# CD11/CD18 and CD14 Share a Common Lipid A Signaling Pathway[1]

**Robin R. Ingalls,[2]* Brian G. Monks,* Ricardo Savedra, Jr.,* William J. Christ,† Russell L. Delude,‡ Andrei E. Medvedev,[3§] Terje Espevik,§ and Douglas T. Golenbock***

**The activation of phagocytes by the lipid A moiety of LPS has been implicated in the pathogenesis of Gram-negative sepsis. While two LPS receptors, CD14 and CD11/CD18, have been associated with cell signaling, details of the LPS signal transduction cascade remain obscure. CD14, which exists as a GPI-anchored and a soluble protein, lacks cytoplasmic-signaling domains, suggesting that an ancillary molecule is required to activate cells. The CD11/CD18 integrins are transmembrane proteins. Like CD14, they are capable of mediating LPS-induced cellular activation when expressed on the surface of hamster fibroblasts Chinese hamster ovary (CHO)-K1. The observation that a cytoplasmic deletion mutant is still capable of activating transfected CHO-K1 argues that CD11/CD18 also utilizes an associated signal transducer. We sought to identify further similarities between the signaling systems utilized by CD14 and CD11/CD18. LPS-binding protein, which transfers LPS to CD14, enhanced both LPS-induced cellular activation and binding of Gram-negative bacteria in CD11/CD18-transfected CHO-K1, thus implying that LPS-binding protein can also transfer LPS to CD11/CD18. When synthetic lipid A analogues were analyzed for their ability to function as LPS agonists, or antagonists, in the CHO transfectants, we found the effects were identical regardless of which LPS receptor was expressed. This supports the hypothesis that a receptor distinct from CD14 and CD11/CD18 is responsible for discriminating between the lipid A of LPS and the LPS antagonists. We propose that this receptor, which is the target of the LPS antagonists, functions as the true signal transducer in LPS-induced cellular activation for both CD14 and CD11/CD18.** ***The Journal of Immunology*****, 1998, 161: 5413–5420.**

Lipopolysaccharide (LPS, endotoxin) is the major constituent of the outermost membrane of Gram-negative bacteria. Intensive research over the last decade has implicated LPS or, more specifically, the lipid A core of LPS, in the pathogenesis of Gram-negative sepsis and septic shock (1–3). The interaction of LPS with its receptors triggers the production of inflammatory mediators, resulting in a systemic response, such as fever. In the case of septic shock, the inflammatory mediators are produced in excess, resulting in hemodynamic instability, activation of the complement and clotting cascades, and multiorgan failure. In spite of great medical advances, the morbidity and mortality associated with Gram-negative sepsis remains high, and the search for effective therapies to modulate the host response continues.

The identification of CD14 as a signaling receptor for complexes of LPS was a seminal event in understanding the mechanism by which LPS-induced cellular activation occurs. CD14, a 55-kDa glycosyl phosphatidylinositol (GPI)[4]-linked protein present on the surface of phagocytic leukocytes, has been shown to bind LPS and to mediate cellular activation (4–6). In addition, a soluble form of CD14 (sCD14) is also capable of binding LPS and activating some CD14-deficient cells, such as endothelial cells (7–10). Several lines of evidence support a role for CD14 in LPS signaling, including: 1) LPS binds membrane and sCD14 as a complex with LPS-binding protein (5, 6, 11, 12); 2) mAbs to CD14 inhibit the ability of LPS to stimulate phagocytes (5, 13–16) and endothelial cells (8); 3) cells that are LPS hyporesponsive or unresponsive become sensitive to LPS when transfected with CD14 (17, 18); and 4) CD14-deficient mice have severely diminished responses to LPS (19).

One interesting aspect of CD14-mediated signal transduction is that it is greatly enhanced by two serum proteins: LPS-binding protein (LBP) and sCD14. Soluble CD14, as described above, acts as an LPS receptor for some non-CD14 bearing cells, such as endothelial cells (7–10). LBP, in contrast, is a lipid transfer protein. Although CD14 can bind LPS in its absence, LBP accelerates the binding of LPS monomers to both membrane and sCD14 (12, 20), thus enhancing the sensitivity of cells to LPS (5, 12, 21, 22).

It is generally agreed that the interaction between lipid A and CD14 is central to cellular activation by LPS; however, details of the downstream signaling events remain obscure. For example, since CD14 lacks a transmembrane domain, it seems probable that CD14 utilizes an accessory receptor to transmit a signal across the plasma membrane. Furthermore, pharmacologic studies with lipid

*Maxwell Finland Laboratory for Infectious Diseases, Department of Medicine, Boston Medical Center and Boston University School of Medicine, Boston, MA 02118; †Eisai Research Institute, Andover, MA 01810; ‡Department of Surgery, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02118; and §Norwegian University of Science and Technology, Trondheim, Norway

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[2] Address correspondence and reprint requests to Dr. Robin R. Ingalls, Maxwell Finland Laboratory for Infectious Diseases, Boston Medical Center, Boston University School of Medicine, 774 Albany Street, Boston, MA 02118.

[3] Current address: Department of Microbiology and Immunology, Uniformed Services of the Health Sciences, Bethesda, MD 20814.

[4] Abbreviations used in this paper: GPI, glycosyl phosphatidylinositol; sCD14, soluble form of CD14; LBP, lipopolysaccharide-binding protein; RSLA, *Rhodobacter sphaeroides* lipid A; CHO, Chinese hamster ovary; NF-κB, nuclear factor-κB; PG, peptidoglycan; $IC_{50}$, 50% inhibitory concentration; EMSA, electrophoretic mobility shift assay; ReLPS, lipopolysaccharide from *Salmonella minnesota* R595; LAM, lipoarabinomannan.



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A antagonists suggest that CD14 activates cells via an ancillary-signaling molecule. For example, the biologically derived lipid A analogues *Rhodobacter sphaeroides* lipid A (RSLA) and lipid $IV_A$ are potent LPS antagonists in LPS-responsive human cells (23–25). In hamster and mouse cells these compounds have very different effects: in hamsters, both compounds are LPS mimetics (26), while in mice lipid $IV_A$ is a LPS mimetic and RSLA is a LPS antagonist (23, 24, 27, 28). Data from transfected cell lines have shown that the species-specific effects of the lipid A-like compounds are determined not by the species of CD14, but by the genome of the host cell on which it is expressed (26). In addition, careful binding studies by Kitchens et al. (15) and Kitchens and Munford (29) demonstrated that these compounds inhibit the ability of LPS to activate cells at concentrations that are too low to inhibit binding of LPS to CD14. Taken together, these data suggest that the inhibitors are not simply competing with LPS for binding to CD14, but that they are antagonizing LPS at a site distinct from CD14. Thus, while CD14 plays a major role in LPS recognition by phagocytes, it is not felt to be a direct signaling receptor.

The CD11/CD18 ($\beta$2) integrins represent a second group of LPS receptors. Although Wright and Jong demonstrated that the integrins were capable of binding unopsonized bacteria and LPS (30), it was initially unclear if this interaction triggered a cellular response independent of CD14. For example, it was demonstrated that PBMCs from CD18-deficient patients responded normally to LPS (31). Studies in our laboratory recently demonstrated that the CD11/CD18 integrins enable LPS-induced signal transduction when transfected into Chinese hamster ovary (CHO) cells, thus demonstrating that CD11/CD18 can enable LPS responsiveness independent of CD14 (32, 33). Although the integrins are transmembrane receptors, the cytoplasmic domains do not appear to be necessary for signaling translocation of nuclear factor-$\kappa$B (NF-$\kappa$B) in response to LPS binding. This is based on the observation that a mutant CD11b/CD18, deficient in the cytoplasmic domains and incapable of internalization of Gram-negative bacteria, is still competent for LPS-induced cellular activation (33). Thus, the integrins, like CD14, may function to transfer LPS to a second receptor that transduces the signal.

We sought to identify further similarities and differences between the signaling systems utilized by CD14 and CD11/CD18. First, we found that LBP was capable of enhancing CD11/CD18-dependent binding of whole Gram-negative bacteria to cells, as well as cellular activation via LPS in the CD11/CD18-transfected CHO cells. In addition, we found that lipid $IV_A$ and two synthetic lipid A analogues demonstrated the same species specificity in CD11/CD18-transfected cell lines as they did in CD14 transfectants. In light of the similarities between CD14- and CD11/CD18-mediated signal transduction, we hypothesize that both LPS receptors form a signaling complex that utilizes a common lipid A recognition molecule that functions as the true signal transducer in LPS-mediated cellular activation.

## Materials and Methods

### *Reagents*

All solutions were guaranteed sterile and pyrogen free by the manufacturer unless otherwise stated. PBS, Ham's F-12, DMEM, and RPMI 1640 were obtained from BioWhittaker (Walkersville, MD). Ex-Cell 301 serum-free medium was obtained from JRH Biosciences (Lenexa, KS). FCS (LPS $\leq$ 10 pg/ml) was obtained from HyClone Laboratories (Logan, UT). Human serum was derived from clotted whole blood from healthy volunteers and heat inactivated in a water bath at 56°C for 20 min. Ciprofloxacin was a gift from Miles Pharmaceuticals (West Haven, CT). G418 was obtained from Sigma (St. Louis, MO). Murine LBP was a gift of Ralf Schumann (Humboldt University, Berlin, Germany); human LBP and soluble CD14 were gifts of Henry Lichenstein (Amgen, Thousand Oaks, CA). LPS from *Salmonella minnesota* R595 (ReLPS) was the gift of Drs. N. Qureshi and K. Takayama (University of Wisconsin, Madison, WI). The generation of compound B287 has been published previously (34). Compound B1287 was prepared at Eisai Research Institute (Andover, MA; patent reference no. WO-9639411-A1). Synthetic lipid $IV_A$ was purchased from ICN (Costa Mesa, CA). Lipids were prepared as 1 mg/ml dispersed sonicates in pyrogen-free PBS and stored at $-20$°C. Prior to use, the suspensions were thawed and sonicated for 3 min in a water bath sonicator (Laboratory Supplies, Hicksville, NY) before diluting to final concentration. Mycobacterial lipoarabinomannan (noncapped araLAM from a rapidly growing mycobacterial species) was provided by Drs. J. Belisle and P. Brennan (Colorado State University, Fort Collins, CO) under National Institutes of Health Contract NO1-A1-25147. The LPS content of this preparation, as assayed by *Limulus* amebocyte lysate assay, was 14 ng/mg of LAM. Peptidoglycan (PG; soluble polymeric peptidoglycan isolated from the cell walls of *Staphylococcus aureus*) was a gift from Dr. R. Dziarski (Indiana University School of Medicine, Gary, IN). PG contained $<$12 pg of LPS/mg as determined by the *Limulus* amebocyte lysate assay.

### *Cell lines*

The following cell lines were obtained from the American Type Culture Collection (Manassas, VA): CHO-K1, a hamster fibroblast cell line; HT1080, human fibrosarcoma cell line; RAW 264.7, an LPS-responsive murine macrophage cell line (35); and THP-1, a human promonomyelocytic line (36). The following stably transfected cell lines were engineered as previously described: HT1080/CD14$^{human}$ and HT1080/CD14$^{murine}$, human and murine CD14-transfected HT1080 lines (26); CHO/Neo, CHO-K1 transfected with the pCDNA1/Neo vector (18); CHO/CD14 human CD14-transfected CHO-K1 (18); and CHO/CD11b, CHO-K1 transfected with full length human CD11b and CD18, and CHO/CD11b$^{mutant}$, which contains a cytoplasmic deletion mutant form of CD11b and CD18 (33). CHO/CD11c, CHO-K1 transfected with human CD11c and CD18, was engineered by the same method as described for CHO/CD11b (33) using human CD11c and CD18 cDNA in $\pi$H3M vectors (37) and pcDNA1/Neo.

### *Cell culture and stimulation conditions*

Cell lines were maintained as follows: RAW 264.7 and HT1080 in DMEM/HG; THP-1 in RPMI 1640; and CHO-K1 cells in Ham's F-12. Tissue culture medium was supplemented with 10% FCS and 10 $\mu$g ciprofloxacin/ml (complete medium). Transfected CHO cell lines were maintained in complete medium supplemented with G418 (500 $\mu$g active drug/ml). Cell lines were grown as adherent monolayers in tissue culture dishes at 37°C in 5% $CO_2$, and passaged twice a week to maintain logarithmic growth. THP-1 cells were differentiated with vitamin $D_3$ (0.1 $\mu$M) for 72 h prior to stimulation (15).

One day prior to stimulation, cells growing as adherent monolayers in tissue culture dishes were trypsinized, resuspended in complete medium, and plated in 6-well tissue culture dishes at a density of $5 \times 10^5$ per well. Plates were incubated overnight at 37°C in 5% $CO_2$. On the day of stimulation, wells were aspirated and washed three times with PBS to remove FCS. For RAW and THP-1 cells, medium was replaced with 1 ml of DMEM or RPMI 1640, respectively, and supplemented with 2% heat-inactivated human serum. For the CHO cells, medium was replaced with Ham's F-12 with 2% FCS or Ex-Cell serum-free medium. When LBP was used in assays, it was added to Ex-Cell for a final concentration of 150 ng/ml. Compound B1287, also diluted in PBS, was added at the same time as the stimulant. Culture dishes were returned to 37°C/5% $CO_2$ for 1 h.

### *Preparation of nuclear extracts*

The procedure used for the preparation of nuclear extracts has been published in detail (38). After stimulation, cells were washed in tissue culture plates with PBS/2% FCS, harvested using a rubber policeman, and pelleted in a microcentrifuge (Beckman Microfuge 11). Cell pellets were resuspended in 0.4 ml buffer I (10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM EGTA, 1 mM PMSF, 10 mM $\beta$-glycerol phosphate, 0.3 M sucrose, and 1.0 $\mu$g/ml each of the following protease inhibitors: aprotinin, antipain, leupeptin, chymostatin, and pepstatin), incubated on ice for 15 min, and lysed by adding Nonidet P-40 to a final concentration of 0.5%. Nuclei were collected by centrifugation and resuspended in 50 $\mu$l of buffer II (20 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 320 mM KCl, 0.5 mM DTT, 0.2 mM EGTA, 0.5 mM PMSF, 10 mM $\beta$-glycerol-phosphate, 25% glycerol, and 1.0 $\mu$g/ml protease inhibitors as above). After a 15-min incubation on ice, the nuclear extracts were cleared by centrifugation and transferred to a new tube. Protein concentration was determined using Bio-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA).

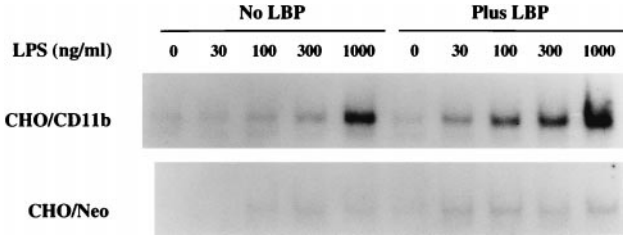


**FIGURE 1.** LBP enhances LPS-induced signaling in CD11b/CD18-transfected CHO cells. CHO cells transfected with CD11b/CD18 (CHO/CD11b) were treated with increasing doses of LPS in the presence or absence of recombinant human LBP (150 ng/ml). Nuclear proteins were prepared, and nuclear levels of NF-κB were measured by EMSA using a κB site-containing probe. Only the band representing NF-κB bound to the κB site-containing probe is shown.

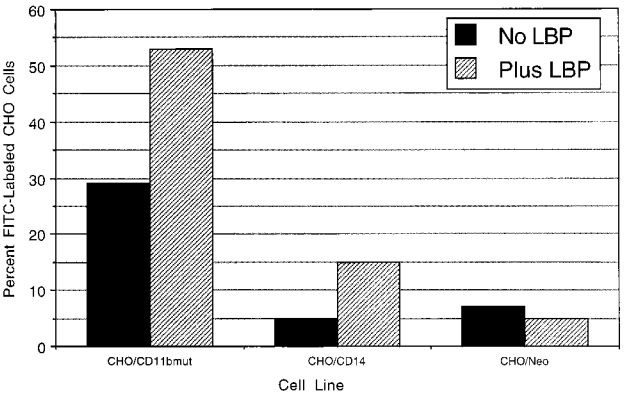


**FIGURE 2.** LBP enhances binding of FITC-*E. coli* to CHO transfectants. Monolayers of CHO transfectants were incubated for 1 h with heat-fixed and FITC-labeled *E. coli*, with or without LBP, and subjected to flow cytometry. For each condition, 104 CHO cells were analyzed for fluorescence. A gate was set for fluorescence above background, defined as the amount of fluorescence generated by *E. coli* binding to the CHO/Neo control line, and results were expressed as the percentage of cells that fell within this gate. Shown above are data from one representative experiment comparing extracellular binding of bacteria in the phagocytosis mutant CHO/CD11b$^{mutant}$, CHO/CD14, and CHO/Neo.

### *Electrophoretic mobility shift assay (EMSA)*

Nuclear extract pellets were assayed for the presence of (NF-κB) as described in detail (38). Briefly, 4 μg of the crude nuclear protein was incubated with $^{32}$P-labeled oligonucleotides containing the consensus sequence for NF-κB binding from the murine Ig κ light chain gene enhancer. The DNA-protein binding reactions were analyzed by nondenaturing gel electrophoresis. Gels were transferred to filter paper, dried, and exposed to x-ray film. Scanning densitometry of the autoradiographs was performed using Sigma Gel (version 1.0; Jandel Scientific, San Rafael, CA).

### *Binding assays*

Binding assays were performed as previously described (33). Briefly, *Escherichia coli* MC1061/P3 were cultured to a density of $1 \times 10^9$/ml, heat fixed, and labeled with FITC (0.1 mg/ml) for 30 min at room temperature. Monolayers of CHO transfectants growing at a density of $1 \times 10^5$ per well were incubated with $10^8$ bacteria at 37°C for 1 h and analyzer for fluorescence signal (18).

## Results

### *LBP enhances the sensitivity of CHO/CD11b to LPS and Gram-negative bacteria*

The serum protein LBP is a lipotransferase, catalytically transferring LPS from aggregates to CD14. LBP does not form detectable complexes with LPS and CD14, and it is not required for LPS-induced cellular activation. However, by facilitating the interaction between LPS and CD14, LBP enhances the sensitivity of cells to LPS (12). In addition to transferring LPS to CD14, LBP is also capable of transferring LPS to lipoprotein particles, such as high density lipoprotein, where it may play a role in LPS neutralization (20).

We asked whether LBP could perform a similar function in the interaction between LPS and CD11/CD18. We had previously reported that, unlike CD14-dependent signaling, activation of CD11c/CD18-transfected CHO cells was not enhanced by the addition of human serum (32). When we attempted to examine this observation further, we found recombinant sCD14 had no effect on LPS-induced activation in either the CHO/CD11b or CHO/CD11c cell lines (data not shown). However, with LBP, the results were quite different. Here we found another similarity between CD11/CD18 and CD14 with respect to signaling: the addition of recombinant human LBP (150 ng/ml) to serum-free medium increased the sensitivity of CHO/CD11b to LPS by approximately 30-fold (Fig. 1). A similar effect was also seen with the cytoplasmic deletion mutant CHO/CD11b$^{mutant}$ (data not shown). This suggested to us that LBP was also capable of transferring LPS to CD11/CD18.

Wright et al. first described the ability of LBP to opsonize LPS-bearing particles, including Gram-negative bacteria, to enhance their recognition by macrophages (11). When we examined the ability of LBP to enhance the binding of Gram-negative bacteria to the CHO transfectants, we found a similar effect. As shown in Figure 2, opsonization of FITC-labeled *E. coli* with recombinant murine LBP (200 ng/ml) resulted in a dramatic increase in binding to CHO cells expressing CD11/CD18. An increase in binding of bacteria to CHO/CD14 could also be seen in the presence of LBP, although overall the binding was much lower than that seen in the CD11b/CD18-transfected CHO cells. In contrast, the control line CHO/Neo cell line failed to bind significant bacteria whether or not LBP was present.

### *Structure of compound B1287*

Several biosynthetic inhibitors of lipid A, such as RSLA and lipid $IV_A$, have been useful in the study of LPS-induced cellular activation. The development of new methodologies for the preparation of synthetic lipid A has greatly benefited the study of LPS antagonists. One such compound, compound B287, is based on the proposed structure of RSLA, and has activity identical to that of natural RSLA when tested in macrophage cell lines and whole human blood ex vivo (34). Christ et al. recently reported on the activity of E5531, a potent LPS antagonist similar in structure to *Rhodobacter capsulatus* lipid A (39). They found that this compound blocked LPS-mediated cellular activation in human macrophages and protected mice from lethality induced by LPS. This compound also inhibits a broad range of effects in LPS-challenged humans (39) (M. Lynn, personal communication), and is currently in human clinical trails as an antiendotoxic agent. The structure of E5531, as well as the nontoxic lipid A moieties from *R. capsulatus* and *R. sphaeroides* lipid A, served as the foundation for the development of compound B1287. Figure 3 depicts the structure of compound B1287 in comparison with compound B287 and ReLPS lipid A. Compound B287 differs from lipid A by the presence of a 3-keto-myristoyl group at the 2 position, a double bond in the acyloxyacyl chain at the 2′ position, and the absence of an oxyacyl chain at the 3′ position. It also has shorter 3 and 3′ acyl groups. Compound B1287 is similar in structure to B287 with the following exceptions: the ester linkage of the fatty acyl side chains at the 3 and 3′ positions have been replaced by ether linkages; the hydroxyl groups at the C-6′ position and on the 3′ acyl chain have been

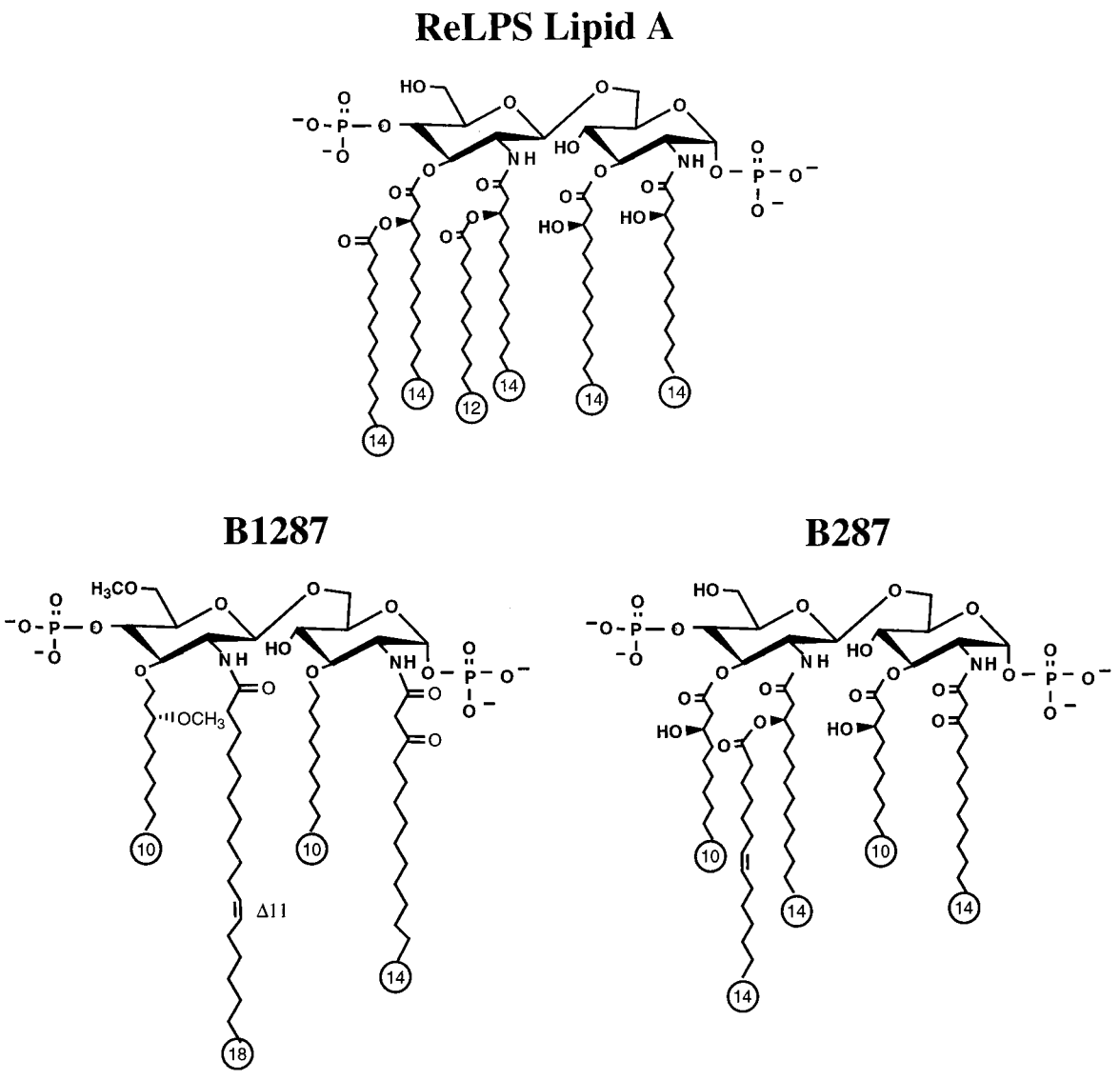


**FIGURE 3.** Structures of the synthetic LPS antagonists: compounds B287 and B1287. The structures of two synthetic lipid A antagonists are shown above in comparison with ReLPS lipid A. The antagonists differ from lipid A in the number and length of their fatty acyl side chains, the presence of a keto moiety on the amide-linked acyl side chain, and a double bond in its acyl constituents. Compound B1287 also has a MeO group in the C-6′ position and on the 3′ acyl chain.

replaced by MeO groups; the oxyacyl chain at the 2′ position has been removed; and a double bond has been introduced in the 2′ acyl chain. In addition, the 2′ acyl chain has been lengthened to C18.

*Compound B1287 blocks LPS in responsive human and murine cell lines*

RAW and vitamin $D_3$-differentiated THP-1 cells were treated with increasing doses of LPS in the presence or absence of compound B1287, and the nuclear extracts were assayed for the presence of NF-κB. As predicted by the activity of RSLA, which is an antagonist in human and murine cells, B1287 blocked LPS-induced NF-κB translocation in both cell lines. A comparison of the sensitivity of the two cell lines by scanning densitometry to the antagonist reveals that THP-1 (Fig. 4) and RAW (Fig. 5) have an $IC_{50}$ of $<1$ ng/ml and 100 ng/ml, respectively. However, in both cases, the ability of B1287 to block LPS-induced signal transduction could be overcome with sufficiently high concentrations of LPS. Similar results were observed with the HT1080 transfectants expressing either human (HT1080/CD14$^{human}$) or murine (HT1080/CD14$^{murine}$) CD14 (data not shown).

*Compound B1287 blocks LAM- and PG-induced cellular activation in human macrophages*

Phagocytic leukocytes respond to a variety of bacterial products, and CD14 has been implicated as a component of the cell-activating receptor system for outer wall microbial components other than LPS. This includes the glycolipid LAM from mycobacterial species (40–45), Gram-positive metabolites, such as PG (41, 46–50), and mannuronic acid polymers (51, 52). The conclusion that LAM activation is CD14 dependent is based, in part, on the ability of anti-CD14 mAbs to inhibit macrophage responses to these substances and the dependence upon the presence of LBP (45). While CD14 appears to be necessary for cellular activation by LAM and PG, it is not sufficient by itself, as CD14-transfected CHO cells fail to respond to either compound (45) (data not shown). Interestingly, lipid A partial structures have been shown to inhibit LAM- (45) and PG-induced cellular activation (48) as well.

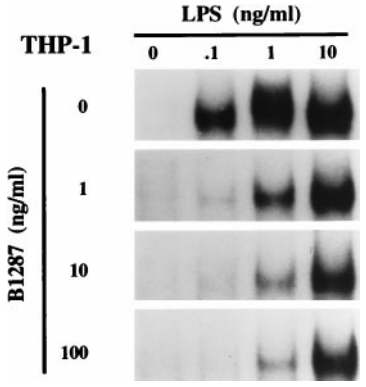


**FIGURE 4.** Compound B1287 inhibited LPS-induced NF-κB translocation in THP-1 cells. Vitamin $D_3$-differentiated THP-1 cells were treated with increasing doses of ReLPS in the presence of increasing doses of compound B1287 for 1 h. Nuclear proteins were prepared, and nuclear levels of NF-κB were measured by EMSA using a κB site-containing probe. Shown above is the portion of the gel-containing DNA probe bound to nuclear NF-κB. By scanning densitometry, half-maximal stimulation occurred with 0.1 ng LPS/ml. The $IC_{50}$ was calculated to be <1 ng B1287/ml.

When the ability of compound B1287 to block LAM-induced NF-κB translocation in THP-1 cells was examined, we found similar results. Like RSLA (45), B1287 was a potent antagonist of NF-κB translocation by LAM (Fig. 6). Similar data was found using B1287 to block PG-induced NF-κB translocation in the same system (data not shown). These data support the hypothesis that LPS, LAM, and PG signal cells through a shared receptor complex, which includes CD14 and a common lipid A recognition molecule (45).

*Compound B1287 blocks LPS-responsive CHO-K1 transfectants*

Based on the ability of the synthetic LPS analogues B287 and lipid $IV_A$ to stimulate hamster macrophages and CD14-transfected CHO cells (26), we predicted that the same species-specific effects would be seen in the CHO cells expressing CD11/CD18. In fact, we found compound B287 to be an LPS mimetic in both CHO/CD11b and CHO/CD11c cells (Fig. 7). Lipid $IV_A$ showed similar

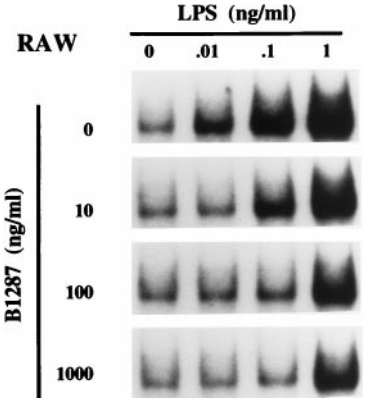


**FIGURE 5.** Compound B1287 inhibited LPS-induced NF-κB translocation in RAW cells. RAW cells were treated with increasing doses of ReLPS in the presence of increasing doses of compound B1287 for 1 h. Nuclear proteins were prepared, and nuclear levels of NF-κB were measured by EMSA using a κB site-containing probe. Shown above is the portion of the gel-containing DNA probe bound to nuclear NF-κB. By scanning densitometry, half-maximal stimulation occurred with 0.01 to 0.1 ng LPS/ml. The $IC_{50}$ was calculated to be 100 ng B1287/ml.

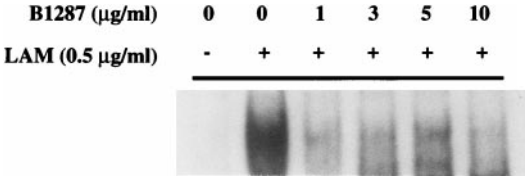


**FIGURE 6.** The LPS antagonist B1287 blocks LAM-induced NF-κB translocation in THP-1 cells. Vitamin $D_3$-differentiated THP-1 cells were treated with 0.5 μg/ml of LAM in the presence or absence of compound B1287 for 1 h. Nuclear proteins were prepared, and nuclear levels of NF-κB were measured by EMSA using a κB site-containing probe. The bands represent DNA probe bound to nuclear NF-κB.

LPS-mimetic activity (data not shown).

In contrast to the LPS-mimetic activity observed with all the prior synthetic LPS analogues, we found B1287 had no LPS-like activity on its own in the transfected CHO lines (data not shown). Furthermore, we found that, when coincubated with LPS, B1287 was a potent antagonist in CHO/CD14 cells, with an $IC_{50}$ of 100 ng/ml by scanning densitometry (Fig. 8). In addition, B1287 was found to be an LPS antagonist in CD11b/CD18-transfected CHO cells (Fig. 9). In contrast to CHO/CD14, which are more sensitive to LPS, CHO/CD11b required higher doses of LPS for stimulation and, consequently, higher doses of B1287 to completely block signaling. We estimate the $IC_{50}$ in CHO/CD11b to be approximately 100 to 1000 ng/ml (data not shown).

## Discussion

The current understanding of the LPS receptor system consists of multiple LPS receptors on the surface of phagocytes which, after interacting with LPS, mediate remarkably diverse events. CD14 is likely the LPS receptor primarily responsible for initiating LPS-induced cellular activation. This is supported, in part, by the observation that CD14-deficient mice are highly resistant to LPS and Gram-negative bacteria (19). In contrast, CD11/CD18 is important for phagocytosis of invading Gram-negative bacteria (53, 54). While it is not essential for cellular responses to LPS (31), expression of CD11/CD18 is sufficient for imparting LPS responsiveness (32, 33). The relative importance of CD11/CD18 in LPS-induced cellular activation of phagocytic cells, which normally express CD14, is unclear at this time. However, it may play a role under certain conditions, such as the cellular activation seen with high doses of LPS in CD14-deficient mice (19).

There are important differences between CD14 and CD11/CD18. In the CHO-K1 system, CD14 appears to be more sensitive to LPS compared with CD11/CD18, and the activation of cells appears to occur more rapidly (32, 33). While sCD14 is capable of

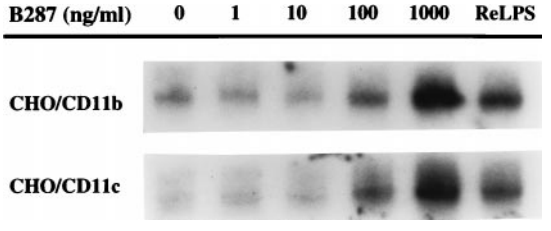


**FIGURE 7.** Compound B287 is an LPS mimetic in CD11/CD18-transfected CHO cells. CHO cells transfected with CD11b/CD18 (CHO/CD11b) or CD11c/CD18 (CHO/CD11c) were treated with increasing doses of compound B287 or 1 μg/ml ReLPS for 1 h. Nuclear proteins were prepared, and nuclear levels of NF-κB were measured by EMSA using a κB site-containing probe. The bands represent DNA probe bound to nuclear NF-κB.

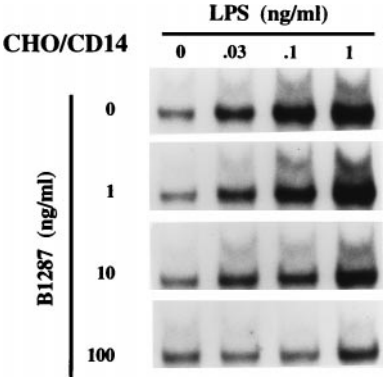


**FIGURE 8.** Compound B1287 is an LPS antagonist in CHO/CD14. CHO cells transfected with human CD14 (CHO/CD14) were treated with increasing doses of LPS in the presence of increasing doses of compound B1287 for 1 h. Nuclear proteins were prepared, and nuclear levels of NF-κB were measured by EMSA using a κB site-containing probe. The bands represent DNA probe bound to nuclear NF-κB. By scanning densitometry, half-maximal stimulation occurred with 0.03 to 0.1 ng LPS/ml. The $IC_{50}$ was calculated to be 100 ng B1287/ml.

activating many non-CD14-bearing cell types, there is no evidence, at least with a soluble form of CD11c/CD18, that the integrins can function in this manner (R. R. Ingalls, M. A. Arnaout, and D. T. Golenbock, unpublished observations). There are also significant similarities between CD14 and CD11/CD18 that suggest a shared signaling system may exist. First, the extracellular LPS-binding domain of the two receptors appears to be the most important feature of the receptor; the mechanism by which it is anchored to the membrane (i.e., GPI-anchored vs transmembrane) does not appear to be relevant. For example, CD14 functions equally as well when it exists as an integral protein as it does when it is GPI anchored (55). In addition, the cytoplasmic domains of CD11/CD18, while essential for functions such as phagocytosis, are not required for LPS-induced signaling (33).

Second, both CD14 and CD11/CD18 appear to interact not only with LPS, but also with LPS complexed to LBP. While it is agreed that LBP functions to move LPS onto CD14 (5, 12, 22, 56), no role for LBP has ever been established with respect to the CD11/18 integrins. Our data support the observation by Wright et al. that

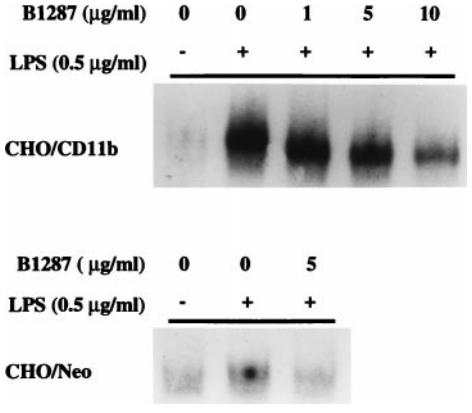


**FIGURE 9.** Compound B1287 is an LPS antagonist in CHO/CD11b. CHO cells transfected with human CD11b/CD18 (CHO/CD11b) or the neomycin-resistant vector alone (CHO/Neo) were treated with LPS (0.5 μg/ml) in the absence or presence of increasing doses of compound B1287 for 1 h. Nuclear proteins were prepared, and nuclear levels of NF-κB were measured by EMSA using a κB site-containing probe. The bands represent DNA probe bound to nuclear NF-κB.

Table I. *Species-specific pharmacology of lipid A-like compounds*[a]

| Cell Species | Cell Line | Lipid $IV_A$ | RSLA | B1287 |
|---|---|---|---|---|
| Human | Macrophage | − | − | NT |
| | HT1080/CD14$^{human}$ | − | − | − |
| | HT1080/CD14$^{murine}$ | − | − | − |
| Mouse | Macrophage | + | − | − |
| | 70Z/3-CD14$^{human}$ | + | − | NT |
| | 70Z/3-CD14$^{murine}$ | + | − | NT |
| Hamster | Macrophage | + | + | NT |
| | CHO/CD14$^{human}$ | + | + | − |
| | CHO/CD14$^{murine}$ | + | + | NT |
| | CHO/CD11b | + | + | − |
| | CHO/CD11c | + | + | NT |

[a] Data show the summary of the work on the species-specific effects of the lipid A analogues (23–28). +, Agonist (LPS-mimetic) activity; −, LPS-antagonist activity; NT, not tested; CD14$^{human}$, human CD14; CD14$^{murine}$, murine CD14; CD11b, human CD11b/CD18; CD11c, human CD11c/CD18; RSLA column also includes data on the synthetic compound B287.

LBP can act as an opsonin for Gram-negative bacteria (11). In addition, we have observed that LBP enhances LPS-induced activation of CD11/CD18-transfected CHO cells by approximately 30-fold. The physiologic relevance of this opsonic function is currently unproved, although one can imagine that this process would not only amplify CD11/18-mediated signaling (resulting in bacterial engulfment, mobilization of anti-bacterial machinery such as toxic radical production), but also increase other aspects of host defenses (e.g., cytokine production) via CD14.

Finally, the species-specific effects of the natural and synthetic lipid A-like compounds appear to be identical in CD14- and CD11/CD18-mediated signaling. Detailed pharmacologic studies with lipid A analogues have provided a framework for understanding how the LPS receptors are coupled to immediate events following LPS binding. Indeed, all proposed models to explain LPS-induced signal transduction must take into account the complex observations made with the LPS-receptor antagonists. The existence of a lipid A recognition molecule, which is capable of discriminating between the lipid A of LPS and the LPS antagonists, has been postulated by many groups (reviewed in Ref. 57). However, the mechanism by which the antagonists would block LPS-induced cellular activation at the level of this postulated lipid A recognition molecule remains unclear. For example, the antagonists could compete directly with LPS for binding to the lipid A recognition molecule. Alternatively, the antagonists could induce a negative signal at the level of the lipid A recognition molecule which rapidly inhibits the ability of LPS to activate cells (15). It is unlikely that these conflicting models will be reconciled until the identification and precise cellular location of this associated effector molecule is determined.

Table I summarizes the work on the species-specific effects of the lipid A analogues (23–28). These data, combined with binding studies (29), provide additional evidence for the hypothesis that the biologic target of lipid A partial structure is neither CD14 nor CD11/CD18, but a second LPS recognition molecule that associates with CD14- or CD11/CD18-bound LPS. Because CD14 is unable to discriminate between agonist and antagonist, it seems unlikely that the antagonists are simply competing with LPS for binding to CD14. Based on our hypothesis that CD11/CD18 utilizes the same signal transduction pathway as CD14, we would support the same model for the antagonist's action in the CD11/CD18 system. We propose that it is this lipid A recognition molecule that is activated (or inhibited) by lipid A, RSLA, lipid $IV_A$, or compound B1287. In our view, the interaction of lipid A with its

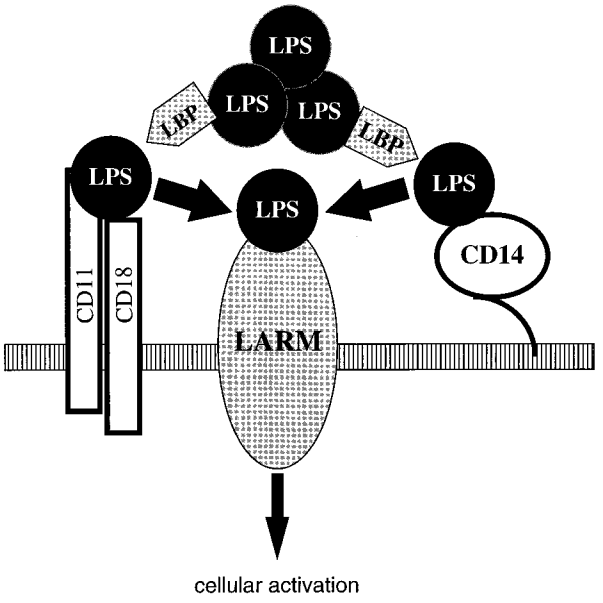


**FIGURE 10.** Proposed model for activity of LPS antagonists in LPS-responsive cell lines. The diagram depicts a model for LPS-induced signal transduction. We propose that the LPS antagonists, such as RSLA, lipid $IV_A$, or compound B1287, inhibit LPS activation of cells by competing for binding to the lipid A recognition molecule (LARM), which is also the signal transducer. Both CD14 and CD11/CD18 are capable of transferring lipid A and the lipid A antagonists to the signal transducer. Based on this model, the LAM-signaling system (not shown) would be predicted to consist of CD14, the lipid A recognition molecule, and an additional factor expressed only by leukocytes (45).

signaling receptor is facilitated when ligand is presented by either CD14 or CD11/CD18. This ligand presentation thus represents the primary role of these LPS-binding receptors in endotoxin-induced signaling (Fig. 10). The ability of B1287 and similar compounds to inhibit a variety of CD14-dependent ligands, such as LPS, PG, and LAM, implies that different bacterial products share CD14 and this putative lipid A recognition molecule as part of their receptor complex (45).

We believe that the putative signal transducer is shared between the LPS receptors, and that cooperation between the various LPS receptors to form a signaling complex may be central to the cellular response to LPS. Such cooperation would be highly favorable to the cell, as it would efficiently direct the diverse intracellular signaling cascades that make up the inflammatory response. This would include activation of transcription factors, phagocytosis, and the generation of cytokines. While we favor the existence of a receptor complex on the surface of LPS-responsive cells, the precise subcellular localization of such a receptor is not critical to this proposed model of the LPS activation pathway.

Compound B1287 is unique among the previously studied antagonists in its ability to block the activation of human, mouse, and hamster LPS-responsive cells. As such, it will be a useful research tool for the study of LPS responses in a variety of animal systems. In addition, the unique ability of compound B1287 to inhibit the inflammatory effects of LPS in cells from multiple animal species implies that animal studies with this antisepsis agent can produce clinically relevant data. This is in contrast to other potentially useful compounds, such as RSLA, to which human and animal responses are very different. More importantly, however, its ability to block diverse bacterial virulence factors such as LPS, LAM, and PG suggest that this form of therapy may be advantageous in the early treatment of sepsis and other life-threatening infections, where the ability to distinguish clinically between Gram-negative, Gram-positive, and mycobacterial infections is often not possible. The ability of B1287 and similar compounds to inhibit diverse bacterial products also implies that the innate immune system has a relatively limited repertoire of proteins involved in bacterial recognition.

The host immune response to infection is necessary to maintain homeostasis and eradicate an invading microorganism. However, it is clear that in many cases the bystander injury produced by the inflammatory response can be more deleterious to the host than the inciting event. This appears to be the case in the sepsis syndrome. By better understanding the complex interactions of the multiple receptors involved in LPS recognition and the subsequent cellular activation, clinically useful therapies can be developed for the treatment of Gram-negative sepsis and septic shock.

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