Lipopolysaccharide (LPS) Recognition in Macrophages

Participation of LPS-binding Protein and CD14 in LPS-induced Adaptation in Rabbit Peritoneal Exudate Macrophages

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Abstract

Exposure of rabbit peritoneal exudate macrophages (PEM) or whole blood to picomolar concentrations of LPS induces adaptation or hyporesponsiveness to LPS. Because of the importance of plasma LPS-binding protein (LBP) and the macrophage cell membrane protein CD14 in recognition of LPS, we examined the effect of LBP on LPS-induced adaptation in PEM. PEM exposed to LPS in the presence of LBP for 8 h were markedly less responsive to subsequent stimulation by LPS than monocytes/macrophages (MΦ) adapted in the absence of LBP. LPS-induced expression of TNF was sharply reduced in LBP–LPS-adapted PEM, but in contrast these cells remained fully responsive to *Staphylococcus aureus* peptidoglycan. We considered that specific hyporesponsiveness in LPS-adapted MΦ or in blood monocytes could be due to decreased expression of CD14 or diminished binding of LBP–LPS complexes to CD14. However, flow cytometry analysis revealed only minimal reduction of CD14 expression or CD14-dependent binding of a fluorescent LPS derivative when normo- and hyporesponsive cells were compared. These results show that complexes of LPS and LBP are more effective than LPS alone in inducing adaptation to LPS, and LPS-induced hyporesponsiveness probably results from changes in cellular elements distinct from CD14 that are involved in either LPS recognition or LPS-specific signal transduction. (*J. Clin. Invest.* 1993. 92:2053–2059.) Key words: LPS • adaptation • LBP • CD14 • macrophage

Introduction

Complications of Gram-negative sepsis include hypotension, disseminated intravascular coagulation, multiple organ failure, and death. A similar set of changes occur in experimental animals after intravenous injection of Gram-negative endotoxin (LPS) (1–5). Monocytic/macrophages (MΦ)[1] play a central role in determining the host response during Gram-negative infection and are stimulated by picomolar concentrations of LPS to undergo phenotypic changes and to secrete a variety of mediators (6–8). In particular, cytokine production has been shown to play an important role in host defense during sepsis (2, 9–14). Unfortunately, cytokine release may also lead to injury (3, 15–17). Thus, regulation of MΦ response to LPS is critical for host survival during Gram-negative sepsis.

In previous studies we observed that after sequential intravenous injections of LPS spaced 5 h apart in rabbits, serum TNF levels were elevated only after the first injection (3). This phenomenon, known as adaptation or LPS tolerance, has also been observed in other species and in humans (18). To determine mechanisms for LPS-induced hyporesponsiveness we established an in vitro model using elicited rabbit peritoneal exudate macrophages (PEM) or MΦ-like cell lines and showed that LPS-specific desensitization or adaptation was observed after a 6-h exposure of MΦ to picomolar concentrations of LPS (19, 20). Because LPS-adapted MΦ released normal levels of cytokine in response to alternative stimuli such as heat-killed *Staphylococcus aureus,* we reasoned that LPS hyporesponsiveness must result from changes in cell surface receptors that specifically recognize LPS or alterations in LPS-specific signal transduction pathways (20).

Recent studies from our laboratory have provided new information about mechanisms of LPS recognition. In blood, a normal plasma protein, LPS-binding protein (LBP), binds with high affinity to LPS via lipid A, and this LPS–LBP complex binds to CD14, which is present on cell surfaces of MΦ (21–25). Although MΦ are responsive to LPS in serum-free medium, in the presence of LBP, MΦ respond to orders of magnitude lower concentrations of LPS with accelerated induction of cytokine message. These changes depend upon binding of LBP–LPS complexes to CD14, suggesting the importance of the LBP-CD14 pathway under physiologic conditions (26, 27). Here we describe studies that examine the role of LBP and CD14 in LPS-induced adaptation. We do this by comparing the properties of MΦ treated with picomolar concentrations of LPS in the presence or absence of LBP and by using flow cytometry to quantitate expression of CD14 and the ability of CD14 to bind complexes of LBP and LPS in normo- and hyporesponsive MΦ.

Methods

Animals. Outbred NZW rabbits (male, 1.8–2.2 kg) were obtained from Western Oregon Rabbit Co. (Philomath, OR) and maintained on a standard pelleted diet for 7–14 d before use. All protocols used in these studies were approved by the Animal Research Committee of The Scripps Research Institute.

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1. *Abbreviations used in this paper:* HKSA, heat-killed *Staphylococcus aureus;* LBP, lipopolysaccharide-binding protein; MΦ, monocyte/macrophage; PEM, peritonial exudate macrophage; PGN, peptidoglycan from *S. aureus.*

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Macrophage activators. Salmonella minnesota Re595 LPS was prepared as described previously (3). FITC-labeled Re595 LPS was prepared as described (28). The molar fluorescein-to-LPS monomer ratio was 0.5:1. A clinical isolate of *S. aureus*, provided by Dr. Theo Kirkland (VA Hospital, San Diego, CA) was grown in trypticase soy broth for 8 h, washed in saline, heated to 100°C for 3 h, resuspended in saline to 10^{11} cells/ml, and stored at 4°C. *S. aureus* cell wall peptidoglycan was a gift from Dr. Roman Dziarski (Indiana University School of Medicine, Gary, IN) (29). Heat-killed *S. aureus* (HKSA) and *S. aureus* peptidoglycan (PGN) induce TNF release in PEM or whole blood independently of the LBP-CD14 pathway of LPS stimulation (J. Mathison, unpublished observation). Stock solutions of LPS (5 mg/ml) or PGN (7.5 mg/ml) were stored at −20°C. Immediately before use, LPS and PGN were thawed and sonicated using a microsonicator (Microson; Heat Systems-Ultrasonics, Farmingdale, NY) and working dilutions were prepared using 10 mM Hepes-buffered sterile, pyrogen-free saline (0.9% sodium chloride irrigation, USP; Travenol Laboratories, Deerfield, IL).

LPS-binding protein. LBP was purified from acute-phase rabbit serum as previously described (24). Working stocks (0.2–0.5 mg/ml) were prepared in 50 mM Hepes and stored at −20°C.

Peritoneal exudate macrophages. Media and solutions for PEM culture were prepared with sterile, tissue culture–grade plasticware or glassware that was acid cleaned and baked overnight at 200°C to inactivate endotoxin. RPMI was obtained as powdered cell culture medium (BioWhittaker, Inc., Walkersville, MD), dissolved in sterile nonpyrogenic water (Travenol Laboratories), and supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM Hepes. Elicited PEM were prepared and cultured in serum-free medium as described previously (3).

Cytolytic assay for TNF. TNF was measured using a cytolytic assay with actinomycin D (1 μg/ml)-treated L929 cells or WEHI clone 13 cells as described elsewhere (3, 30). In this assay 1 U of activity is defined as the amount of TNF producing 50% lysis in 96-well plates containing 5×10^4 cells/well. Each plate included TNF standard (conditioned medium from LPS-treated RAW 264.7 cells, 5×10^4 U/ml), which was calibrated using human recombinant (r)TNF standard obtained from the National Institute for Biological Standards and Control (Hertfordshire, England). The coefficient of variation (SD/mean) for the assay was 0.1–0.15. Supernatants from MΦ cultures were assayed in duplicate, and all experiments were replicated at least three times.

Measurement of CD14 expression and LBP-dependent binding of FITC-LPS. Adherent MΦ cultured in 75-cm² flasks containing serum-free medium were exposed to LPS in the presence or absence of LBP for 8 h at 37°C and recovered by scraping and resuspension in HBSS (phenol red free; Sigma Chemical Co., St. Louis, MO) containing 10 mM Hepes.

Aliquots of PEM (10^6 cells in 100 μl) were exposed for 30 min at 0°C to anti–human CD14 mAb (FITC-MY4, or the isotopic control FITC-MsIgG2b; Coulter Immunology, Hialeah, FL), goat anti–rabbit CD14 mAb (IgG fraction isolated from serum of goats immunized with purified recombinant rabbit CD14 and provided by V. Kravchenko and J. D. Lee in our laboratory), or anti–human CD18 mAb (IB4; a gift from Dr. K. E. Arfors; Pharmacia Experimental Medicine, La Jolla, CA) isotype control IgG2a (anti–human chorionic gonadotropin α subunit; Biodesign, Pasadena, CA) or control (nonimmune) goat IgG. The MΦ were then washed twice and resuspended in 0.5 ml HBSS containing FITC conjugate (either mouse anti–goat IgG; Accurate Chemical & Scientific Co.) or goat anti–mouse IgG (Gibco Laboratories, Grand Island, NY). After 30 min propidium iodide was added (2 μg/ml final concentration, and the fluorescence was quantitated using a FACScan® flow cytometer with the LYSIS II software (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Quantitation of LBP-dependent binding of LPS to MΦ was performed at room temperature (21–23°C) using 0.5-ml aliquots of MΦ (10^6 cells/ml in HBSS containing 0.05% sodium azide and 0.1% BSA, fraction V; Sigma Chemical Co., St. Louis, MO). Nonimmune goat IgG (250 μg/ml final concentration) was added, followed 5 min later by saline or goat anti-CD14 IgG. After 30 min, LBP (0.5 μg/ml) or saline was added, followed immediately by FITC-LPS (30 ng/ml) for 30 min. Propidium iodide (2 μg/ml) was added to the samples 5 min before analysis using the FACScan® flow cytometer, and propidium iodide–stained (nonviable) MΦ were excluded from analysis.

Data are expressed as mean ± SE where three or more experiments are reported. Statistical significance of LBP-dependent binding of FITC-LPS to MΦ was evaluated using a two-sample *t* test, and analysis of variance was used to evaluate levels of expression of CD14, CD18, and control IgG binding in resting and adapted MΦ.

RNA preparation and Northern blotting analysis. PEM in 75-cm² flasks (2×10^7 PEM, 10 ml serum-free medium) were dosed with LPS±LBP, and at 0, 0.75, 1.5, 4.5, or 10 h the conditioned medium was removed, and total cellular RNA was prepared for Northern blot analysis of TNF as described previously (20).

Ex vivo adaptation in whole blood. Rabbit blood was collected by cardiac puncture, anticoagulated with 10 U/ml heparin (Heparin sodium injection, [USP], 5,000 U/ml; Lyphomed, Deerfield, IL), and incubated 10 h at 37°C with gentle mixing in the presence or absence of LPS. The blood cells were then washed three times (400 *g*) using serum-free RPMI 1640 (described above) resuspended in 50% plasma, challenged with LPS or HKSA for 4 h, followed by measurement of TNF cytolytic activity in the supernatant using the WEHI clone 13 assay. CD14 expression in resting and adapted blood cells was evaluated in mononuclear cells prepared using Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) or in whole blood cells prepared by ammonium chloride lysis of red blood cells (1 part blood/14 parts 140 mM NH_4Cl, 17 mM Tris, pH 7.2; 5 min at room temperature) followed by resuspension in HBSS. The cell preparations were stained with FITC-labeled antibodies as described above, and live gating was used in the cell sorter to analyze antigen expression in monocytes or neutrophils.

Results

A previous report from our laboratory described an in vitro model of LPS-induced adaptation where rabbit PEM were exposed to a primary dose of LPS followed by washing and addition of a second, challenge dose of LPS or alternative agonist (20). One of our criteria for adaptation derived from the initial studies is marked reduction in TNF mRNA in MΦ treated with the primary LPS alone. In the present studies we exposed cells to a primary LPS dose in the presence and absence of LBP and then compared the kinetics of the TNF mRNA response to the challenge LPS dose in the presence or absence of LBP. As shown in Fig. 1, when resting (unadapted) MΦ were exposed LPS in serum-free medium TNF steady-state mRNA levels were increased 1.5 and 4.5 h postchallenge. However, when LBP was added to the cultures immediately before LPS challenge, the kinetics of TNF mRNA expression were accelerated with elevated steady-state levels observed at 0.75 and 1.5 h followed by substantially reduced levels at 4.5 h. When MΦ were pretreated with LPS for 8 h, washed, and challenged with LPS, TNF mRNA steady-state levels were markedly reduced compared with the response in resting MΦ (Fig. 1). Addition of LBP to the culture medium during the adapting (primary dose) and challenge phase of this experiment led to hyporesponsiveness to LPS that was even more pronounced than when the MΦ were adapted in the absence of LBP (Fig. 1). When MΦ were adapted to LPS in the absence of LBP followed by LPS challenge in the presence of LBP, TNF mRNA expression was accelerated and partially restored, whereas TNF mRNA expression was undetectable in MΦ adapted in the pres-

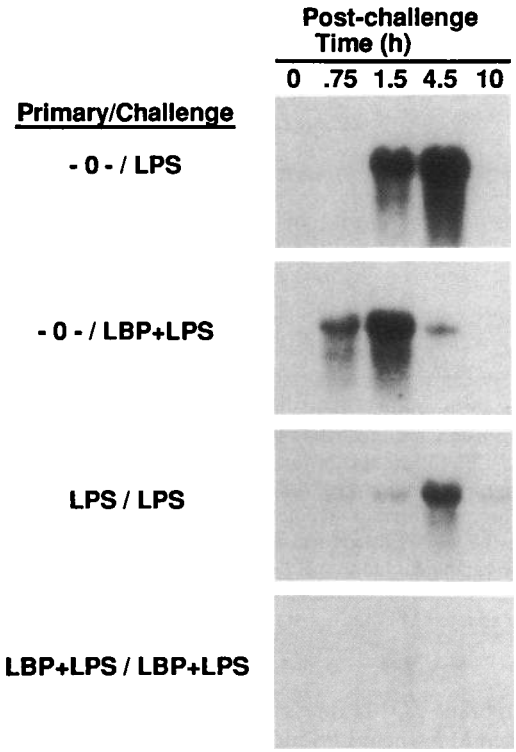

Figure 1. TNFα expression in PEM that were adapted to LPS and challenged with LPS in the presence and absence of LBP. After an 8-h exposure to serum-free medium in the presence or absence of LPS (100 pg/ml) and LBP (0.1 μg/ml), the cells were washed, replenished with serum-free medium, and challenged with LPS (300 pg/ml) in the presence or absence of LBP. At various times after LPS challenge, total cellular RNA was prepared and evaluated by Northern blot analysis using a synthetic cDNA probe for rabbit TNFα.

ence of LBP and challenged with LPS alone (data not shown). These results demonstrate that complexes of LPS and LBP are more effective than LPS alone at inducing hyporesponsiveness as assessed by levels of LPS-stimulated TNF mRNA.

We next examined release of TNF cytolytic activity in PEM that were cultured in serum-free medium, adapted, and challenged with LPS in the presence or absence of LBP. As shown in Table I, when resting MΦ were challenged with LPS in the absence of LBP, TNF release was 7,000 U/ml. MΦ pretreated with LPS in the absence of LBP were hyporesponsive to LPS challenge (840 U/ml); however, inclusion of LBP with the challenge LPS partially restored responsiveness (7,200 U/ml). In contrast, when MΦ were exposed to 100 pg LPS in the presence of LBP, the cells were hyporesponsive to LPS challenge, even in the presence of LBP. Thus, LBP partially restored LPS responsiveness to LPS-adapted MΦ only when the cells were adapted in the absence of LBP. MΦ exposed to 100 ng LPS for 8 h (±LBP) were uniformly unresponsive to LPS challenge. This LPS-induced hyporesponsiveness to LPS, induced in the presence or absence of LBP, is specific for LPS because in all cases the MΦ remained fully responsive to PGN.

In previous studies we demonstrated that TNF mRNA half-life was not changed in LPS-adapted MΦ (20). Moreover, we showed that LPS-adapted MΦ remain fully responsive to alternative stimuli, including heat-killed *S. aureus*. These results indicate that the pathways required for TNF transcription and release of mature protein remain intact in LPS-adapted MΦ. Thus, likely mechanisms for the reduced responsiveness of these cells to LPS include defective recognition of LPS or deficiency in LPS-specific signal transduction pathways. Because of the importance of CD14 in cellular recognition of LPS, we considered the possibility that specific hyporesponsiveness to LPS could be due to reduced expression of CD14 or to a defect in binding of LBP–LPS to cell surface C14.

To evaluate expression of CD14 and also specific binding of LBP–LPS to CD14 in resting and adapted MΦ, PEM were cultured in serum-free medium with two different primary LPS doses in the presence or absence of LBP. After 8 h the MΦ were resuspended and processed for flow cytometry as described in Methods. Specifically, aliquots of the PEM were stained with antibodies to quantitate surface expression of CD14, and in addition, LBP-dependent binding of FITC-LPS was measured in the presence or absence of excess blocking anti-CD14 antibody. As shown in Fig. 2*A* and Table II, CD14 expression was somewhat increased over control levels when MΦ were exposed to 100 pg LPS in serum-free medium for 8 h. However, exposure to 100 pg LPS in the presence of LBP resulted in CD14 expression slightly below control levels. Exposure to 100

Table I. Effect of LBP on LPS-induced Adaptation in PEM

	TNF release			
			Challenge supernatant* (20 h)	
Primary LPS	Primary supernatant (8 h)	Challenge (LPS or PGN)	LPS	PGN
	U/ml		*U/ml*	
0	<30		7,000	7,100
0	<30	+LBP	21,000	
100 pg	1,300		840	9,700
100 pg	1,300	+LBP	7,200	
LBP + 100 pg	6,500		350	7,300
LBP + 100 pg	6,500	+LBP	1,400	
100 ng	11,000		<30	9,600
100 ng	11,000	+LBP	150	
LBP + 100 ng	6,400		40	8,800
LBP + 100 ng	6,400	+LBP	200	

PEM were cultured in serum-free medium±LBP and LPS for 8 h followed by washing and challenge with 300 pg LPS in the presence or absence of LBP. Some of the PEM were stimulated with *S. aureus* PGN (1 μg/ml) as an alternative challenge. Conditioned medium was harvested at 8 h (primary supernatant) and 20 h (12 h after addition of LPS). TNF cytolytic activity was measured using the L929 cell assay as described in Methods. Residual TNF release from unchallenged MΦ (i.e., MΦ that were exposed to LPS±LBP for 8 h, washed, and incubated for 12 h in serum-free medium without LPS or LBP) was subtracted from the total TNF observed in supernatants of MΦ challenged with LPS±LBP. Residual release (TNF; U/ml) was < 30 for unstimulated and 100 pg LPS-stimulated MΦ, 360 for LBP + 100 pg LPS-stimulated cells, 2,700 for 100 ng LPS-stimulated cells, and 2,200 for 100 ng LPS + LBP–stimulated MΦ. PGN-induced TNF release was not affected by LBP.

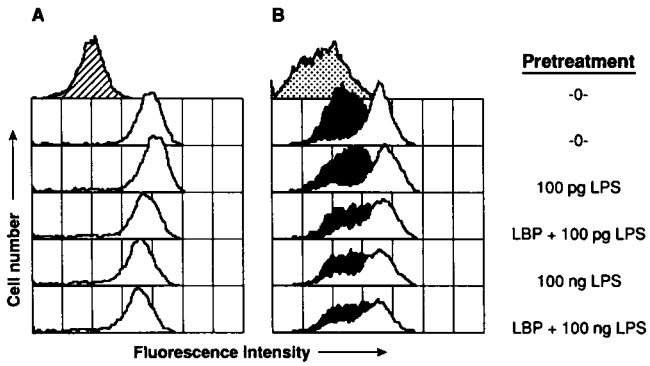

Figure 2. CD14 expression (*A*) and binding of FITC-LPS (*B*) in PEM that were adapted to LPS in the presence or absence of LBP (0.1 μg/ml). After an 8-h pretreatment, the MΦ were resuspended and labeled with FITC antibody or FITC-LPS and evaluated by flow cytometry as described in Methods. (*A*) Cross-hatched tracing represents cells stained with control (nonimmune IgG); open tracings represent cells stained with anti-CD14. (*B*) Stippled tracing represents cells stained with FITC-LPS in the absence of LBP; filled tracings represent cells blocked with anti-CD14 followed by staining with FITC-LPS in the presence of LBP; open tracings represents cells stained with FITC-LPS in the presence of LBP.

ng LPS in the presence or absence of LBP resulted in even lower CD14 expression (75% of control levels). In these same cells expression of CD18 was increased above control levels after exposure to LPS ± LBP. Because of the modest changes in CD14 expression these results suggested that LPS hyporesponsiveness in LPS-adapted MΦ was not due simply to decreased expression of CD14. Using specific cDNA probes for rabbit CD14 in Northern blotting analysis, we have not observed any changes in steady-state levels of CD14 mRNA in resting and adapted MΦ (data not shown).

Table II. Effect of LPS-induced Adaptation on CD14 Expression in PEM

	Mean channel number (% of unadapted cells)		
Pretreatment	CD14	CD18	Control IgG
0	100	100	100
100 pg	114±6	146±6	109±4
LBP + 100 pg	85±8	139±8	108±4
100 ng	75±6	128±12	103±2
LBP + 100 ng	76±9	140±13	113±4

PEM were exposed to serum-free medium or LPS±LBP for 8 h followed by resuspension and quantitation of CD14 (MY4 mAb) and CD18 (IB4 mAb) using flow cytometry. Comparable fluorescence for CD14 was observed when cells were stained with IgG fraction of goat anti-rabbit CD14 antiserum. Fluorescent mean channel numbers for control (unadapted) MΦ were: 14±2 (control IgG); 79±9 (CD14); and 87±18 (CD18); percent change was calculated from the mean channel number of the unadapted MΦ and expressed as mean percent±SE. Mean channel numbers for CD14 and CD18 were significantly increased over the control IgG ($P < 0.05$); the decrease in CD14 channel number in adapted MΦ was not statistically significant.

Table III. Effect of LPS-induced Adaptation on FITC-LPS Binding in PEM

	Mean channel number (% of unadapted cells)	
Pretreatment	FITC-LPS	LBP + FITC-LPS
0	19 (100)	75 (100)
100 pg	19 (102±6)	76 (102±9)
LBP + 100 pg	18 (95±8)	70 (93±7)
100 ng	18 (97±9)	58 (78±4)
LBP + 100 ng	19 (102±8)	57 (76±2)

PEM were exposed to serum-free medium ±LBP±LPS for 8 h followed by resuspension and incubation at room temperature for 30 min with FITC-LPS (30 ng/ml) in the presence or absence of LBP (0.5 μg/ml). Samples were analyzed by flow cytometry as described in Methods. Fluorescent mean channel number for control MΦ (100%) was 19±0.5 for FITC-LPS and 75±10 for LBP + FITC-LPS; $n = 3$ (SE). LBP-dependent binding of LPS was significantly increased over LPS alone, $P < 0.05$; the decreased channel number for binding of LBP–LPS to adapted MΦ was not statistically significant.

Thus, membrane CD14 expression did not change substantially in adapted MΦ where LPS-induced TNF release was decreased >90%. Also, we considered the possibility that antigenically detectable CD14 remained on the cell surface, but that these molecules have altered binding properties for LPS. To evaluate binding of LPS to resting and adapted MΦ, we used FITC-labeled LPS and flow cytometry to quantitate binding of LPS to PEM in the presence or absence of LBP. In three separate experiments we observed markedly enhanced binding of FITC-LPS to PEM in the presence of LBP (Fig. 2 and Table III). LBP-dependent binding of FITC-LPS was prevented by pretreatment of cells with anti-CD14 antibody (MY4). When LBP-dependent binding of FITC-LPS was compared in resting and adapted PEM, the binding in cells adapted to 100 pg LPS/ml was not changed compared to control levels, and binding was only moderately decreased in 100 ng adapted PEM (Fig. 2 and Table III).

In previous studies we observed that when sequential intravenous injections of LPS, spaced 5 h apart, were made in rabbits, serum TNF levels were elevated only after the first injection. LPS injection in this model leads to acute leukopenia; thus, monocytes and neutrophils, which express CD14 and are LPS responsive, are absent from the peripheral circulation after the first LPS injection. Therefore, to evaluate phenotypic changes in peripheral blood cells we used a model of ex vivo tolerance in which heparinized blood was exposed to primary LPS for 10 h followed by washing, resuspension in 50% plasma, and challenge with LPS. As shown in Fig. 3, hyporesponsiveness was induced by as little as 3 pg LPS/ml, and adaptation was more pronounced after exposure to 10–100 pg LPS/ml. We also evaluated CD14 expression in blood cells after a 10-h exposure to LPS, and as shown in Fig. 4 and Table IV, CD14 expression in monocytes and neutrophils was not changed. 70% of the cells included in the monocyte gate were CD14 positive, whereas 90% were CD18 positive. Comparable results were observed in three separate experiments with rabbit blood and also with human blood exposed to as much as 10 ng LPS/

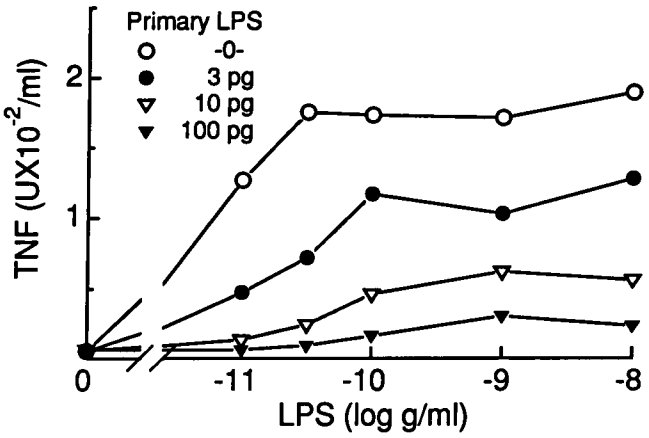

Figure 3. LPS-induced adaptation in rabbit blood ex vivo. Heparinized blood was exposed to various doses of LPS 10 h at 37°C followed by washing, resuspension in RPMI 1640 containing 50% autologous plasma, and challenge with LPS for 4 h. TNF cytolytic was measured using WEHI clone 13 cells.

Table IV. Effect of Ex Vivo LPS-induced Adaptation in Whole Blood on CD14 Expression in Monocytes and Neutrophils

	Mean channel number			
	Monocytes		Neutrophils	
Antibody	-0-	LPS	-0-	LPS
Anti-CD14	617	601	57	55
Control IgG2b	138	158	16	20
Anti-CD18	182	204	44	23
Control IgG2a	8.4	9.8	6.1	5.2

Rabbit blood anticoagulated with 10 U/ml heparin was incubated for 10 h at 37°C in the presence or absence of 10 pg LPS/ml, and mononuclear cells were isolated by centrifugation over Ficoll-Paque, whole blood leukocytes were prepared by ammonium chloride lysis of red blood cells, and the cells were processed for flow cytometry as described in Methods. Live gating was used to select monocytes in the mononuclear cell preparation and also to select neutrophils in the whole blood leukocyte preparation.

ml (S. Steinemann, unpublished observations). To determine if LPS-induced hyporesponsiveness in whole blood was specific for LPS, LPS-pretreated blood cells were challenged with HKSA, and in contrast to the observations in PEM, responsiveness to HKSA was found to be decreased in a dose-dependent manner (Table V).

Discussion

Herein we show that complexes of LBP and LPS are more effective inducers of adaptation than LPS alone. We also show that complexes of LPS–LBP can partially restore LPS responsiveness in adapted MΦ when LPS alone is added as the primary dose. Because CD14 functions as receptor for LBP–LPS and antibody to CD14 has been shown to block cellular responses to LPS, we considered that decreased expression of CD14 could be responsible for the LPS hyporesponsiveness of adapted MΦ. However, flow cytometry analysis demonstrated that CD14 expression was not substantially changed in MΦ or in blood monocytes that were adapted by LPS pretreatment.

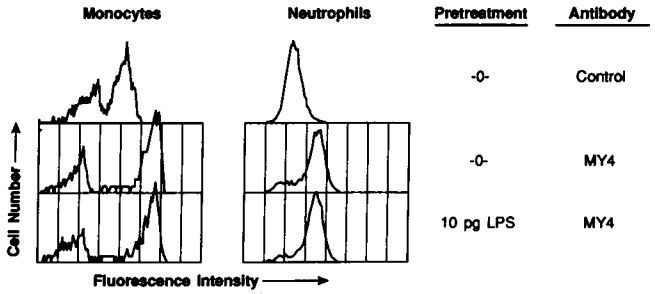

Figure 4. CD14 expression in resting and LPS-adapted blood cells. Rabbit blood was exposed to 10 pg LPS for 10 h followed by washing and separation of mononuclear cells using Ficoll-Paque or preparation of whole blood leukocytes by ammonium chloride lysis. CD14 expression was assessed using flow cytometry as described in Methods. Live gating was used to select monocytes or neutrophils.

We further considered that antigenically intact CD14 on the cell surface might be altered somehow so that specific binding of LPS would be hindered, thus causing hyporesponsiveness. However, specific binding of FITC-LPS to LPS-adapted MΦ, determined by flow cytometry, was not substantially different from that observed in resting MΦ. MΦ adapted to LPS in the presence or absence of LBP remained fully responsive to stimulation by *S. aureus* peptidoglycan, releasing normal levels of TNF, whereas blood cells adapted to LPS ex vivo were hyporesponsive to *S. aureus* challenge.

The LBP-CD14 pathway greatly amplifies the sensitivity of MΦ to LPS, and resulting cytokine release provides a mechanism for bringing other elements of host defense into action (23, 26, 31, 32). However, it is now clear that in addition to host defense, cytokine release may also lead to tissue injury (1). Thus, regulatory mechanisms must exist to limit MΦ responses to LPS during infection. The adaptive responses of MΦ to LPS that we and others have demonstrated provide a way for regulating cytokine release during endotoxemia (20, 33–36). In the present studies we have shown that LBP promotes LPS-induced adaptation, as MΦ adapted using LPS–LBP com-

Table V. Effect of Ex Vivo LPS-induced Adaptation in Whole Blood on HKSA-induced TNF Release

	Primary LPS (pg/ml)			
	0	3	10	100
TNF (U/ml)	140	73	34	9

Rabbit blood anticoagulated with 10/ml heparin was incubated for 10 h at 37°C in the presence of various concentrations of LPS followed by washing, resuspension of blood cells using diluted plasma, and challenge with heat-killed *S. aureus* (10^7/ml, final concentration) for 4 h at 37°C. TNF cytolytic activity was measured using the WEHI clone 13 assay.

plexes compared with LPS alone are less responsive to LPS challenge, as assessed by induction of cytokine message and protein release. The concentration of LBP used in these experiments was 0.1 μg/ml. It seems likely that adaptation in the presence of LBP more likely reflects conditions under which tolerance is induced in vivo because plasma LBP levels range from 5 to 10 μg/ml (D. Leturcq and P. Tobias, unpublished data). That CD14 expression and LBP-promoted binding of FITC-LPS are not abolished in adapted MΦ suggests that other, yet unidentified components of LPS recognition/transduction must regulate responsiveness in LPS adapted MΦ. Data from other laboratories also suggest the existence of additional components in LPS recognition. For example, Kitchens et al. (37) showed that LPS partial structures could block cell responses to LPS without interfering with CD14-dependent uptake of LPS. Recent studies of Lee et al. (38) used the murine pre-B cell line, 70Z, which is induced by high concentrations of LPS to express surface Ig. Transfection of 70Z cells with human CD14 resulted in a 1,000-fold increase in sensitivity of these cells to LPS. It was surprising that increased sensitivity of CD14-transfected cells was observed under serum-free conditions and that addition of LBP led to even greater sensitivity (38). Thus, several lines of evidence indicate that CD14 plays a central role in cellular recognition of LPS. Moreover, the data suggest additional LPS recognition elements that are functional, though inefficient, in the absence of CD14 and LBP.

Studies with CD14-transfected murine pre-B cells (70Z/3-hCD14) provide a clear demonstration of the importance of CD14 in cellular recognition of LPS (38). Using a ^{125}I-F(ab') binding assay 70Z/3-hCD14 cells were found to have 13,000 sites per cell compared with undetectable levels in 70Z/3-RSV cells (P. Tobias, unpublished observations). The 70Z/3-hCD14 cell line is a useful model for quantifying levels of CD14 expression required for recognition of LBP–LPS complexes and, in addition, may provide information about other components of LPS recognition that are critically changed in LPS-adapted cells. In the present study LPS responsiveness in LPS-adapted MΦ was reduced > 90% while CD14 expression and binding of LBP-LPS complexes in these cells remained normal. Compared with the LPS-induced marked, rapid loss of MΦ TNF receptors (11), the alterations in CD14 expression in LPS-treated MΦ are not important in determining cellular responsiveness to LPS. Studies are currently under way in our laboratory to identify cellular components for LPS recognition and signal transduction that are critically altered in LPS adapted MΦ.

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