Anti-endotoxic Activity of Murine Monoclonal Antibody (E5) 
Assessed by Inhibition of Priming of Human Phagocytes by Endotoxin

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Summary

The in vitro activity of a murine monoclonal antibody (E5) reactive with endotoxin was examined in human whole blood by measuring the luminol-chemiluminescence (CL) activity in response to phorbol myristate acetate (PMA) as an index of the priming effect of lipopolysaccharide (LPS) on the release of reactive oxygen species. Whole blood samples obtained from healthy adults showed a significantly enhanced CL response to PMA after incubation with LPS (100 ng/ml, \textit{Escherichia coli} O111:B4) for 10 min at 37\(^\circ\)C, as compared with untreated blood samples, though no CL response was induced by LPS itself. This priming effect of LPS varied from person to person. Similarly, various degrees of the priming effect were observed with other LPS preparations derived from \textit{E. coli} O55:B5, \textit{Klebsiella pneumoniae}, \textit{Serratia marcescens} and \textit{Salmonella typhimurium}. However, the priming effects of these LPS or a synthetic lipid A (LA-15-PP) of \textit{E. coli} were significantly prevented to various degrees when such endotoxins were treated with E5 for 30 min at 37\(^\circ\)C prior to being added to blood samples. The inhibitory effect E5 was dose-dependent and was most potent against the LPS of \textit{E. coli} O111:B4.

These results indicate that E5 suppresses the priming effect of LPS on oxygen radical release from human whole blood, and therefore suggest that E5 may be a useful drug for supportive therapy in patients with gram-negative septicemia or endotoxemia, especially in a case involving serious neutrophil-mediated organ injury caused by excessive release of oxygen free radicals.

Introduction

Lipopolysaccharide (LPS or endotoxin), a complex glycolipid, is the major component of the outer membrane of gram-negative bacteria. It is largely responsible for the tissue damage, serious clinical sequelae, and mortality in patients with gram-negative bacteremia and septic shock\(^{19}\). The clinical syndrome of gram-negative bacterial septicemia or endotoxemia appears to result primarily from excessive stimulation of the host immune system by LPS\(^{29}\). LPS induction of cytokine release, particularly tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin-1, is probably the central event in the pathophysiology of gram-negative septicemia or endotoxemia, although many additional immunological events accompany cytokine release\(^{30}\). LPS and some cytokines such as TNF-\(\alpha\) cause the priming of polymorphonuclear leukocytes

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(PMN) for subsequent oxygen radical release, phagocytosis and bactericidal activity\(^3,4,5\). This activations of PMN at the sites of gram-negative infection is favorable for the host defense against invading bacteria. However, during sepsis, high levels of circulating LPS and/or released cytokines may lead to the unwanted activation and recruitment of PMN into uninfected remote organs, resulting in serious PMN-mediated organ injury caused by excessive release of toxic products such as oxygen free radicals\(^6,7\).

It has been suggested that anti-LPS monoclonal antibodies (MAbs) may provide protection against clinical gram-negative sepsis\(^8,9\). E5, a murine monoclonal immunoglobulin M antibody directed against LPS, was obtained from mice immunized with whole cells of the J5 rough mutant of Escherichia coli O111:B4. E5 has been shown to react in vitro with a variety of LPS, specifically with the lipid A moiety, in previous extensive studies\(^8,10,11\).

The present study was undertaken to clarify the in vitro ability of E5 to inhibit LPS activity with human phagocytic cells as targets. Exposure of PMN and monocytes/macrophages to LPS in vitro primes the cells to respond to subsequent stimulation by enhanced release of reactive oxygen species\(^3,4\). This metabolic response of phagocytic cells produces chemiluminescence (CL) which can be amplified and measured in vitro by the addition of luminol\(^12\). The luminol-enhanced system permits the use of a small amount of whole blood to measure CL\(^13\). The CL response of whole blood reflects the ability of blood granulocytes to generate oxygen species upon stimulation by a particulate or soluble stimulus\(^13\). We used the LPS-primed CL response of whole blood as a parameter for endotoxic activity and examined whether priming of whole blood could be blocked by preincubating a lipid A preparation or various LPS preparations with E5.

### Materials and Methods

#### Blood samples

Four milliliters of fresh heparinized blood (10 U/ml) was drawn each volunteer into a sterile plastic tube, and blood samples from 38 healthy volunteers (20 males, 18 females, mean age 28.4 ± 5.3 years, range 19–39) were used in this experiment.

#### Monoclonal antibody

A murine-derived IgM MAb against LPS (E5) was kindly provided by Pfizer Pharmaceuticals Inc., Tokyo, Japan, in an injection form of 2 mg of E5/ml. This antibody has previously been reported to possess potent anti-endotoxin activity in experimental studies\(^10,11\).

#### LPS and lipid A preparations

The following five LPS and lipid A preparations were used: LPS isolated from *E. coli* O111:B4, *E. coli* O55:B5, *Klebsiella pneumoniae*, *Serratia marcescens* and *Salmonella typhimurium* (Sigma Chemical Co., St., Louis, MO, USA) and synthetic lipid A (LA-15-PP) of *E. coli* (Daiichi Kagaku Co., Tokyo).

#### Chemicals and media

Dulbecco's modified Eagle's medium (MEM, Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 25 m mol of HEPES and L-glutamine 0.3 g/L, pH 7.4, was used to dilute blood samples. Luminol (Tokyo Kasei Kougyo Co., Tokyo) was dissolved in phosphate buffered saline at 20 µg/ml. Phorbol myristate acetate (PMA) (Sigma Chemical Co.) was dissolved in dimethyl sulfoxide at 100 µg/ml.

#### Priming effect of LPS or lipid A on CL response of whole blood

Luminol-dependent CL assay was performed by using 10-fold diluted whole blood. Initially the whole blood was incubated with 25 µl (100 ng) of LPS or lipid A solution or pyrogen-free 0.9% saline for 10 min at 37°C in a total volume of 1 ml. This reaction mixture contained 100 µl of whole blood, 25 µl of LPS or lipid A solution or 0.9% saline for control, and 20 µl of an 11.3 mM luminol solution, with adjustment of the total volume to 1 ml with MEM. The 10-min preincubation time was sufficient for LPS priming to give a

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significant CL response. After the preincubation, 5 µl (0.5 µg) of PMA solution was added to the mixture and the CL activity was continuously measured for 20 min with a six-channel Biolumat LB 9505 device (Berthold Co., Bad Wildbad, Germany). The priming effect of LPS or lipid A was determined as the mean difference of the CL index between LPS (or lipid A)-treated and saline-treated (control) blood samples. The CL index was calculated by dividing the integral CL value of LPS (or lipid A)-treated blood samples by the integral CL value of saline-treated blood sample.

In the experiments with E5, LPS or lipid A solution (25 µl, 100 ng) or pyrogen-free 0.9% saline (25 µl) was initially incubated with various concentrations of E5 (0.1–20 µg) in a total 100-µl volume of MEM for 30 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 addition of PMA. All these assays were conducted in duplicate.

Statistical analysis
Data are shown as mean ± SD of several assays performed in duplicate samples from multiple healthy volunteers, and analyzed statistically by using Student’s t-test.

Results

CL response of human whole blood primed with LPS and lipid A

Figure 1 shows a typical pattern of the priming effect of LPS (100 ng/ml) of *E. coli* O111:B4 or lipid A (100 ng/ml) of *E. coli* on the CL response to PMA in human whole blood. LPS or lipid A alone did not induce the CL response of the blood samples, but increased the CL response to PMA following 10 min preincubation, resulting in about 1.3-fold enhancement of the integral CL value above the response of the untreated blood sample. This priming effect of the LPS varied from person to person (Fig. 2).

Inhibition by E5 of the enhanced CL in human whole blood

As shown in Fig. 3, E5 completely suppressed the enhanced CL response to PMA in the blood samples primed with LPS (100 ng/ml) of *E. coli* O111:B4, when the LPS was incubated with E5 for 30 min prior to
Fig. 3 Inhibition of the enhanced chemiluminescence response of whole blood by E5; CL response of whole blood primed with pyrogen-free 0.9% saline (control) or LPS O111:B4 (100 ng) preincubated with 0.1-20 μg of E5. The CL-index was determined as described in Materials and Methods. Data are shown as means and SDs (n=15). p: significantly difference between the CL-indexes with and without pretreatment with E5. NS: nonsignificant.

Fig. 4 Inhibition of the enhanced chemiluminescence response of whole blood by E5; CL response of whole blood primed with pyrogen-free 0.9% saline (control) or Lipid A (100 ng) preincubated with 0.1-20 μg of E5. The CL-index was determined as described in Materials and Methods. Data are shown as means and SDs (n=10). p: significantly difference between the CL-indexes with and without pretreatment with E5. NS: nonsignificant.

Fig. 5 Inhibition of the enhanced chemiluminescence response of whole blood by E5; CL response of whole blood primed with pyrogen-free 0.9% saline (control) or various LPS preparations (100 ng) preincubated with 20 μg of E5. The CL-index was determined as described in Materials and Methods. Data are shown as means and SDs (n=7-15). There are significant differences between the CL-indexes with and without pretreatment with E5.
the addition of the whole blood to the mixture. This inhibitory effect of E5 was dose-dependent with a statistically significant difference at concentrations of 10 μg/ml or higher. Similar suppression by E5 of the enhanced CL response was observed with lipid A of \textit{E. coli} (100 ng/ml) (Fig. 4) and complete inhibition was achieved at 20 μg of E5 per ml. In these experiments, E5 alone failed to affect the CL response to PMA in whole blood samples at the highest concentration (20 μg/ml) examined.

In separate experiments, the different types of LPS derived from \textit{E. coli} O55:B5, \textit{K. pneumoniae}, \textit{S. marcescens} and \textit{S. typhimurium} also exhibited the ability to enhance the CL response to PMA in human blood samples (Fig. 5), although the magnitudes of the response were different. The priming effects of all these LPS were variably, but significantly, suppressed by the preincubation of the LPS with 20 μg of E5 per ml. The inhibitory effect of E5 was most potent against the LPS of \textit{E. coli} O111:B4.

\textbf{Discussion}

It has been shown that LPS primes various PMN-functions involving the production of reactive oxygen species, in terms of the CL response\textsuperscript{14}. The same investigators reported that rough LPS preparations cause higher CL responses of PMN than the smooth LPS preparations, suggesting that the difference may be due to the more lipophilic character of rough LPS\textsuperscript{14}. In the present study, however, we used whole blood CL instead of PMN-CL as a parameter for endotoxic activity, and five types of smooth LPS and one lipid A as the CL enhancers. These various types of LPS and the lipid A showed clearly the priming effect on the CL response of whole blood after stimulation with PMA. The priming time of whole blood CL was shorter than that of PMN-CL in the absence of serum, 10 min vs. 60 min. Such fast onset of the priming effect of LPS in whole blood may be due to activation of the complement cascade by LPS and/or the enhanced affinity for the cell surface receptors of phagocytic cells through the formation of complexes of LPS with serum LPS-binding protein. However, the priming effect of LPS varied from donor to donor when the same LPS preparation was used. Furthermore, the degree of priming depended on the type of LPS. Nevertheless, these data indicate that human whole blood can be primed for the luminol-dependent CL response by pretreatment with various types of LPS or a synthetic lipid A (LA-15-PP).

These priming effects of LPS or lipid A in human whole blood were significantly suppressed by preincubation of each endotoxin with an anti-LPS MAb, E5. The inhibitory effect of E5 was most potent against LPS of \textit{E. coli} O111:B4 and was dose-dependent, with less, but statistically significant, inhibition of the priming by other LPS derived from \textit{E. coli} O55:B5, \textit{K. pneumoniae}, \textit{S. marcescens} and \textit{S. typhimurium}.

Although the underlying mechanism by which LPS primes PMN is still unknown, the lipid A portion is known to mediate most of the pathologic effects of the toxicity of endotoxin\textsuperscript{15}. Wood and colleagues\textsuperscript{10,11} have reported that E5 binds to various rough and smooth types of LPS preparations, including LPS isolated from species of \textit{Escherichia}, \textit{Klebsiella}, \textit{Proteus}, \textit{Pseudomonas}, \textit{Salmonella}, \textit{Serratia} and \textit{Yersinia} by using standard immunologic techniques such as enzyme-linked immunosorbent assay, radioimmunoassay and antibody capture assay using immobilized antibody and a chromogenic \textit{Limulus} amebocyte lysate detection system. In addition, E5 has been shown to bind with avidity to a variety of natural and synthetic lipid A preparations.

Accordingly, our finding that E5 blocked the priming effect of a lipid A preparation on the CL response supports the idea that E5 may bind to an epitope of the hydrophobic lipid A region and thereby prevent the attachment of lipid A to its receptors on the target cell membrane to block the priming effect of the endotoxin on the CL response, because E5 and LPS (or lipid A) were incubated prior to being added to the whole blood samples in this study.

In fact, in a multicenter randomized placebo-controlled clinical trial, E5 was shown to reduce significantly the mortality and morbidity of patients with gram-negative sepsis\textsuperscript{16}. The results of recent
clinical studies may support the idea that the MAb E5 binds to the endotoxin and facilitates its elimination or detoxification, thereby improving the clinical outcome. At present, it is not known whether the inhibition by E5 of the priming effect of LPS in human whole blood, that is, the inhibition of oxygen radical release, may contribute to the clinical efficacy of E5. However, such activity of E5 seems likely in vivo, since E5 was shown to suppress the synthesis of hydrogen peroxide in the lungs of rats receiving a single injection of LPS.[17]

In conclusion, the present study indicates that E5 has the ability to inhibit the enhanced CL response in human whole blood samples primed with different types of LPS or lipid A. It may be useful for supportive therapy in patients with gram-negative infection such as septicemia and endotoxemia, especially in a case involving serious PMN-mediated organ damage caused by excessive release of toxic products such as oxygen free radicals.

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References

マウス抗リポポリサッカライドモノクローナル抗体（E5）の
抗エンドトキシン活性：エンドトキシンによるヒト食細胞に
に対するプライミング効果の抑制効果による評価

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要 旨
マウス抗リポポリサッカライド（LPS）モノクローナル抗体（E5）のエンドトキシンに対する in vitro の反応活性を、活性酸素放出能に対するLPSのpriming 効果を指標として、phorbol myristate acetate（PMA）刺激によるルミノール依存性全血化学発光（CL）を測定することで検討した。健常成人より得た全血試料は、100ng/mlのEscherichia coli（E. coli）O111：B4由来LPSで37°Cで10分間前処理された後には、PMAに対するCL反応が、未処理の場合と比較して有意に増強した。しかし、CL反応はLPSそれ自体では誘導されなかった。このようなLPSのpriming 効果は健康成人の間でも異なっていた。同様に、程度の差はあるものの、E. coli O55：B5, Klebsiella pneumoniae, Serratia marcescens, Salmonella typhimurium 由来の他のLPSを用いた場合においてもpriming 効果が認められた。しかし、これらLPS やE. coli型合成lipid A（LA-15-PP）のpriming 効果は、これらエンドトキシンと血液検体を接触させる前に、E5で30分間37°Cで前処理しておくと程度の差はあら有意に抑制された。
このE5の抑制効果は濃度依存的で、E. coli O111：B4由来LPSに対して最も強かった。

以上の成績より、E5はヒト全血の活性酸素放出能に対するLPSのpriming 効果を抑制することが示唆され、そしてそれ故にE5は、グラム陰性桿菌敗血症やエンドトキシン血症を伴う患者、特に食細胞由来の活性酸素の過剰産生により引き起こされる好中球を介する細菌傷害をきたす様な症例に対して、補助療法として有効な治療法となるかもしれない。