Inhibition by lipid A-specific monoclonal antibodies of priming of human polymorphonuclear leucocytes by endotoxin

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Summary. Exposure to lipopolysaccharide (LPS) primes polymorphonuclear leucocytes (PMNL) for enhanced release of oxygen metabolites after subsequent stimulation. The metabolic response of human PMNL primed with LPS and stimulated with formyl-methionyl-leucyl-phenylalanine (FMLP) was measured by chemiluminescence (CL) as a parameter for endotoxic activity. Polymyxin B (PMB) and monoclonal antibodies (MAbs) with specificity for lipid A were tested for inhibition of the priming effect of Re LPS of *Salmonella minnesota* R595, Rc LPS of *Escherichia coli* J5 and smooth LPS of *E. coli* O111. The CL response of PMNL primed with Re LPS or Rc LPS was higher than that of PMNL primed with smooth LPS. Pre-incubation of rough or smooth LPS with PMB caused dose-dependent inhibition of priming of PMNL. Two IgM MAbs, 8-2 and 26-20, which recognise different epitopes on the hydrophobic part of lipid A, also completely prevented priming of PMNL by either rough or smooth LPS. The dose-dependent inhibitory effect of both MAbs was similar to the inhibition by PMB. These results indicate that the binding of MAbs to the hydrophobic part of lipid A is important in blocking lipid A-mediated effects.

Introduction

It is now firmly believed that the pathophysiological features of gram-negative septic shock are caused by lipopolysaccharide (LPS, endotoxin).¹ The lipid A structure, which is highly conserved among different gram-negative bacilli, is believed to be the toxic principle of endotoxin.² Antibodies directed against the lipid A core-oligosaccharide part of LPS may show cross-reactivity with LPS from various gram-negative bacilli.^{3–5} There is some evidence that these antibodies may protect against gram-negative septicemia,^{6–8} although conflicting results have been reported.⁹

We have produced several monoclonal antibodies (MAbs) directed against the 2-keto-3-desoxyoctulosonic acid (KDO)/lipid A inner core structure of LPS.¹⁰ Two IgM MAbs, 8-2 and 26-20, bind to different epitopes within the lipid A region.^{10, 11} Both MAbs showed broad cross-reactivity with heterologous LPS preparations. In the present study the capacities of MAb 8-2 and 26-20 to inhibit LPS activity were tested *in vitro* with human polymorphonuclear leucocytes (PMNL) as targets. The exposure of PMNL to endotoxin *in vitro* primes the cells to respond to subsequent stimulation by enhanced release of oxygen metabolites and elastase secretion.^{12–14} This metabolic response of PMNL, the so-called respiratory burst, produces chemiluminescence, which can be amplified and measured *in vitro* by the addition of

luminol.¹⁵ PMNL primed by LPS *in vivo* may even exhibit an increased resistance to infection.¹⁶ However, this priming may also result in increased oxidative tissue damage, as in conditions such as the adult respiratory distress syndrome associated with septic shock.¹⁷

We used LPS-primed PMNL chemiluminescence as a parameter for endotoxic activity. It was examined whether priming of PMNL could be blocked by pre-incubating LPS with either MAb 26-20 or MAb 8-2. The inhibitory effect of both MAbs was compared with the well-known inhibitor polymyxin B (PMB), an antibiotic with potent anti-endotoxin activity.¹⁸

Materials and methods

LPS preparations

Three LPS preparations were used: Re LPS of *Salmonella minnesota* R595, which contains lipid A and the KDO molecule; Rc LPS of *Escherichia coli* J5, which contains the KDO/lipid A portion and part of the core oligosaccharide; and smooth LPS of *E. coli* O111:B4. Smooth LPS contains about 10-fold (w/w) less lipid A than rough LPS. Therefore, in our experiments, 1 µg of smooth LPS was considered to be equivalent to 100 ng of rough LPS. Re LPS of *S. minnesota* R595 and Rc LPS of *E. coli* J5 were extracted by the phenol-chloroform-petroleum ether method as described by Galanos *et al.*¹⁹ LPS of *E. coli* O111 was

extracted and purified, as described previously, with hot phenol-water and sequential ultracentrifugation.²⁰ Lyophilised LPS was dissolved in pyrogen-free water at 1 mg/ml, sonicated and kept at -20°C . Before each test, LPS was freshly diluted in pyrogen-free phosphate-buffered saline (PBS), sonicated again for 3 min, and the pH was adjusted to 7.0 with triethylamine.

Monoclonal antibodies

The production and characterisation of IgM MABs 8-2 and 26-20 were described previously.¹⁰ The MABs were produced by hybridomas of BALB/c mice spleen cells and the mouse myeloma cell line Sp2/o-Ag14. MAB 8-2 was induced by the Re mutant of *S. typhimurium* and MAB 26-20 by synthetic lipid A. A Semliki Forest virus-specific IgM MAB (UM 8-4) was used as control.²¹

MABs 8-2 and 26-20 and the control IgM MABs were purified from mouse ascitic fluids by size-exclusion chromatography,²² through a Sephacryl S-300 column (Pharmacia, Uppsala, Sweden). IgM-positive peak fractions were estimated to be >90% pure by chromatography and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was measured by the method of Lowry *et al.*²³

Isolation of PMNL

Heparinised venous blood was obtained from healthy donors. PMNL were purified to >97% purity by dextran sedimentation followed by Ficoll-Paque (Pharmacia) gradient centrifugation. Residual erythrocytes were lysed by hypotonic shock and PMNL were washed and resuspended in pyrogen-free PBS.

Chemiluminescence of PMNL primed with LPS

Primed PMNL were obtained by mixing 5×10^6 cells with either 100 ng of rough LPS or 1 μg of smooth LPS in 900 μl of pyrogen-free PBS for 60 min at 37°C . Luminol (50 μl) was added to the cell suspension to a final concentration of 10^{-5} M. PMNL were stimulated with 10^{-6} M formyl-methionyl-leucyl-phenylalanine (FMLP) in a total volume of 1 ml. FMLP was dissolved in dimethylsulphoxide (DMSO) and stored at -20°C . Before use, this stock was diluted in pyrogen-free PBS. Immediately after adding FMLP, chemiluminescence (CL) was measured every 10 s for 3 min in a luminometer (Packard Instruments, Brussels, Belgium) at 37°C . Inhibition experiments were performed by pre-incubation of 100 ng of LPS with 10 μg of MAB or 1 μg of PMB (Sigma) in 110 μl of pyrogen-free PBS for 30 min at 37°C . Subsequently, PMNL were primed for 60 min with these mixtures and CL was measured after addition of FMLP.

Limulus amoebocyte lysate assay

All reagents used were diluted in pyrogen-free PBS

and tested for LPS contamination by the kinetic turbidimetric Limulus Amoebocyte Lysate (LAL) assay.²⁴ The LAL used for this study was lot 99-01-347 (Associates of Cape Cod, Inc.) and the LAL-5000 (Pyrotell/Bentho, Inc.) was used to read the test. All reagents contained less than 300 pg of LPS/ml.

Results

Chemiluminescence of PMNL primed with LPS

Priming of human PMNL (5×10^6) with 100 ng of LPS in 1 ml of pyrogen-free PBS for 60 min at 37°C resulted in a clearly enhanced CL response after subsequent stimulation with FMLP (10^{-6} M) when compared with PMNL pre-incubated with buffer only. Different LPS preparations were tested for their ability to prime PMNL for an enhanced CL response. PMNL were primed with Re LPS of *S. minnesota* R595 (100 ng/ml), Rc LPS of *E. coli* J5 (100 ng/ml) or smooth LPS of *E. coli* O111 (1 $\mu\text{g}/\text{ml}$). As shown in the table and fig. 1, both smooth and rough LPS preparations primed PMNL, although the magnitude of the response depended on the kind of LPS used. Priming of PMNL with smooth LPS of *E. coli* O111 produced peak values of CL two to three times higher than peak values produced by control PMNL incubated with pyrogen-free PBS. Priming with Rc LPS of *E. coli* J5 or Re LPS of *S. minnesota* R595 was more effective and generated higher peak values than priming with smooth LPS. The ratio of CL peak values produced by PMNL primed with rough LPS and control PMNL incubated with pyrogen-free PBS varied from four to 20. Furthermore, the priming effect of LPS on PMNL varied from donor to donor (table).

Inhibition of enhanced chemiluminescence by polymyxin B

Incubation of LPS (100 ng) with PMB (1 μg) before

Fig. 1. Enhanced CL response of PMNL primed with LPS; mean values ($n=2$) and range of variation of CL of PMNL from one donor primed with LPS of *E. coli* O111 (□), Rc LPS of *E. coli* J5 (△), Re LPS of *S. minnesota* (○), and pyrogen-free PBS (●), followed by stimulation with FMLP.

Table. CL of human PMNL from three donors primed by different LPS forms and stimulated with FMLP

Donor	Mean (n=2) CL peak values* (cpm $\times 10^3$) after priming with			
	PBS	Re LPS	J5 LPS	O111 LPS
1	50 (2)	1148 (34)	801 (120)	213 (2)
2	52 (12)	186 (25)	144 (2)	113 (18)
3	69 (25)	1284 (53)	838 (216)	324 (1)

* Results are expressed as mean peak values (range of variation).

priming PMNL abolished the enhanced CL response after FMLP stimulation. The inhibitory effect of PMB was dose dependent, as shown in fig. 2 for priming with Rc LPS of *E. coli* J5. Complete inhibition of the priming effect of 100 ng of LPS was obtained with 1 μ g of PMB; inhibition reduced sequentially when LPS was pre-incubated with 10^{-4} , 10^{-5} , and 10^{-6} mg of PMB respectively, in 100 μ l of PBS.

Inhibition of enhanced chemiluminescence by MAbs 8-2 and 26-20

To establish whether MAbs directed against lipid A prevent LPS priming of PMNL, the three LPS preparations (100 ng) were pre-incubated with either MAb 8-2 (10 μ g) or MAb 26-20 (10 μ g) in 110 μ l of pyrogen-free PBS for 30 min at 37°C. Pre-incubation with either MAb completely prevented the priming of PMNL by Re LPS of *S. minnesota* R595 (fig. 3A), Rc LPS of *E. coli* J5 (fig. 3B) and LPS of *E. coli* O111 (fig. 3C). Inhibition by both MAbs was dose-dependent, as shown in fig. 4 for MAb 8-2 against priming with Rc LPS of *E. coli* J5. A control IgM MAb directed against Semliki Forest virus (MAb UM 8-4) did not prevent priming of PMNL with either rough or smooth LPS (fig. 3A–C). The enhanced CL response of PMNL primed with LPS pre-incubated with MAb

Fig. 2. Inhibition of enhanced CL response by PMB; CL response of PMNL primed with pyrogen-free PBS (●), LPS of *E. coli* J5 (○) and J5 LPS pre-incubated with (3) 10^{-3} mg, (4) 10^{-4} mg, (5) 10^{-5} mg and (6) 10^{-6} mg of PMB (Δ).

Fig. 3. Inhibition of enhanced CL response by MAb 8-2 or 26-20; CL response of PMNL primed with (A) Re LPS of *S. minnesota*, (B) Rc LPS of *E. coli* J5 or (C) smooth LPS of *E. coli* O111. The LPS preparations were pre-incubated with MAb 8-2 (□), MAb 26-20 (◇), control MAb UM 8-4 (▽), PMB (Δ) or pyrogen-free PBS (○); PMNL incubated with pyrogen-free PBS without LPS served as negative control (●).

Fig. 4. Inhibition of enhanced CL response by MAb 8-2; CL response of PMNL primed with Rc LPS of *E. coli* J5 pre-incubated with (2) 10^{-2} , (3) 10^{-3} , (4) 10^{-4} or (5) 10^{-5} mg of MAb 8-2 (□) or pyrogen-free PBS (○) and of PMNL incubated with pyrogen-free PBS without LPS (●).

UM 8-4 equalled the CL response of PMNL primed with LPS pre-incubated with pyrogen-free PBS.

In further experiments to examine whether the abolition of enhanced CL was due to the MABs acting as scavengers, the MABs were added after priming of PMNL with LPS, which requires at least 30–60 min.¹⁸ Subsequent stimulation of PMNL with FMLP produced peak values of CL, as high as CL peak values of PMNL primed with LPS and not treated further. The inability of the control IgM MAB UM 8-4 to abolish the enhanced CL response (fig. 3) also argues against a possible scavenging effect.

Discussion

This study has shown that human PMNL can be primed for an enhanced respiratory burst by exposure to both smooth and rough LPS. The priming of PMNL by LPS was prevented by pre-incubation of LPS with anti-lipid A MABs. The inhibitory effect of MABs on LPS was similar to that of the well-known inhibitor PMB.

When PMNL were primed with 100 ng of LPS from *E. coli* J5 (Rc LPS), 1 µg of LPS from *E. coli* O111 (smooth LPS) or 100 ng of LPS from *S. minnesota* R595 (Re LPS), stimulation with FMLP caused an enhanced CL response when compared with untreated cells. Although the LPS dose seems relatively high, when compared with concentrations *in vivo*, it was needed in order to observe a clear difference between positive and negative controls. As reported earlier, the priming effect varied from donor to donor.¹³ Furthermore, the degree of priming depended on the type of LPS. The CL response after stimulation with FMLP was higher when PMNL were primed with rough LPS. A two-to four-fold increase of CL after stimulation was observed when PMNL were primed with smooth LPS of *E. coli* O111, whereas rough LPS

produced a four-to twenty-fold increase. Our findings are consistent with the results of Kapp *et al.*²⁵ and Henricks *et al.*²⁶ who measured CL of PMNL after direct stimulation with LPS. However, the different efficacies of the LPS types on FMLP-induced CL are in contrast with the findings of Fittschen *et al.*,¹² who showed similar enhancement of PMNL granular secretion for both smooth and rough types of LPS. Kapp *et al.*²⁵ examined 10 types of smooth and rough LPS and showed that the observed difference between rough and smooth LPS was not due to a different degree of aggregation. They suggested that the difference may be due to the more lipophilic character of rough LPS, which may render it more susceptible to interaction with PMNL membranes.

The mechanism by which LPS primes PMNL is still unknown. Lipid A is believed to be primarily responsible for the toxic effects of endotoxin.² It appears that lipid A mediates the binding of LPS to host cells. Many workers have postulated the existence of a specific cell receptor for LPS.^{27–29} Lei and Morrison have isolated a membrane protein from murine splenocytes that binds LPS specifically,^{29,30} but its functional activity remains to be elucidated. Danner *et al.*³¹ demonstrated that priming of PMNL can be prevented by lipid X, a precursor of lipid A, and by 3-aza-lipid X, a diamino-analogue of lipid X. The latter molecule also exerted an agonist effect, supporting the possibility of a specific receptor-ligand interaction.

Pre-incubation of rough or smooth LPS with PMB resulted in dose-dependent inhibition of the enhanced CL response of PMNL after FMLP stimulation. PMB is a cyclic polypeptide antibiotic with potent anti-endotoxin properties, because it binds to the lipid A part of the LPS molecule.³² PMB inhibits priming of PMNL by Re LPS, neutralises the Schwartzman phenomenon, prevents endotoxin lethality in mice and inhibits LPS-induced interleukin-1 secretion.^{18,33,34} Our results support the role of lipid A as the toxic principle of endotoxin responsible for priming of PMNL.

The inhibitory capacity of IgM MABs 8-2 and 26-20 was evaluated and compared with PMB. Both MABs neutralised the priming effect of the three kinds of LPS tested—Rc LPS of *E. coli* J5, Re LPS of *S. minnesota* R595 and smooth LPS of *E. coli* O111. The protective capacity of both MABs was dose-dependent and both equalled the maximum inhibitory effect of PMB.

MABs 26-20 and 8-2 are each specific for a distinct epitope within the hydrophobic lipid A region,¹¹ and we have demonstrated previously that the epitopes are obscured after membrane incorporation of lipid A in passive haemolysis and that they are concealed when PMB binds to lipid A in ELISA.¹⁰ These MABs may protect PMNL against priming by LPS by preventing the attachment of lipid A to the cell membrane in a similar manner to PMB. The hydrophobic interaction of PMB and endotoxin appears to be necessary for the

neutralisation of lipid A-mediated effects.³¹ Both MABs bind to the hydrophobic part of lipid A and may, therefore, mimic the inhibitory activity of PMB. Chia *et al.*³⁵ reported that MABs directed against lipid A failed to inhibit LPS-induced secretion of tumour necrosis factor by macrophages. The MABs described by Chia *et al.*³⁵ and Pollack *et al.*⁴ reacted predominantly with the hydrophilic elements of lipid A, which could account for the lack of inhibition. Our results emphasise the importance of interaction of MABs with the hydrophobic part of lipid A.

In conclusion, two IgM MABs, 8-2 and 26-20, directed against separate epitopes within the hydrophobic lipid A part of LPS, inhibited the enhanced CL response of PMNL primed with different forms of LPS. Their protective capacity was as potent as the inhibition produced by PMB. Although data from in vitro studies cannot be translated to the in-vivo situation, these results indicate that in-vivo protection experiments are worthy of further investigation.

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