline	1.00	1.00
PMNs+IL-1 (1 ng)	1.02±0.05 NS	1.00±0.08 NS
PMNs+IL-1 (10 ng)	1.05±0.07 NS	1.06±0.09 NS
PMNs+IL-1 (100 ng)	1.08±0.12 NS	1.07±0.13 NS
PMNs+saline	1.00	1.00
PMNs+IL-6 (1 ng)	0.97±0.06 NS	0.98±0.05 NS
PMNs+IL-6 (10 ng)	1.02±0.04 NS	1.01±0.08 NS
PMNs+IL-6 (100 ng)	0.99±0.05 NS	1.02±0.09 NS

CL-index was determined as described in Materials and Methods. Data are shown as means±SD (n=10). Significantly different (no significance: NS) from control without each cytokine.

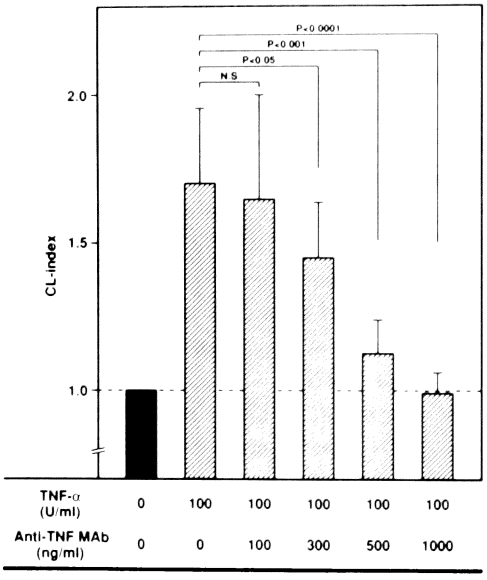

Fig. 6. Inhibition of enhanced chemiluminescence (CL) response of whole blood by anti-tumor necrosis factor-α monoclonal antibody (anti-TNF-αMAb); CL response of whole blood primed with pyrogen-free 0.9% saline (control) of TNF-α (100 U) preincubated with 100—1,000 ng of anti-TNF-αMAb. CL-index was determined as described in Materials and Methods. Data are shown as means± SD (n=9). Significantly different between the CL-index with or without pretreatment of anti-TNF-αMAb.

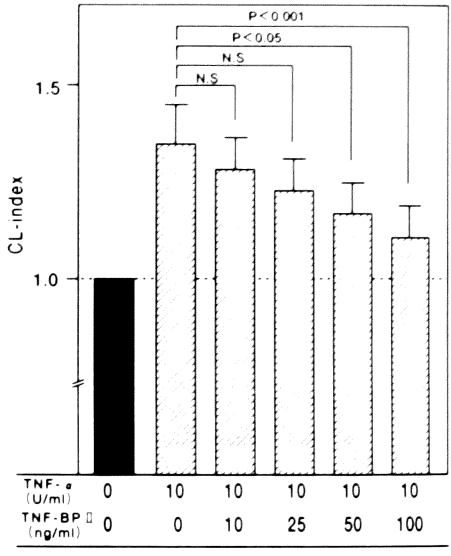

Fig. 7. Inhibition of enhanced chemiluminescence (CL) response of whole blood by tumor necrosis factor binding-protein II (TNF-BPII); CL response of whole blood primed with pyrogen-free 0.9% saline (control) or TNF-α (10 U) preincubated with 10—100 ng of TNF-BPII. CL-index was determined as described in Materials and Methods. Data are shown as means±SD (n=8). Significantly different between the CL-indexes with or without pretreatment of TNF-BPII.

In these experiments, anti-TNF-α MAb or TNF-BPII alone failed to affect the CL response to PMA in whole blood samples at the highest concentrations examined: 1 μg/ml and 100 ng/ml, respectively.

Discussion

When phagocytes interact with soluble or particulate stimuli, the cells respond with a burst in oxidative metabolism which generates reactive oxygen species such as superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2)[15,16]. This activation, which is an essential step in host defense against invading microorganisms, is accompanied by light emission or chemiluminescence (CL) by the cells[17]. The addition of luminol to the system induces a large amount of CL, a result with potential clinical application[17~19], since this system permits testing of very few cells to evaluate the activity of granulocytes in blood[20~22]. It was suggested that light generation in luminol-dependent CL is totally dependent on the myeloperoxidase (MPO)-H_2O_2 system[23]. Whole blood CL reflects the activity of blood granulocytes to generate reactive oxygen species upon stimulation and the opsonic activity of serum[21,24]. In this study, I compared luminol-dependent CL of whole blood and that of isolated PMNs from patients in the acute phase of gram-negative septicemia with specimens from normal subjects. The results showed that the CL responses of whole blood and PMNs induced by non-opsonized zymosan or PMA were higher in most patients' blood than in normal controls, indicating that increased PMNs in patients' peripheral blood were primed for enhanced active oxygen release. To determine what kind of cytokine primes PMNs in these patients, I evaluated the priming effects of inflammatory cytokines including TNF-α, IL-1β and IL-6 on the CL response of human phagocytes and compared them with that of LPS, a well-known CL primer, *in vitro*. The CL response of whole blood and PMNs primed with TNF-α was greater than that of LPS after stimulation with non-opsonized zymosan or PMA. Clinically detectable serum levels of TNF-α which could prime the CL response of PMNs were found in patients with gram-negative septicemia (as described below). Some investigators have reported that IL-1α[11,12] and IL-6[25] activate PMNs to induce an oxidative burst response of the cells. However, other investigators have reported that IL-1β[26,27] and IL-6[28] did not affect superoxide anion production from PMNs. In my study, IL-1β and IL-6 failed to show significant priming effects on the CL response at concentrations ranging from 1—100 ng/ml. These results suggest that the biological effect of IL-1β on PMN function may differ from that of IL-1α. This discrepancy in the priming effect of these cytokines on superoxide anion production may result from differences in methodology.

Clinical and experimental evidence implicates TNF-α as central to the pathogenesis of septic shock[4,5]. Elevated TNF-α serum levels are frequently detected in severe septic patients[29~32]. A recent study by Bone and his colleague[31] reported that the patients with gram-negative septicemia had detectable plasma levels of TNF-α (median, 120±81 pg/ml, range, nondetectable to 1,000 pg/ml), IL-1β (median, 189±70 pg/ml, range, nondetectable to 2,850 pg/ml) and IL-6 (median, 780±239 pg/ml, range, nondetectable to 2,380 pg/ml). In addition, the higher serum levels of TNF-α, IL-1β and IL-6 were found in patients with septic shock; TNF-α level ranged from 100 to 5,000 pg/ml with mean of 701±339 pg/ml[29], IL-1β level ranged from <150 to 3,260 pg/ml with median 480 pg/ml[30] and IL-6 level ranged from less than 0.1 to 305 ng/ml with median 3.5 ng/ml[32]. There was a correlation between the serum level of TNF-α and severity of illness as well as mortality rates[29,30,32].

These findings together with the data in this study suggest that one way to prevent the organ damage caused by toxic substances such as oxygen free radicals released from activated PMNs may be to neutralize the biological activities of TNF-α, because TNF-α shows the most powerful priming effect on the CL

response of PMNs.

Recently, with the availability of monoclonal antibody hybridoma technology, the feasibility of blocking the deleterious effects of TNF has been realized. Several investigators have demonstrated the efficacy of passive immunization with MAbs against TNF from the effects of endotoxin or bacterial challenge in various animal models[33,34]. There was a reduction in mortality in *in vivo* animal models with anti-TNF-MAb, and the circulating levels of TNF were significantly lower in animals receiving anti-TNF-MAb compared with control animals[34,35]. In this *in vitro* study, I used the CL response of human whole blood as a parameter of biological activity of TNF-α. The priming effect of TNF-α on CL response was significantly suppressed by preincubating TNF-α with a murine anti-TNF-α MAb. A previous study has also shown that this antibody completely blocks cytotoxic activity using the TNF-α-susceptible cell line L 929 (Dr. A. Nii personal communication).

These results suggest that anti-TNF-α MAb may be of value as adjuvant therapy in the treatment of critically-ill patients with septic shock or septic acute respiratory distress syndrome (ARDS) in which circulating TNF-α is elevated and PMN function is excessively activated.

Recently, several laboratories have detected heterogeneity in TNF receptor preparations and have proposed that at least two distinct cell-surface molecules bind TNF-α[36~38]. In addition, both receptors appear to be released from cells in soluble form, as TNF-binding proteins of 30 kDa (TNF-BP I) and 27 kDa (TNF-BPII) have been isolated from urine and serum[37~39]. These soluble extracellular domains retain the capacity to bind ligand with affinity, and therefore may act as inhibitors *in vivo*[40~42] To determine whether one of the TNF-BPs, TNF-BPII, can block the biological activity of TNF-α, I used TNF-α-primed whole blood CL in the same way. TNF-BPII prevented the priming of CL response with TNF-α in a dose-dependent fashion. This result indicates that TNF-BPII is also able to suppress the excessive release of oxygen free radicals from PMNs.

In conclusion, these *in vitro* studies showed that anti-TNF-α MAb or TNF-BPII has the ability to prevent the enhanced CL response in human whole blood samples primed with TNF-α. These findings may be useful in improving supportive therapy in patients with gram-negative septicemia and septic shock, as they point out a potential therapy for reducing PMN-mediated organ damage due to excessive release of toxic products such as reactive oxygen species. However, it remains to be determined whether or not the findings in this study can be applied to *in vivo* clinical situations.

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References

1) Dunn D L: Immunotherapeutic advances in the treatment of gram-negative bacterial sepsis. World J. Surg. 11: 233~240, 1987
2) Lynn W A, Golenbock D T: Lipopolysaccharide antagonists. Immunology Today 13: 271~276, 1992
3) Bone R C: The pathogenesis of sepsis. Ann. Intern. Med. 115: 457~469, 1991
4) Cerami A: Inflammatory cytokines. Clin. Immun. Immunopathol. 62: S 3~S 10, 1992
5) Spooner C E, Markowits N P, Saravolats L D: The role of tumor necrosis factor in sepsis. Clin. Immun. Immunopathol. 62: S 11 ~S 17, 1992
6) Wilson M E: Effects of bacterial endotoxins on neutrophil function. Rev. Infect. Dis. 7: 404~418, 1985
7) Klebanoff S J, Vadas M A, Harlan J M, Sparks L H, Gamble J R, Agosti J M, Waltersdorph A M.: Stimulation of neutrophils by tumor necrosis factor. J. Immunol. 136: 4220~4225, 1986
8) Steinbeck M J, Roth J A: Neutrophil ac-

tivation by recombinant cytokines. Rev. Infect. Dis. 11: 549~568, 1989
9) Glauser M P, Zanetti G, Baumgartner J D, Cohen J: Septic shock: pathogenesis. Lancet 338: 732~736, 1991
10) Zimmerman J J, Dietrich K A: Current perspectives on septic shock. Pediatr. Clin. North Am. 34: 131~163, 1987
11) Ferrante A, Nandoskar M, Walz A, Goh D H B, Kowanko I C: Effects of tumor necrosis factor-α and interleukin-1 alpha and beta on human neutrophil migration, respiratory burst and degranulation. Int. Arch. Allergy Appl. Immumol. 86: 82~91, 1988
12) Ozaki Y, Ohashi T, Kume S: Potentiation of neutrophil function by recombinant DNA-produced interleukin-1-α. J. Leukocyte Biol. 42: 621~627, 1987
13) Heinrich P C, Castell J V, Andus T: Interleukin-6 and the acute phage response. Biochem. J. 265: 621~636, 1990
14) Ziegler E J, Fischer C J, Sprung C L, et. al.: Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. N. Engl. J. Med. 324: 429~436, 1991
15) Babior B M: Oxygen-dependent microbial killing by phagocytes. N. Engl. J. Med., 298: 659~668, 1978
16) Makino R, Tanaka T, Iizuka T, Ishimura Y, Kanegasaki S: Stoichiometric conversion of oxygen to superoxide anion during the respiratory burst in neutrophils. J. Biol. Chem. 261: 11444~11447, 1986
17) Allen R C, Stjernholm R L, Steel R H: Evidence for the generation of an electronic state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Commun. 47: 679~684, 1972
18) Allen R C, Loose L D: Phagocytic activation of a luminol dependent chemiluminescence in rabbit alveolar and peritoneal macrophages. Biochem. Biophys. Res. Commun. 69: 245~252, 1976
19) Steel R W: Clinical applications of chemiluminescence of granulocytes. Rev. Infect. Dis. 13: 918~925, 1991
20) Kato T, Wokalek H, Schopf E, Eggert H, Ernst M, Rietschel E, Fischer H: Measurement of chemiluminescence in freshly drawn human blood. 1. Role of granulocytes, platelets and plasma factor in zymosan induced chemiluminescence. Klin. Wochenschr. 59: 203~221, 1981
21) Faden H, Maciejewski N: Whole blood luminol-dependent chemiluminescence. J. Reticuloendothel. Soc. 30: 219~226, 1981
22) Lindena J, Burkhardt H, Dwenger A: Mechanisms of non-opsonized zymosan-induced and luminol-enhanced chemiluminescence in whole blood and isolated phagocytes. J. Clin. Chem. Clin. Biochem. 25: 765~778, 1987
23) Darlgren C, Briheim G: Comparison between the luminol-dependent chemiluminescence of polymorphonuclear leukocytes and of the myeloperoxidase-HOOH system: Influence of pH, cations and protein. Photochem. Photobiol. 41: 605~610, 1985
24) Ono Y, Kunii O, Kobayashi K, Kanegasaki S: Evaluation of opsonophagocytic dysfunctions in severely burned patients by luminol-dependent chemiluminescence. Microbiol. Immumol. 37: 567~571, 1993
25) Borish L, Rosenbaum R, Albury L, Clark S: Activation of neutrophils by recombinant interleukin 6. Cellular Immunol. 121: 280~289, 1989
26) Kharazmi A, Nielsen H, Bendtzen K: Recombinant interleikin 1 alpha and beta prime human monocyte superoxide production but have no effect on chemotaxis and oxidative burst response of neutrophils. Immunobiol. 177: 32~39, 1988
27) Georgilis K, Schaefer C, Dinarello C A, Klempner M S: Human recombinant interleikin 1 beta has no effect on intracellular calcium or on functional responses of human neutrophils. J. Immunol. 138: 3403~3407, 1987
28) Yuo A: Neutrophil functions and hematopoietic growth factors. Zouketu-inshi. 3: 327~333, 1992
29) Damas P, Reuter A, Gysen P, Demonty J, Lamy M, Franchimont P: Tumor necrosis factor and interleukin-1 serum levels during severe sepsis in humans. Clitical Care Medicine 17: 975~978, 1989
30) Calandra T, Baumgartner J D, Grau G E, Wu M M, Lambert P H, Schellenkens J, Verhoef J, Glauser M P: Prognostic values of tumor necrosis factor/cachectin, interleukin-1, interferon-α, and interferon-γ in the serum of patients with septic shock. J. Infect. Dis. 161: 982~987, 1990
31) Casey L C, Balk R A, Bone R C: Plasma cytokines and endotoxin levels correlate with survival in patients with the sepsis syndrome. Ann. Intern. Med. 119: 771~778, 1993
32) Calandra T, Gerain J, Heumann D, Baumgartner J D, Glauser M P: High cir-

culating levels of interleukin-6 in patients with septic shock: Evolution during sepsis, prognostic value, and interplay with other cytokines. Am. J. Med. 91: 23~29, 1991

33) Bautler B, Milsark I W, Cerami A C: Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science 229: 869~871, 1985

34) Opal S M, Cross A S, Kelley N M, Sadoff J C, Bodmer M W, Palardy J E, Victor G H: Efficacy of a monoclonal antibody directed against TNF in protecting neutropenic rats from lethal infection with *P. aeruginosa*. J. Infect. Dis. 161: 1148~1152, 1990

35) Silva A T, Bayston K F, Cohen J: Prophylactic and therapeutic effects of a monoclonal antibody to TNF-alpha in experimental gram-negative shock. J. Infect. Dis. 162: 421~427, 1990

36) Loetscher H, Pan Y C E, Lahm H W, Gentz R, Brockhaus M, Tabuchi H, Lesslauer W: Molecular cloning and expression of the human 55 kd necrosis factor receptor. Cell 61: 351~359, 1990

37) Seckinger P, Isaaz S, Daer L M: Purification and biologic characterization of a specific tumor necrosis factor-inhibitor. J. Biol. Chem. 264: 11966~11973, 1989

38) Engelmann H, Novick D N, Wallach D: Two tumor necrosis factor-binding proteins purified from human urine: Evidence for immunological crossreactivity with cell surface tumor necrosis factor receptors. J. Biol. Chem. 265: 1531~1536, 1989

39) Gray P W, Barrett K, Chantry D, Turner M, Feldmann M: Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein. Proc. Natl. Acad. Sci. USA. 87: 7380~7384, 1990

40) Kohno T, Brewer M T, Baker S L, et. al.: A second tumor necrosis factor receptor gene product can shed a naturally occurring tumor necrosis factor inhibitor. Proc. Natl. Acad. Sci. USA 87: 8331~8335, 1990

41) Van Zee K J, Kohno, T, Fischer E, Rock C S, Moldawer L L, Lowry S T: Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor a *in vitro* and *in vivo*. Proc. Natl. Acad. Sci. USA 89: 4845~4849, 1992

42) Higuchi M, Aggarwal B B: Modulation of two forms of tumor necrosis factor receptors and their cellular response by soluble receptors and their monoclonal antibodies: J. Biol. Chem. 267: 20892~20899, 1992

敗血症患者における食細胞の化学発光反応
—エンドトキシンと炎症性サイトカインによるプライミング効果—

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グラム陰性桿菌による敗血症患者20名の末梢血の全血と好中球の活性酸素産生能を非オプソニン化zymosanやphorbol myristate acetate（PMA）を刺激物としてルミノール依存性化学発光（CL）法を用いて検討した。10倍希釈した全血と好中球のCL反応はこれら患者では，健常人と比較して有意に増強していた。末梢血中の顆粒球数も多くの症例で増加していた。敗血症患者の食細胞のCL反応が何故増強しているのかを明らかにするために，健常人の食細胞のCL反応におよぼすtumor necrosis factor-α（TNF-α），interleukin-1β（IL-1β），IL-6などの炎症性サイトカインとlipopolysaccharide（LPS: endotoxin）のプライミング効果について検討した。これらのサイトカインやLPSは，重症のグラム陰性桿菌敗血症患者の血清中においてしばしば検出可能である。全血と好中球を種々の濃度の各種サイトカインやLPSと共に37℃で10分間ないし60分間保温し，zymosanやPMAにより誘導されるCL反応を20分間の積算値で測定し，サイトカインやLPSのCL反応におよぼす効果を比較した。TNF-αやLPSであらかじめ保温しておくと，好中球のCL反応の増強がそれぞれ1 U/ml，100 ng/ml以上の濃度で認められた。同様の成績は，希釈した全血においても得られた。一方，IL-1βやIL-6においては，好中球を1～100 ng/mlの種々の濃度で10分間ないし60分間保温しても有意なプライミング効果は見られなかった。TNF-αを全血試料にさらす前に，あらかじめマウス抗TNF-αモノクローナル抗体（TNF-α MAb）やTNF結合蛋白II（TNF-BPII）で10分間37℃で前処理しておくと，TNF-αによるプライミング効果が有意に抑制され，これらの抑制効果は濃度依存性に認められた。本研究では，TNF-αによりプライミングされるヒト全血のCL増強効果をTNF-α MAbやTNF-BPIIが抑制する能力があることを明らかにした。これらの知見は，グラム陰性桿菌敗血症や敗血症性ショックを伴う患者，特に活性酸素のような有毒物質の過剰放出により引き起こされる好中球を介する臓器傷害を来たすような症例において，補助療法として役立つかもしれないということを示している。

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