

Competition between Bactericidal/Permeability-Increasing Protein and Lipopolysaccharide-Binding Protein for Lipopolysaccharide Binding to Monocytes

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The bactericidal/permeability-increasing protein (BPI) inhibits the lipopolysaccharide (LPS)-mediated activation of monocytes. Due to its inhibitory activity for various LPS, BPI has therapeutic potential in endotoxic shock. To be efficient *in vivo*, BPI should overcome the action of LPS-binding protein (LBP), a serum molecule that increases the expression of LPS-inducible genes via CD14 of monocytes. rBPI₂₃, a recombinant fragment of BPI, prevented in a dose-dependent manner the binding and the internalization of LPS mediated by LBP. Consequently, rBPI₂₃ also inhibited LPS-induced tumor necrosis factor (TNF α) synthesis from monocytes. LPS- and LBP-mediated activation of monocytes was totally inhibited when LPS was preincubated with rBPI₂₃. Adding rBPI₂₃ at the same time as LBP resulted in an important but partial inhibition of TNF α release, but this inhibition vanished with delaying the time of addition of rBPI₂₃. These studies suggest that the inhibitory activity of BPI is related to its ability to compete with LBP for LPS.

Living organisms have developed several protective mechanisms against toxic lipopolysaccharide (LPS) of gram-negative bacteria. Very recently, two members of a family of proteins have been recognized that possess LPS-binding sites. Indeed, the LPS-binding protein (LBP) and the bactericidal/permeability-increasing protein (BPI) share the ability to bind to smooth and rough LPS as well as to lipid A [1, 2]. There is a striking homology in the DNA sequence of the two human, rabbit, or bovine proteins [3]. However, despite their structural similarities, the functions of LBP and BPI appear antagonistic.

Most circulating LPS binds to plasma LBP, and LPS linked to LBP is transferred to CD14 on the surface of cells of the monocytic lineage and neutrophils, resulting in increased expression of LPS-inducible genes [3, 4]. LBP-mediated processes include increased tumor necrosis factor- α (TNF α) production by monocytes [3–5], accelerated priming of neutrophils for the oxidative response to FMLP [6], and increased adhesive capacity of complement receptor CR3 of neutrophils [7]. LBP was originally described as an acute-phase reactant in the rabbit [8], but its concentration in normal serum of rabbits, calves, mice, and humans [5] suggests

that constitutive LBP is sufficient to bind concentrations of LPS usually found in infected hosts [4].

BPI, a 55-kDa cationic protein found essentially in the azurophilic granules of polymorphonuclear leukocytes [9], is specifically cytotoxic for gram-negative bacteria [10]. Soluble BPI, recombinant BPI, or its 25-kDa N-terminal fragment have been shown to inhibit endotoxin activity by neutralizing LPS, as detected by the inhibition of the proteolytic cascade in the limulus amoebocyte lysate assay, by the inhibition of the priming of neutrophils by LPS, and by the inhibition of LPS-mediated production of TNF α by monocytes [11–13].

Because of the apparent antagonistic properties of these two proteins that bind LPS, it is important to better define the respective roles of LBP and BPI in the LPS-induced activation process, inasmuch as BPI has therapeutic potential in endotoxic shock. We characterized *in vitro* the direct competition between LBP and BPI for binding of LPS to cells of the monocytic lineage and investigated the competition between these two molecules in the activation process of monocytes resulting in TNF α release.

Materials and Methods

Materials. rBPI₂₃ [2], a human recombinant NH₂-terminal fragment of BPI possessing LPS-neutralizing activity [14], was obtained from XOMA (Berkeley, CA). Thaumatin (XOMA), used as a control protein, has a molecular weight and isoelectric point similar to rBPI₂₃ [2]. Murine LBP was prepared from serum. Murine LBP is a 61-kDa molecule that shares NH₂-terminal sequence homology and functional properties with rabbit LBP and is able to present LPS to human monocytes [15]. Rab-

Received 17 November 1992; revised 8 February 1993.

Grant support: Fonds National Suisse de la Recherche Scientifique (32-30265.90); XOMA Corp., Berkeley, California.

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The Journal of Infectious Diseases 1993;167:1351–7

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0022-1899/93/6706-0013\$01.00

bits were immunized with purified murine LBP, and immune IgG was purified by protein G chromatography. A control preparation of IgG was obtained from a polyclonal rabbit antiserum raised against murine recombinant interleukin-1.

LPS isolated from *Escherichia coli* O111, either unlabeled or labeled with fluorescein (FITC-O111 LPS), was purchased from Sigma (St. Louis). MY4, a specific monoclonal antibody (MAb) recognizing human CD14 [16], was purchased from Coulter Immunology (Luton, UK).

Plasma was prepared from a pool of donors. The concentration of LBP in this pool was 20 $\mu\text{g}/\text{mL}$ as measured by ELISA [17] (done by P. Tobias, Scripps Clinics, La Jolla, CA).

Test for LPS binding to monocytes. Heparinized human blood samples obtained from normal donors were used to prepare peripheral blood mononuclear cells (PBMC) by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden). PBMC (10^6) were incubated with 1 μg (alternatively, 10 or 100 ng) of FITC-O111 LPS in 1 mL of RPMI enriched with 10% plasma or with 4% albumin as control. For CD14 blockade, monocytes were pretreated with 10 $\mu\text{g}/\text{mL}$ MY4 MAb at 37°C. After incubation for 1 h, the cells were washed twice with cold RPMI and analyzed using a FACScan flow cytometer with the LYSYS software package (Becton Dickinson, Basel, Switzerland). To restrict the analysis to monocytes, side scatter parameters were used for gating of the signal [18].

Confocal laser microscopy. Human monocytes were purified from PBMC by adherence to plastic in 10% human serum for 1 h and recovered using a rubber policeman. Purity was >95% as assessed by morphology and nonspecific esterase staining. Monocytes were incubated with 1 $\mu\text{g}/\text{mL}$ FITC-O111 LPS in RPMI containing 4% albumin or 10% plasma for 30 min. After two washes, they were spun down using a cytocentrifuge (Cytospin 2; Shandon, Basel, Switzerland) for 3 min at 700 rpm. Slides were mounted in TRIS-glycerin-saline buffer and directly examined by fluorescence microscopy. Optical slices were generated in a Laser-Sharp MRC 500 confocal microscope (Bio-Rad Laboratories, Glattbrugg, Switzerland).

TNF α assays. PBMC (0.5×10^6) in 200 μL of RPMI enriched with 10% plasma were added to each well of 96-well flat-bottom microtiter plates (Becton Dickinson). These preparations were stimulated with unlabeled O111 LPS at various concentrations. Thaumatin or rBPI₂₃ at 10 $\mu\text{g}/\text{mL}$ was added at various times relative to LPS exposure in plasma. Supernatants were collected for TNF α measurements after 4 h of culture at 37°C. Under the conditions used in this study, TNF α release by lymphocytes present in the PBMC fraction was negligible [19]. The cytotoxic activity of TNF α was measured on WEHI clone 13 cells as described [20, 21].

Results

Effects of rBPI₂₃ on the LBP-mediated binding of FITC-O111 LPS to monocytes. We previously reported that plasma enhanced the binding of FITC-O111 LPS to human monocytes compared with binding observed with albumin alone. The enhancement in LPS binding was due to plasma LBP and was blocked by the MY4 anti-CD14 MAb [5]. It

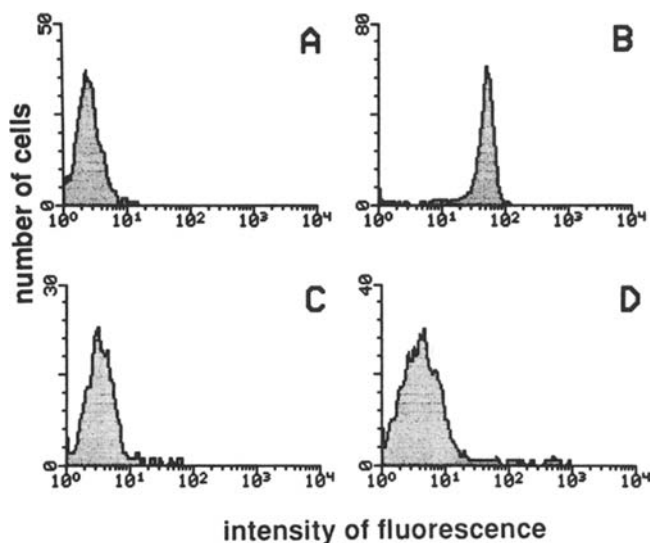


Figure 1. Effects of recombinant bactericidal/permeability increasing protein (rBPI₂₃) on LPS-binding protein-mediated binding of fluorescein-labeled *Escherichia coli* O111 LPS by human peripheral blood mononuclear cells. Incubation medium contained 4% albumin (A), 10% plasma (B), 10% plasma after CD14 was blocked on monocytes with anti-CD14 monoclonal antibody (C), or 10% plasma with 10 μg of rBPI₂₃ (D). Fluorescence was assessed by flow cytometry.

was thus of interest to evaluate whether LPS-BPI complexes would also bind to CD14 on monocytes or would interfere with the LBP-mediated binding of LPS through CD14. Human monocytes incubated with 1 $\mu\text{g}/\text{mL}$ FITC-O111 LPS in medium containing 4% albumin showed only a modest fluorescence (figure 1A). In contrast, when cells were incubated with FITC-O111 LPS in the presence of 10% plasma, monocytes showed a distinctly brighter fluorescence (figure 1B). Preincubation of monocytes with MAb MY4 completely suppressed the plasma-mediated LPS binding (figure 1C) to the level of control monocytes incubated in the presence of albumin.

In our observations with 10% plasma, the concentration of LBP was 2 $\mu\text{g}/\text{mL}$. We added rBPI₂₃ to 10% plasma at a final concentration of 10 $\mu\text{g}/\text{mL}$ together with FITC-O111 LPS. The presence of rBPI₂₃ resulted in a decreased fluorescence (figure 1D) compared with that observed with 10% plasma alone, indicating that less LPS had bound to monocytes. We then tested the effect of increasing concentrations of rBPI₂₃ in 10% plasma containing LBP on FITC-O111 LPS binding to monocytes, using FITC-O111 LPS concentrations from 10 ng/mL to 1 $\mu\text{g}/\text{mL}$, as described [5] (table 1). The concentration of rBPI₂₃ required for an efficient inhibition of the plasma-mediated binding of FITC-O111 LPS to monocytes was dependent on the LPS concentration. With a weight ratio of 10:1 (10 $\mu\text{g}/\text{mL}$ rBPI₂₃ with 1 $\mu\text{g}/\text{mL}$ FITC-O111 LPS or 1 $\mu\text{g}/\text{mL}$ rBPI₂₃ with 100 ng/mL FITC-O111), a 75% decrease in fluorescence intensity compared to control without

Table 1. Effect of recombinant bactericidal/permeability increasing protein (rBPI₂₃) on the LPS binding protein (LBP)-mediated binding of fluorescein-labeled *Escherichia coli* O111 LPS to human peripheral blood mononuclear cells.

Medium, rBPI ₂₃ (μg/mL)	LPS concentration (ng/mL)		
	10	100	1000
4% albumin	2.0	2.3	2.4
10% plasma			
0	4.2	16.2	40.1
0.1	4.1	16.6	39.9
1.0	2.8	3.6	25.7
10.0	2.0	2.5	9.7
10% plasma + anti-CD14 monoclonal antibody			
0	2.3	2.2	2.5
10	2.1	2.3	2.5

NOTE. Data are mean fluorescence units measured on monocytes in one experiment. Experiment was repeated many times with similar observations (data not shown).

rBPI₂₃ was observed. By increasing the weight ratio to 100:1 (10 μg/mL rBPI₂₃ with 100 ng/mL FITC-O111 LPS), the plasma-mediated binding of LPS to monocytes was abolished.

In the presence or absence of rBPI₂₃, the observed LPS binding was totally suppressed by CD14 blockade by anti-CD14 MAb. Control thaumatin did not affect the LBP-mediated binding of FITC-O111 LPS by human monocytes (data not shown).

Effect of rBPI₂₃ on the binding of FITC-O111 LPS to human monocytes after LBP blockade in plasma. The observations reported above indicated that rBPI₂₃ inhibited the plasma-mediated binding of LPS by monocytes in a dose-dependent manner. When FITC-O111 LPS was used at a 1-μg/mL concentration, residual binding was found despite the presence of 10 μg/mL rBPI₂₃ (table 1). This binding was suppressed by CD14 blockade. We then investigated whether this residual binding was due to LBP or to BPI. We used murine plasma for these experiments, because we had an anti-murine LBP antibody readily available and because murine LBP is able to present LPS to human CD14 in a fashion identical to that of human LBP [5, 15]. LBP was blocked in murine plasma with a rabbit anti-murine LBP IgG fraction. An irrelevant rabbit IgG fraction directed against murine interleukin-1 was used as control. In the presence of control murine plasma, LBP was able to increase the binding of FITC-O111 LPS to human monocytes over that to control monocytes incubated in albumin (figure 2). This binding was suppressed by antibody-mediated LBP blockade and, to a lesser extent, by the addition of rBPI₂₃. The preincubation of monocytes with anti-CD14 MAb MY4 suppressed LBP-mediated binding of FITC-O111 LPS. No increase in binding of FITC-O111 LPS to monocytes over that

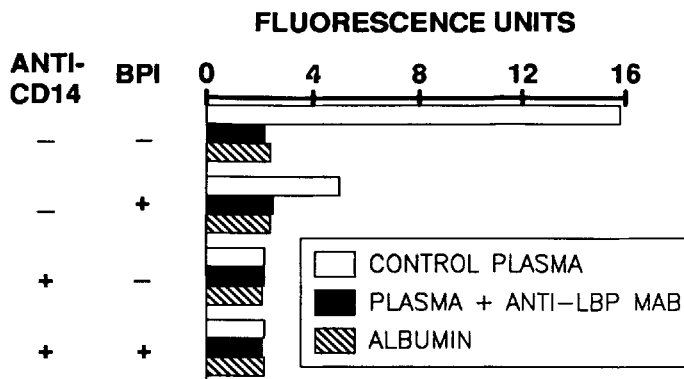


Figure 2. Effects of recombinant bactericidal/permeability increasing protein (rBPI₂₃) on binding of fluorescein-labeled *Escherichia coli* O111 LPS to human peripheral blood mononuclear cells after antibody-mediated blockade of LPS binding protein (LBP) in plasma. Anti-LBP MAB, rabbit IgG directed against mouse LBP. Data are fluorescence units measured on monocytes.

observed in albumin was seen when rBPI₂₃ was added after antibody-mediated LBP blockade in plasma. These experiments thus confirmed that rBPI₂₃ did not promote the binding of FITC-O111 LPS to monocytes but rather interfered with the LBP-mediated binding of FITC-O111 LPS to monocytes.

Effects of competition between purified murine LBP and rBPI₂₃ for the binding of FITC-O111 LPS to monocytes. We further investigated whether rBPI₂₃ could interfere with purified LBP for FITC-O111 LPS binding to monocytes. We used purified murine LBP for these experiments (table 2). When 1 μg of rBPI₂₃ (43 nM) and 1 μg of purified murine LBP (16 nM) were added to monocytes in the presence of 100 ng/mL LPS, rBPI₂₃ suppressed the LBP-mediated binding of FITC-O111 LPS to monocytes.

Effects of rBPI₂₃ on the internalization of FITC-O111 LPS into human monocytes. To distinguish between cell surface binding and intracellular localization of LPS, preparations of adherent monocytes were incubated with FITC-O111 LPS

Table 2. Effect of recombinant bactericidal/permeability increasing protein (rBPI₂₃) on the binding to peripheral blood mononuclear cells of fluorescein-labeled *Escherichia coli* O111 LPS mediated by purified murine LPS-binding protein (LBP).

	Experiment	
	1	2
4% albumin	3.2	3.1
1 μg murine LBP	15.5	11.8
1 μg murine LBP + 100 ng rBPI ₂₃	13.9	12.3
1 μg murine LBP + 1 μg rBPI ₂₃	3.1	3.2

NOTE. Data are mean fluorescence units measured on monocytes by flow cytometry in two separate experiments.

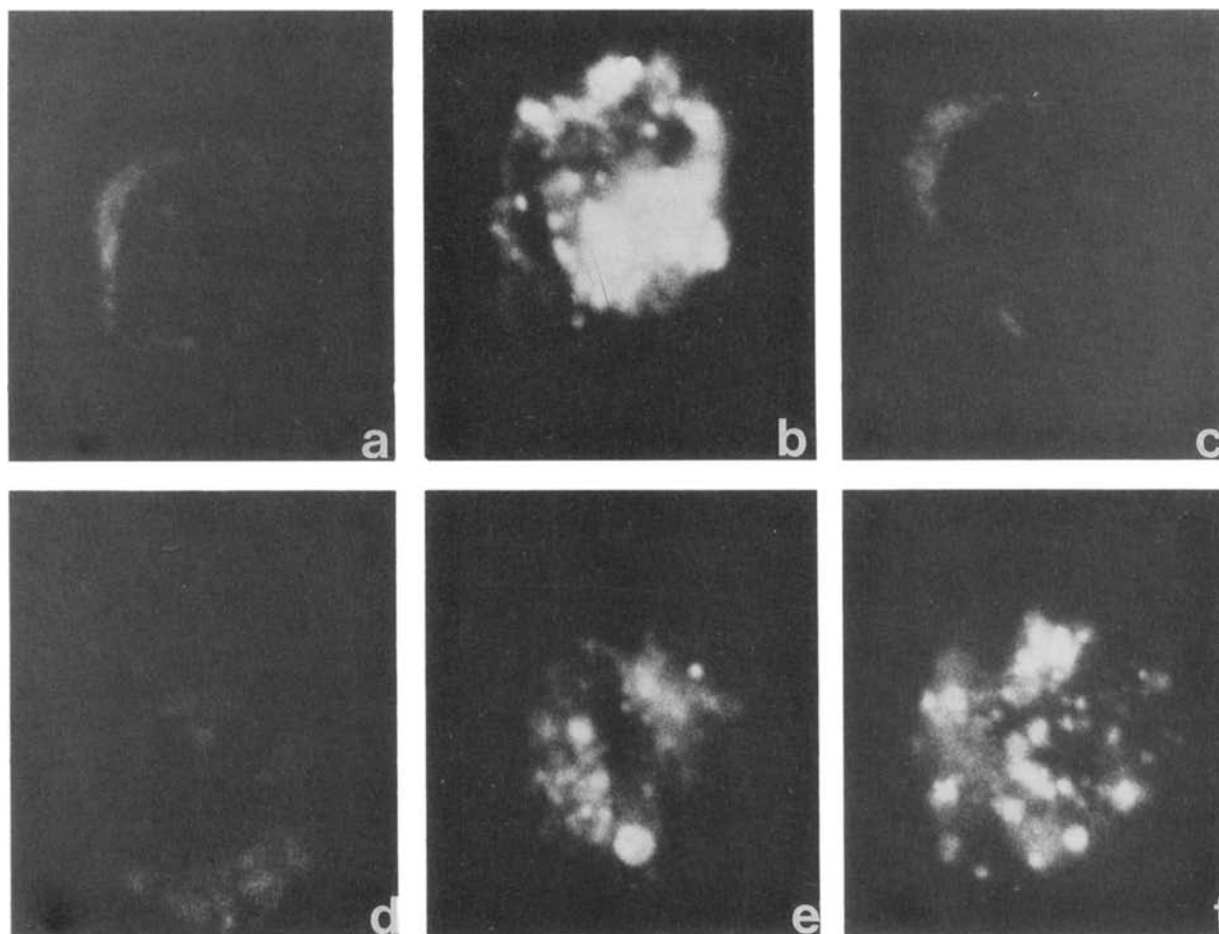


Figure 3. Effects of recombinant bactericidal/permeability increasing protein (rBPI₂₃) on LPS binding protein (LBP)-mediated internalization of fluorescein-labeled *Escherichia coli* O111 LPS by human monocytes. Incubation medium contained 4% albumin (a); 10% human plasma (b); 10% plasma after pretreatment of monocytes with anti-CD14 monoclonal antibody (c); 10% human plasma and rBPI₂₃ that was preincubated with LPS for 5 min (d); rBPI₂₃ added concurrently with 10% plasma and monocytes (e); rBPI₂₃ added 5 min after mixture of monocytes, 10% plasma, and LPS (f). Fluorescence of monocytes was visualized in confocal microscope after 1 h of incubation.

and analyzed by confocal microscopy, which allows observation of subsurface structures. Monocytes were scanned in depth at various focal planes. Through-focus images were similar, and figure 3 is representative of one focal plane. When monocytes were incubated for 1 h with 1 $\mu\text{g}/\text{mL}$ FITC-O111 LPS in 4% albumin (figure 3a), no fluorescence was detectable. When incubation medium contained 10% human plasma, the fluorescence observed was cytoplasmic (figure 3b). When FITC-O111 LPS was added to anti-CD14-treated monocytes in the presence of 10% plasma, fluorescence was abolished (figure 3c). When FITC-O111 LPS was preincubated with 10 $\mu\text{g}/\text{mL}$ rBPI₂₃ before adding human plasma as a source of LBP, no fluorescence was detectable (figure 3d). However, when rBPI₂₃ was added at the same time as plasma LBP (figure 3e) or 5 min thereafter (figure 3f), the uptake of FITC-O111 LPS into monocytes was only partially prevented. At concentrations <10 $\mu\text{g}/\text{mL}$,

rBPI₂₃ was unable to suppress the fluorescence in these experiments (data not shown).

Time-dependent inhibition by rBPI₂₃ of the LBP-mediated binding of FITC-O111 LPS to human monocytes. We previously reported that the LBP-mediated binding of FITC-O111 LPS by monocytes is a rapid process largely completed in 5–15 min [5]. Thus, in the next set of experiments, we added rBPI₂₃ at various times relative to FITC-O111 LPS exposure in plasma (figure 4). When LPS was first preincubated with rBPI₂₃ 30 min or 5 min before being added to monocytes in medium containing plasma, LBP-mediated binding of FITC-O111 LPS to monocytes was virtually abolished. When LPS and rBPI₂₃ were added without preincubation together with monocytes in 10% plasma, rBPI₂₃ was still able to inhibit LPS binding to monocytes. The addition of rBPI₂₃ from 5 to 30 min after the initiation of the reaction of plasma LBP with FITC-O111 LPS progressively reduced the

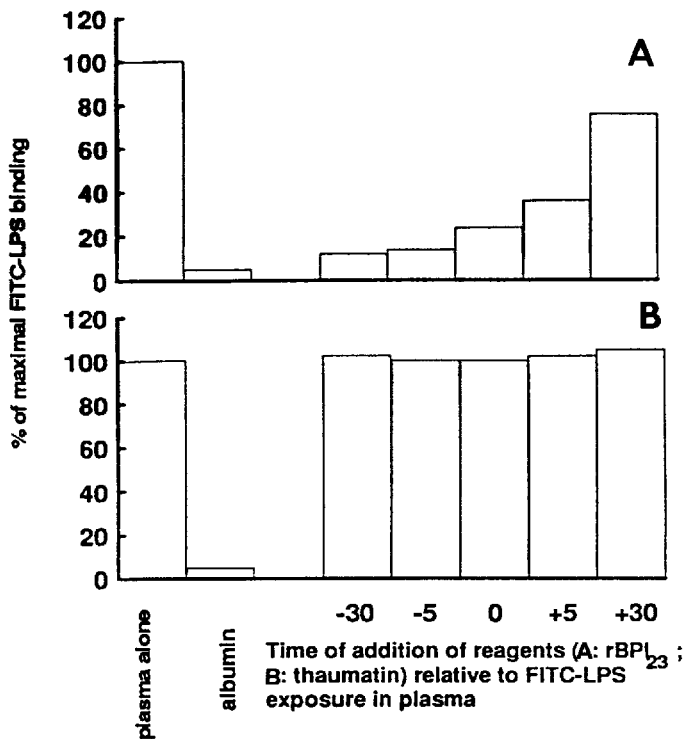


Figure 4. Time-dependent inhibition of LPS binding protein (LBP)-mediated binding of fluorescein-labeled *Escherichia coli* O111 LPS (FITC-LPS) by human peripheral blood mononuclear cells (PBMC) in presence of recombinant bactericidal/permeability-increasing protein (rBPI₂₃). Binding was assessed by flow cytometry after 1 h of incubation with FITC-LPS; results are % of maximal FITC-O111 LPS binding in plasma alone.

inhibitory effect of rBPI₂₃. Furthermore, rBPI₂₃ could not displace the LBP-mediated binding of FITC-O111 LPS. This was shown by adding rBPI₂₃ for a period of 1 h, 1 h after FITC-O111 LPS had reacted with monocytes in 10% plasma. In this setting, the addition of rBPI₂₃ for a prolonged period did not modify the fluorescence pattern (data not shown). Control thaumatin had no effect on the LBP-mediated binding of FITC-O111 LPS by human monocytes.

Effects of rBPI₂₃ on the LPS-mediated TNF α release by human monocytes in medium containing plasma. Having observed that rBPI₂₃ efficiently blocked LPS binding in a time-dependent manner, we added rBPI₂₃ to 10% plasma at various times relative to LPS exposure, and TNF α was measured in the supernatants after 4 h (figure 5). When LPS (either 100 pg/mL or 1 ng/mL) was first incubated with rBPI₂₃ for 5 min before being added to plasma, virtually no TNF α was detectable in the supernatant after 4 h. rBPI₂₃ was still able to inhibit TNF α production, although partially, when added together with LPS. This inhibition of TNF α vanished when LPS concentrations >1 ng/mL were used. Delaying the time of addition of rBPI₂₃ for 5 min (or more,

data not shown) after exposure to LPS resulted in unhampered TNF α release compared with that seen in controls incubated with thaumatin.

Discussion

The focus of this investigation was to assay the direct competition between BPI and LBP for LPS binding to monocytes. Indeed, since BPI appears to be an efficient neutralizing agent of LPS [11–13], it should *in vivo* be able to overcome the action of LBP, which amplifies the effects of LPS on both monocytes and neutrophils [3–7].

The experiments were first conducted in plasma containing LBP using a high LPS concentration (1 μ g/mL). By both flow cytometry and confocal microscopy, we observed that rBPI₂₃, a recombinant NH₂-terminal fragment of BPI [2, 14], reduced LPS binding to monocytes when added to normal plasma containing LBP. Low residual LPS binding was observed, which was suppressed by CD14 blockade. Since binding of LPS to monocytes in the presence of plasma is mediated by the formation of LPS-LBP complexes that bind to CD14 receptors [3], these observations demonstrate that the small residual LPS binding in the presence of a suboptimal concentration of rBPI₂₃ was most likely due to LBP. Thus, rBPI₂₃ was able to prevent the formation of LPS-LBP complexes and their subsequent binding to human monocytes, although the inhibition was not complete at a high concentration of 1 μ g/mL LPS. Furthermore, when LBP in plasma was functionally blocked by specific antibodies, rBPI₂₃ was not able to present LPS to CD14 of monocytes. Confocal microscopy, which allows the observation of subsurface structures, confirmed that rBPI₂₃ prevented the LBP-mediated internalization of LPS through CD14.

At LPS concentrations of 100 and 10 ng/mL, a 100:1 weight ratio of rBPI₂₃ to LPS appeared necessary to suppress the LBP-mediated binding of LPS in plasma. The relative concentrations of LPS, BPI, and LBP appeared thus to play an important role. When similar amounts of rBPI₂₃ (43 nM) and purified murine LBP (16 nM) were added to monocytes in the presence of 100 ng/mL LPS, rBPI₂₃ appeared able to completely suppress the LBP-mediated binding of LPS to monocytes. These data indicate that a 3-fold molar excess of rBPI₂₃ has a potent inhibitory effect on LPS in direct competition with LBP. The concentration of LBP in pooled plasma used in these experiments was 20 μ g/mL, as measured by ELISA [17]. In our observations with 10% plasma, the concentration of LBP was 2 μ g/mL, that is, 33 nM. To completely suppress the LBP-mediated binding of 10 ng/mL LPS, 10 μ g/mL rBPI₂₃, or 430 nM, was necessary. This observation thus suggests that in plasma at least a 10-fold molar excess of rBPI₂₃ should be necessary to overcome totally the action of LBP.

Our experiments showed in addition that the rBPI₂₃-me-

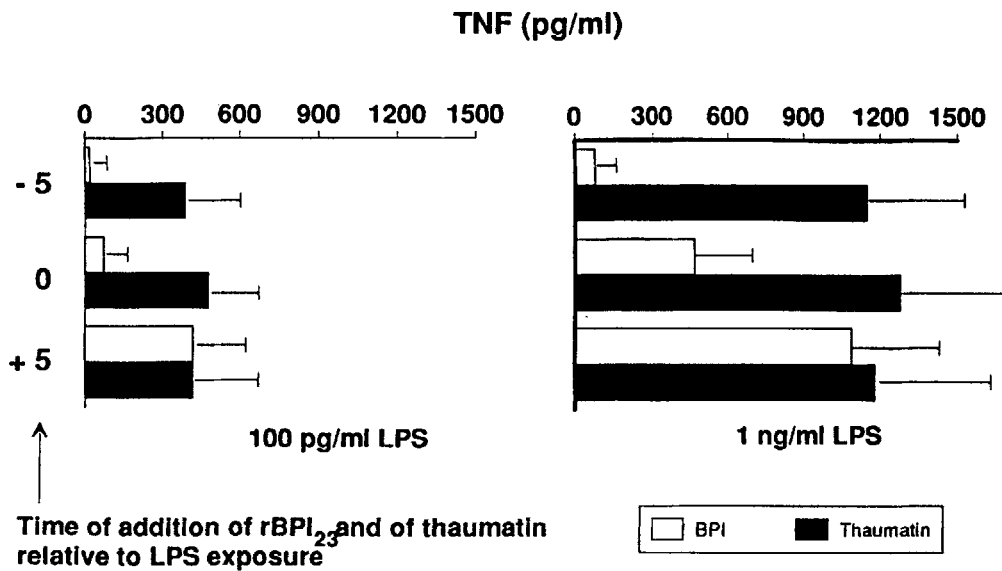


Figure 5. Effects of recombinant bactericidal/permeability increasing protein (rBPI₂₃) and of thauMATIN on tumor necrosis factor (TNF) release of human peripheral blood mononuclear cells stimulated with LPS. TNF was measured by bioassay in supernatants after 4 h of culture. Data are mean + SD of 5 experiments.

diated inhibition of LBP-LPS binding to monocytes was more efficient when rBPI₂₃ could interact with LPS before interference with LBP. Indeed, rBPI₂₃ was unable to reverse the LBP-mediated binding of LPS to monocytes. This was observed in experiments using flow cytometry, confocal microscopy, and monocyte activation measured by TNF α release, thus extending and confirming a similar study with the native nonrecombinant protein [12]. This observation indicated that a concentration of 10 μ g/mL rBPI₂₃ was sufficient to totally inhibit the production of TNF α mediated by either 100 pg/mL or 1 ng/mL LPS. rBPI₂₃ was still able to inhibit TNF α release when added at the same time as LBP, but the inhibition was not complete. However, delayed addition of rBPI₂₃ relative to LBP failed to inhibit TNF α release, an observation consistent with the hypothesis that a few minutes of interaction of the LPS-LBP complex with monocyte CD14 could be sufficient to trigger cytokine production.

In conclusion, under the conditions of our assays—10% plasma containing LBP—we can conclude that rBPI₂₃ competed with but did not totally suppress the LBP-mediated effects of LPS. Indeed, if rBPI₂₃ appeared able to block the LBP-mediated binding of LPS to monocytes as revealed in our binding assays, this apparent blockade did not totally suppress TNF α production. Additional studies should be done in vitro using whole blood or other sources of LPS to confirm these observations. Thus, our studies showed that rBPI₂₃ was very effective as an inhibitor of LPS on monocytes, provided it was used prophylactically. Further studies in vivo are needed to investigate whether rBPI₂₃ could overcome the LPS-mediated activation of the host.

Acknowledgments

We thank G. Centeno, J. Smith, and C. Knabenhans for technical assistance.

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