Bactericidal/Permeability-Increasing Protein Inhibits Induction of Macrophage Nitric Oxide Production by Lipopolysaccharide

Sally Betz Corradin, Didier Heumann, Philippe Gallay, Josiane Smith, Jacques Mauël, and Michel Pierre Glauser

Institute of Biochemistry, University of Lausanne, Epalinges, and Division of Infectious Diseases, Department of Medicine, CHUV, Lausanne, Switzerland

A recombinant (r) NH_2-terminal fragment of bactericidal/permeability-increasing protein, $rBPI_{23}$, was shown to inhibit murine macrophage nitric oxide (NO) production elicited by lipopolysaccharide (LPS) plus interferon-γ (IFN-γ). Normal mouse plasma amplified NO synthesis (measured as NO_2^- release) at LPS concentrations of 1–10 ng/mL, and antibody to the plasma LPS-binding protein (LBP) partially inhibited NO_2^- release in the presence of normal mouse plasma. $rBPI_{23}$ (1 μg/mL) effectively inhibited LPS-dependent NO_2^- release in the presence or absence of normal mouse plasma. Fifty percent inhibition of IFN-γ/LPS-elicited NO_2^- production or of binding of fluoresceinated LPS was obtained with ~0.2 μg/mL $rBPI_{23}$. These results provide a basis for studies of $rBPI_{23}$ effects on NO synthase activity in murine models of gram-negative sepsis.

Lipopolysaccharide (LPS), either as part of the outer membrane of gram-negative bacteria or when released into the circulation upon bacterial lysis, is a potent stimulus of macrophage cytokine production and mediator release [1, 2]. Despite extensive investigation, the precise mechanism(s) by which LPS stimulates macrophage function is still unknown. Recently, a plasma protein, LPS-binding protein (LBP), was shown to bind to smooth and rough LPS [3–5]. Macrophage cell surface protein CD14 was subsequently shown to recognize the LBP-LPS complex [6], and several studies have now demonstrated dramatic enhancement of both binding of and functional responses to LPS by purified LBP [6–11]. Furthermore, transfection of cDNA encoding CD14 into a murine pre–B lymphocyte cell line, which does not normally express this gene, strongly increased the sensitivity of those cells to low concentrations of LPS in the presence of LBP [12]. Although such results do not imply that CD14 alone can initiate signal transduction, they provide evidence for an important role of LBP in modifying host responses to LPS.

Another LPS-binding protein, the neutrophil granule bactericidal/permeability-increasing protein (BPI), binds to LPS in the outer membrane of gram-negative bacteria, causing permeability and cell death [13–16]. Although BPI shows striking sequence homology to LBP [7, 17], the two proteins exert different effects on LPS activity. BPI, its NH_2-terminal fragment, and recombinant (r) BPI inhibit LPS activity in various in vitro systems, including activation of limulus amebocyte lysate [18], priming for neutrophil leukotriene release [18] or LPS-elicited complement receptor expression [19], and induction of tumor necrosis factor (TNF) secretion by human monocytes [18, 20]. Recently, a recombinant protein, $rBPI_{23}$, corresponding to the amino-terminal region of human BPI was produced and purified [21]. $rBPI_{23}$ showed high affinity binding to lipid A and various types of LPS and LPS-neutralizing activity [16, 22]. We have recently demonstrated that $rBPI_{23}$ inhibits the plasma-mediated binding of LPS to human monocytes in a dose-dependent manner [23]. Moreover, antibody to murine LBP inhibited LPS binding in normal mouse plasma (NMP) [11, 23], suggesting that binding occurs through the CD14/LBP pathway and that $rBPI_{23}$ must compete with LBP to be an efficient inhibitor of LPS.

Although certain macrophage responses such as TNFα secretion occur with LPS alone, other activities, including the induction of cytotoxic activity toward tumor cells or intracellular pathogens and synthesis of nitric oxide (NO), are greatly enhanced by costimulation of LPS with interferon-γ (IFN-γ) [24–27]. NO and its stable derivatives NO_2^- and NO_3^- are derived from L-arginine via the action of a group of enzymes termed NO synthases [28, 29]. The constitutive isoenzyme present in endothelial and neuronal cells is reversibly regulated by free calcium ions and can rapidly generate small amounts of NO. Moreover, a cytokine-inducible NO synthase, most extensively studied in murine macrophages, is a calcium-independent enzyme capable of producing much higher levels of NO over a prolonged period. Although constitutive production of NO is believed to play a role in the maintenance of vascular tone, increased production or indiscriminate release occurring on activation of inducible NO synthase could contribute to the hypotension observed during septic shock [28, 30].

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Reprints or correspondence: Dr. Sally Betz Corradin, Institute of Biochemistry, University of Lausanne, Chemin des Boveresses 155, 1066 Epalinges, Switzerland.

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We recently reported that purified rabbit LBP significantly enhanced NO production by murine bone marrow–derived macrophages stimulated with LPS plus IFN-γ [9]. We have now examined the effects of $rBPI_{23}$ on NO production (measured as NO_2^-) using a homologous system in which murine macrophages were stimulated in the presence of NMP as a source of LBP and other plasma factors that might modulate LPS function.

Materials and Methods

Animals. CBA female mice were obtained from IFFA-CREDO (Saint Germain-sur-l'Arbresle, France) and used at 5–10 weeks of age.

Reagents. A recombinant NH_2-terminal fragment of BPI ($rBPI_{23}$) was obtained from XOMA (Berkeley, CA). rIFN-γ (lot no. 2309-24, produced by Genentech, South San Francisco) was supplied by Boehringer Ingelheim (Vienna). Polymyxin B, phenol-extracted LPS, and fluorescein-labeled LPS (FITC-LPS) from *Escherichia coli* O111 were purchased from Sigma (St. Louis). Recombinant human TNFα was a gift from BASF/Knoll (Ludwigshafen, Germany). Polyclonal antibody to purified murine LBP was raised in rabbits and purified by protein A affinity chromatography as previously described [11]. High-titered rabbit polyclonal antibody to *E. coli* O111 LPS was obtained by intravenous injection of heat-killed stationary phase bacteria (5×10^9 three times weekly for 2 weeks). Pooled heat-inactivated (56°C, 30 min) serum was stored at −20°C until use. Preimmune normal rabbit serum and purified IgG served as control antibodies.

Macrophage cultures. Macrophages were obtained by in vitro differentiation of bone marrow precursor cells [31]. Day 10 or 11 macrophages were detached by pipetting, suspended in Dulbecco's MEM (DMEM; Seromed, Munich) supplemented with 10% fetal calf serum (FCS; Seromed), and distributed in 96-well microculture plates (75,000/well).

Infection of macrophage cultures. Macrophages were infected with *Leishmania enriettii* promastigotes as previously described [32] at a parasite-to-macrophage ratio of 16:1.

Macrophage activation. Infected or noninfected macrophages were washed with Hanks' balanced salt solution to remove serum and then cultured overnight with IFN-γ plus LPS or TNFα in the presence or absence of $rBPI_{23}$ or 1% pooled NMP or both. Alternatively, macrophages were primed with 10 units/mL IFN-γ for 24 h prior to stimulation with LPS (with or without $rBPI_{23}$) for an additional 24 h.

TNF assay. Secretion of TNF was determined by biologic assay of supernatants collected 4 h after addition of LPS [33]. WEHI 164 clone 13 cells in 10% FCS–supplemented RPMI medium (10,000 cells/90 μL) were added to microtiter wells containing 90 μL of serial dilutions of test supernatant or standard rTNFα. Plates were incubated at 37°C for 48 h; 20 μL of 3-(1,4 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; United States Biochemicals, Cleveland; 5 mg/mL in RPMI medium) was then added. After 4 h at 37°C, supernatants were removed, and reduced dye was solubilized with 0.2 mL of 20 m*M* HCl in isopropanol containing 3% SDS. Optical density was determined at 570 nm using a microELISA reader fitted with a 620-nm reference filter. Preliminary experiments demonstrated that $rBPI_{23}$ at the concentrations added to macrophage cultures had no effect on WEHI target cell viability either in the presence or absence of TNFα (data not shown).

Table 1. Inhibition of lipopolysaccharide (LPS)-elicited tumor necrosis factor-α (TNFα) production by recombinant (r) BPI_{23}.

	TNF units/mL		
LPS (ng/mL)	Control	BPI	PB
3	2	0*	0
10	8	0	0
30	20	5	0
500	12	10	5

NOTE. BPI_{23} or polymyxin B (PB), 1 μg/mL. 50% inhibition was obtained with 0.08 units/mL TNFα. Results are average of duplicate supernatants and represent 4 independent experiments.

* Undetectable in 1:5 dilutions of macrophage supernatant (<0.4 units/mL).

NO_2^- release. Twenty-four–hour macrophage supernatants (100 μL) were assayed for NO_2^- by the Griess reaction according to a recently described microassay [34]. Briefly, an equal volume of Griess reagent (1% sulphanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H_3PO_4) was incubated with macrophage supernatants for 10 min at room temperature, and absorbance was measured at 550 nm in a microELISA reader using a 620-nm reference filter. NO_2^- concentration (nmol/well) was determined using $NaNO_2$ as a standard. The SE of replicate determinations was <10%.

Measurement of intracellular parasite killing. After a 24-h incubation in the presence or absence of various activators, macrophages were lysed by exposure to 0.01% SDS as described [32]. The wells were then supplemented with DMEM plus FCS and hemin, and parasite growth was recorded by measuring [^{3}H]-thymidine incorporation [32].

Fluorescence-activated cell sorting analysis of LPS binding. Binding of fluoresceinated LPS was determined as previously described [8]. Macrophages (10^6/mL of RPMI containing 4% bovine serum albumin and 1% NMP) were incubated with 10 or 100 ng of FITC-LPS in the presence or absence of $rBPI_{23}$ for 60 min at 37°C. Cells were then washed twice with cold PBS and resuspended in RPMI (10^6/mL). Macrophage fluorescence was analyzed using FACScan (Becton Dickinson, Mountain View, CA) flow cytometry.

Results

Inhibition of LPS-elicited TNF production by $rBPI_{23}$. Previous studies showed that purified or recombinant BPI inhibits LPS-elicited TNF production by human peripheral blood mononuclear cells or whole blood cultures [18, 20]. We therefore examined the ability of $rBPI_{23}$ to inhibit TNF production by the murine bone marrow–derived cultures to be used in studies of NO induction. As shown in table 1, 1

Table 2. Inhibition of macrophage NO_2^- production by recombinant (r) BPI_{23} is specific for lipopolysaccharide (LPS)-induced activation.

	NO_2^- (nmol/well)	
	Alone	Plus $rBPI_{23}$
1% NMP		
IFN-γ		
3 units/mL + LPS, 10 ng/mL	3.3	0.7
100 units/mL +		
LPS, 1 ng/mL	5.9	0.4
TNFα, 1000 units/mL	2.1	2.0
No NMP		
IFN-γ		
3 units/mL + LPS, 10 ng/mL	0.6	0
100 units/mL +		
LPS, 1 ng/mL	0.4	0
TNFα, 1000 units/mL	2.0	1.9

NOTE. $rBPI_{23}$, 1 μg/mL. Nitrite (NO_2^-) represents mean of triplicate wells (SE, ≤0.1 nmol/well). Interferon-γ (IFN-γ) alone, <0.1 nmol of NO_2^-/well. Data are from a typical experiment and represent 4 independent experiments. NMP, normal mouse plasma; TNFα, tumor necrosis factor-α.

μg/mL $rBPI_{23}$ effectively inhibited TNF secretion induced by 3 or 10 ng/mL LPS in the presence of 1% NMP. This inhibition was overcome at higher LPS concentrations. LPS activity was also inhibited by polymyxin B at 1 μg/mL. TNF was undetectable in control untreated cultures (not shown).

Inhibition of nitrite production in response to IFN-γ and LPS. We then determined if $rBPI_{23}$ could inhibit NO synthesis (measured as NO_2^-) by murine macrophages under conditions similar to those described above for TNF production. Since LPS alone is a relatively poor stimulus for NO_2^- production, macrophages were cultured with LPS plus IFN-γ as a priming signal. NO_2^- release elicited by IFN-γ plus LPS was much higher in the presence of 1% NMP than in the plasma-free cultures (table 2). Increasing the concentration of NMP did not further promote NO_2^- production under these conditions (data not shown). Addition of 1 μg/mL $rBPI_{23}$ effectively inhibited NO_2^- production in the presence or absence of plasma. To ensure that the inhibitory effect of $rBPI_{23}$ was specific, macrophages were alternatively stimulated with IFN-γ plus TNFα. NO_2^- release elicited by IFN-γ plus TNFα was independent of the presence of plasma and was not inhibited by $rBPI_{23}$ (table 2). These results demonstrate that $rBPI_{23}$ did not exert nonspecific toxicity or inhibitory effects on murine bone marrow–derived macrophages resulting in failure to induce NO synthetic capacity. Moreover, no differences were seen in the amount of cell-associated protein in each well at the end of the incubation (not shown), further evidence that $rBPI_{23}$ was not toxic for these cultures.

Figure 1 presents the dose-dependency of the $rBPI_{23}$ effect. For these experiments, macrophages were primed with IFN-γ for 24 h, extensively washed, and then stimulated with LPS in the presence of 1% NMP with or without $rBPI_{23}$. Half-maximal inhibition was obtained with 0.1–0.3 μg/mL $rBPI_{23}$ and nearly complete inhibition with 0.5 μg/mL. With a 10-fold higher concentration of LPS (100 ng/mL), 1 μg/mL $rBPI_{23}$ still inhibited NO_2^- production by >50% (not shown).

Inhibition of NO_2^- production by antibody to LBP. The ability of $rBPI_{23}$ to inhibit macrophage activation by LPS may depend in part upon its capacity to compete with plasma LBP. NMP contains ~2 μg/mL LBP as determined

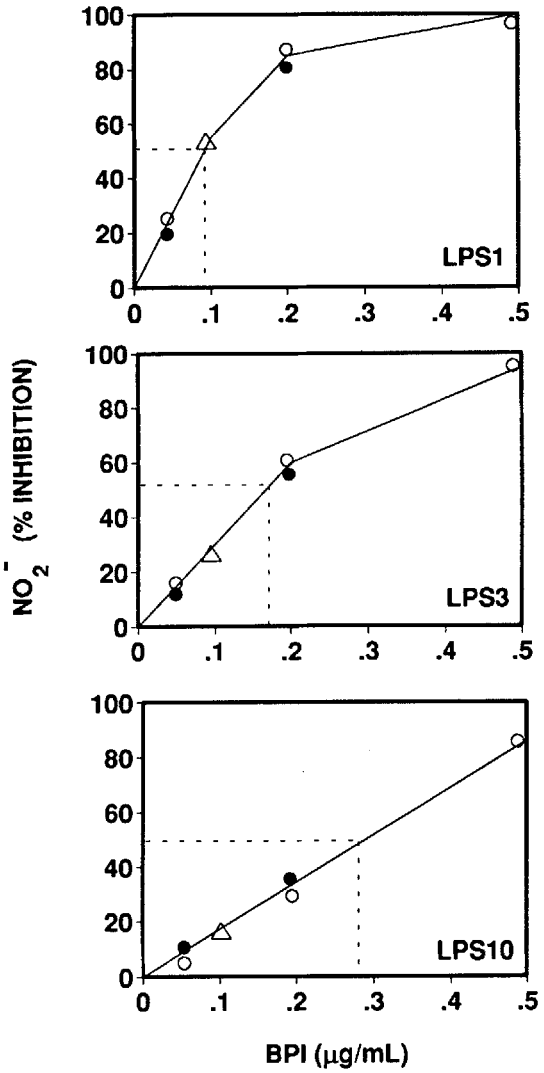

Figure 1. Dose-dependent inhibition by recombinant (r) BPI_{23} of lipopolysaccharide (LPS)-elicited nitrite (NO_2^-) production. Interferon-γ–primed macrophages were washed and stimulated with 1, 3, or 10 ng/mL LPS and 1% normal mouse plasma immediately after addition of various concentrations of $rBPI_{23}$. Each point represents % inhibition of control NO_2^- release in absence of $rBPI_{23}$ calculated as mean of three determinations. Data are from three independent experiments (○, ●, △). Absolute nmol/well NO_2^- release in control cultures (mean of three experiments ± SE): 1 ng/mL LPS, 1.0 ± 0.1; 3 ng/mL LPS, 2.0 ± 0.2; 10 ng/mL LPS, 4.0 ± 0.7.

Table 3. Inhibition of lipopolysaccharide (LPS)-dependent NO_2^- production by anti-LPS-binding protein (LBP) and anti-LPS.

	% inhibition of NO_2^- production			
	LPS 1	LPS 3	LPS 10	TNFα
Anti-LPS	100	ND	89	7
	ND	100	87	0
	100	97	83	0
Anti-LBP	88	27	ND	0
	79	32	ND	0

NOTE. LPS, 1–10 ng/mL; tumor necrosis factor-α (TNFα), 1000 units/mL in the presence of 100 units/mL interferon-γ (IFN-γ); anti-LPS (1:500 dilution), anti-LBP (400 μg IgG/mL). Absolute nmol/well nitrite (NO_2^-) in control cultures (mean ± SE): LPS 1, 0.7 ± 0.1; LPS 3, 2.1 ± 0.2; LPS 10, 3.8 ± 0.1; IFN-γ/TNFα, 1.9 ± 0.3. ND, not determined.

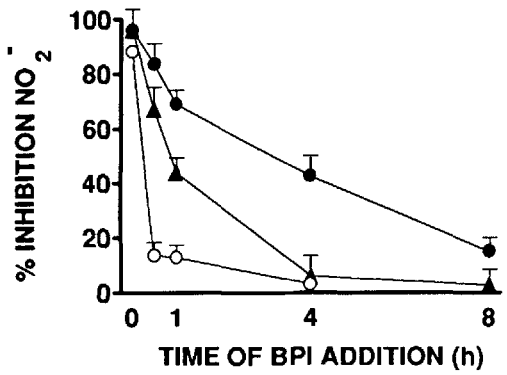

Figure 2. Inhibition of macrophage nitrite (NO_2^-) production by recombinant (r)BPI_{23} added at various times after lipopolysaccharide (LPS) exposure. rBPI_{23} (1 μg/mL) was added to primed macrophages at various times relative to addition of LPS at 1 (●), 3 (▲), or 10 ng/mL (○). Data are % inhibition of NO_2^- production; each point represents mean ± SE of at least three independent experiments. Absolute nmol/well NO_2^- release in control cultures (mean of 3 or 4 experiments ± SE): 1 ng/mL LPS, 0.7 ± 0.1; 3 ng/mL LPS, 2.2 ± 0.3; 10 ng/mL LPS, 3.2 ± 0.2.

by RIA (unpublished data). The recent availability of purified polyclonal antibody directed against murine LBP [11] allowed us to examine its effect on NO_2^- production stimulated by LPS in the presence of 1% NMP using IFN-γ–primed macrophages.

Macrophages were alternatively stimulated with TNFα plus IFN-γ, and rabbit polyclonal antiserum directed against *E. coli* O111 LPS was used as a positive control for inhibition of LPS-dependent stimulation. Anti-LPS (three experiments) inhibited NO_2^- production elicited by LPS but not by IFN-γ/TNFα (table 3). In two experiments, anti-LBP similarly inhibited only LPS-induced NO_2^- release. No inhibition was observed with normal rabbit serum or purified preimmune rabbit IgG (data not shown). These results are consistent with the NMP-dependent enhancement of NO_2^- release as well as with results from a previous study [9], demonstrating that purified rabbit LBP enhances NO_2^- production by LPS-stimulated murine macrophages.

Inhibition of LPS-mediated NO_2^- production as a function of time of rBPI_{23} addition. The effect of time of rBPI_{23} addition on its inhibition of NO production was examined using IFN-γ–primed macrophages. rBPI_{23} strongly inhibited NO_2^- release when added up to 1 h after 1 or 3 ng/mL LPS plus 1% NMP (figure 2). Moreover, rBPI_{23} consistently inhibited the response to 1 ng/mL LPS even when added as late as 4 h. Induction of NO synthesis appeared to be an even slower process in the absence of plasma since inhibition of 3 ng/mL LPS was obtained when rBPI_{23} was added 8 h after LPS (data not shown). When the concentration of LPS was increased to 10 ng/mL (1% NMP), rBPI_{23} did not inhibit NO_2^- release when added 30 min after stimulation (figure 2). We therefore examined the effects of delaying the addition of inhibitor for shorter periods. In three experiments, addition of rBPI_{23} 10 min after 10 ng/mL LPS inhibited NO_2^- release by 40.7% ± 4.2% (mean ± SE).

Inhibition of LPS binding to murine macrophages by rBPI_{23}. We previously reported that purified LBP or acute-phase rabbit serum enhanced the binding of 1 μg/mL FITC-LPS to bone marrow–derived macrophages [9]. Furthermore, anti-LBP inhibited LPS binding in the presence of rabbit or murine sera [9, 11]. Since NO_2^- production is induced by much lower levels of LPS than those used in the binding experiments, we have now examined the binding of 10 or 100 ng/mL FITC-LPS to bone marrow–derived macrophages using 1% NMP. In addition, primed and unprimed macrophages were compared since both populations were used to study the effects of rBPI_{23} on NO production, and it was possible that IFN-γ pretreatment might modify the expression of macrophage CD14 or other putative LPS receptor molecules. As shown in table 4, 1 μg/mL rBPI_{23} strongly inhibited binding at either LPS concentration. Furthermore, virtually no differences were detectable between the primed and unprimed populations. Inasmuch as low but reproducible binding of 10 ng/mL LPS was detectable in these experi-

Table 4. Recombinant (r) BPI_{23} inhibition of lipopolysaccharide (LPS) binding by primed or unprimed murine macrophages.

	Mean fluorescence (arbitrary units)	
Macrophage status, LPS concentration (ng/mL)	Without rBPI_{23}	With rBPI_{23}
Unprimed		
10	5.2	2.6
100	17.6	3.5
Primed		
10	5.2	2.6
100	13.2	3.3

NOTE. Binding in 4% albumin (negative control) was 2.5–2.6 fluorescence units.

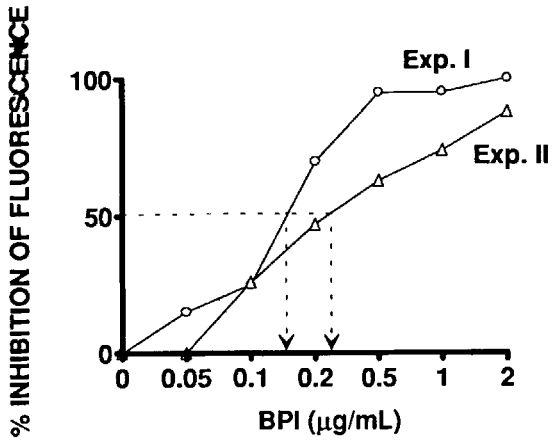

Figure 3. Dose-dependent inhibition of lipopolysaccharide (LPS) binding to murine macrophages by recombinant (r)BPI_{23}. Interferon-γ–primed macrophages (10 units/mL, 24 h) were incubated with 10 ng/mL fluorescein isothiocyanate–LPS (FITC-LPS) and 1% normal mouse plasma with or without increasing concentrations of $rBPI_{23}$ for 1 h at 37°C. LPS binding measured as FITC-LPS fluorescence is presented as % of control binding in absence of $rBPI_{23}$. Mean fluorescence (arbitrary units) of 10,000 cells in control: experiment (exp) I (○), 4.8; exp II (△), 4.7.

ments, we could directly compare the capacity of $rBPI_{23}$ to inhibit binding with its effect on NO_2^- release. When binding of 10 ng/mL FITC-LPS was examined in the presence of 1% NMP (figure 3), half-maximal inhibition of macrophage fluorescence was obtained with ~0.2 μg/mL $rBPI_{23}$ (i.e., concentrations similar to those for NO_2^- production). To further examine the relationship between LPS binding and NO production, $rBPI_{23}$ was added 10 min after 10 ng/mL FITC-LPS. In three experiments, delaying the addition of $rBPI_{23}$ decreased the inhibition of macrophage fluorescence from 95.3% ± 3.8% ($rBPI_{23}$ at time 0) to 29.0% ± 4.4% ($rBPI_{23}$ at 10 min).

Inhibition of intracellular leishmanicidal activity. Since $rBPI_{23}$ could inhibit the induction of NO_2^- release, it was of interest to examine its effects on an in vitro system in which NO is known to have biologic activity. An excellent correlation exists between murine macrophage activation for NO production and the ability to kill intracellular *Leishmania* species [35–37]. We therefore examined the effects of $rBPI_{23}$ on leishmanicidal activity stimulated by LPS. Macrophages infected with *Leishmania* species 24 h before stimulation generally produce higher levels of NO_2^- than do uninfected cells (unpublished data). Addition of IFN-γ plus LPS or IFN-γ plus TNFα induced NO_2^- secretion and leishmanicidal activity (table 5). In cultures stimulated with IFN-γ and LPS, BPI inhibited NO_2^- production and abolished intracellular killing. $rBPI_{23}$ did not inhibit cytotoxic activity nonspecifically since intracellular killing induced by IFN-γ plus TNFα was unaffected by $rBPI_{23}$. Furthermore, the ability of control macrophages (DMEM or IFN-γ alone) to support *Leishmania* infection in the presence of $rBPI_{23}$ is further evidence for the lack of toxicity of this molecule under the in vitro conditions used in this study.

Discussion

Production of a large array of cytokines and inflammatory mediators by LPS-activated macrophages undoubtedly contributes to the pathologic manifestations of gram-negative sepsis. In particular, current evidence suggests that rapid release of TNFα in response to picogram concentrations of LPS is responsible for many of the clinical symptoms associated with lethal endotoxemia [38]. However, high levels of serum TNF are not necessarily associated with mortality [39]. Circulating levels of IFN-γ are also detectable in patients and in experimental models of endotoxic shock and, recently, we [40] and others [41, 42] have demonstrated that monoclonal antibodies to IFN-γ can significantly decrease lethality in vivo.

As previously shown for macrophage cytolytic activity, efficient induction of NO production by either LPS or TNFα requires costimulation by IFN-γ [26, 34]. Although it has been suggested that induction of the Ca^{2+}-independent NO synthase pathway is important in the pathogenesis of endotoxic shock, recent attempts to correlate toxicity of bacterial products with enzyme induction in vivo have been unsuccessful [43, 44]. Since in vivo models designed to test the

Table 5. Recombinant (r) BPI_{23} inhibits intracellular leishmanicidal activity of bone marrow–derived macrophages stimulated by interferon (IFN)-γ plus lipopolysaccharide (LPS).

Experiment no., conditions	$rBPI_{23}$	Parasite survival (cpm ± SE)	NO_2^- (nmol/well)
Experiment I			
DMEM	−	53,646 ± 827	0.1
	+	55,066 ± 130	0.1
IFN-γ, 10 units/mL +	−	61,392 ± 2652	0.4
	+	57,426 ± 573	0.2
LPS, 1 ng/mL	−	374 ± 95	4.3
	+	58,953 ± 943	0.2
LPS, 3 ng/mL	−	498 ± 142	6.5
	+	57,060 ± 40	0.8
Experiment II			
DMEM	−	39,222 ± 725	0
	+	42,940 ± 522	0
IFN-γ, 100 units/mL	−	36,988 ± 717	0
	+	42,623 ± 630	0
IFN-γ, 10 units/mL + LPS,	−	70 ± 5	6.5
3 ng/mL	+	47,351 ± 992	0.2
IFN-γ, 100 units/mL + TNFα,	−	5456 ± 176	2.4
1000 units/mL	+	2647 ± 302	2.5

NOTE. Data are from 2 independent experiments and represent 5 experiments. DMEM, Dulbecco's MEM; IFN-γ, interferon-γ; TNFα, tumor necrosis factor-α. $rBPI_{23}$, 1 μg/mL. Nitrite (NO_2^-), SE ≤0.3 nmol/well (n = 3).

efficiency of BPI in inhibiting the lethal consequences of endotoxic shock should consider possible effects on NO production, we first assessed the capacity of $rBPI_{23}$ to inhibit NO_2^- release by macrophages stimulated in vitro by IFN-γ plus LPS or by IFN-γ plus TNFα.

Preliminary studies on LPS-elicited TNF secretion by bone marrow–derived macrophages demonstrated that $rBPI_{23}$ inhibited release of this mediator at concentrations equivalent to those previously reported by Marra et al. [20] for human monocytes. In those studies, LPS was preincubated with purified BPI. In our experiments, LPS was added to cell cultures containing $rBPI_{23}$ and 1% NMP was immediately added. Using a similar protocol with IFN-γ–primed macrophages, we found that $rBPI_{23}$ inhibited LPS-triggered NO production with an approximate IC_{50} of 0.1–0.3 μg/mL (4–12 n*M*). These concentrations are nearly identical to those reported by Ooi et al. [18] for the inhibition by an NH_2-terminal BPI fragment of TNF secretion in whole blood elicited by 1 ng/mL LPS. Furthermore, on a molar basis, $rBPI_{23}$ was at least as effective as polymyxin B (data not shown), in agreement with results of Marra et al. [20] on effects of polymyxin B and soluble BPI on in vivo induction of TNFα secretion. When we later examined the effect of $rBPI_{23}$ on macrophage binding of FITC-LPS, half-maximal inhibition was observed at concentrations similar to those obtained for inhibition of NO production. These concentrations are also similar to the reported K_d of $rBPI_{23}$ for various LPS [21].

As shown in control experiments, NO_2^- production elicited by IFN-γ plus TNFα is unaffected by $rBPI_{23}$, demonstrating specificity for LPS-activated macrophages. Of note, under the conditions used in these studies (i.e., low IFN-γ and LPS concentrations and assay of NO_2^- at 24 h), TNF is not an important cofactor for LPS-induced NO production. The amount of bone marrow macrophage TNF secreted is much less than the concentrations used here to trigger NO production (these studies and [9]). Furthermore, antibody to murine TNFα did not inhibit NO_2^- production by cultures stimulated with IFN-γ plus LPS [45].

Interestingly, $rBPI_{23}$ inhibited NO_2^- production when added several hours after LPS. Similar results were obtained with polymyxin B or rabbit anti-O111 LPS (not shown). Elicitation of NO production is a relatively slow process, with NO_2^- release first detectable 4–8 h after stimulation. Our results demonstrate that induction occurs more quickly at 10 ng/mL LPS than at a 10-fold lower concentration, suggesting that low concentrations of LPS stimulate certain macrophage responses such as NO synthase activity by a slow accumulation of multiple signal-transducing events. The ability of $rBPI_{23}$ to inhibit NO production when added after LPS contrasts with our previous results on inhibition of TNF production by human monocytes [23]. It remains to be seen whether LPS recognition and signal-transduction events leading to NO production and TNFα secretion are significantly different.

Alternatively, plasma or cellular components involved in LPS-dependent macrophage activation may vary between the human and murine systems. As shown here, antibody to murine plasma LBP inhibited NO_2^- release, consistent with our previous results using purified LBP [9]. However, it is also clear that NO production elicited by higher LPS concentrations can occur in the absence of plasma or plasma LBP. Furthermore, $rBPI_{23}$ inhibits NO_2^- release in the presence or absence of NMP, and thus of LBP.

$rBPI_{23}$ also inhibited macrophage leishmanicidal activity induced by IFN-γ plus LPS but not by IFN-γ plus TNFα. These experiments underline the specificity of $rBPI_{23}$ for LPS and provide a sensitive in vitro model to demonstrate lack of toxicity for macrophages since control unstimulated cells support an intracellular microbial infection equally well in the presence or absence of $rBPI_{23}$.

Taken together, these results suggest that $rBPI_{23}$ might be an effective inhibitor of NO production in vivo where concentrations of LPS rarely exceed picogram per milliliter levels [46, 47]. Moreover, inhibition of LPS-elicited TNF production by $rBPI_{23}$ could provide a further level of control of inducible NO synthase activity in gram-negative shock. The relative role of this pathway in patients or in murine models of endotoxemia remains a controversial issue that deserves further study.

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