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CD14 enhances cellular responses to endotoxin without imparting ligand-specific recognition

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ABSTRACT **Binding of the lipid A portion of bacterial lipopolysaccharide (LPS) to leukocyte CD14 activates phagocytes and initiates the septic shock syndrome. Two lipid A analogs, lipid IV_A and *Rhodobacter sphaeroides* lipid A (RSLA), have been described as LPS-receptor antagonists when tested with human phagocytes. In contrast, lipid IV_A activated murine phagocytes, whereas RSLA was an LPS antagonist. Thus, these compounds displayed a species-specific pharmacology. To determine whether the species specificity of these LPS antagonists occurred as a result of interactions with CD14, the effects of lipid IV_A and RSLA were examined by using human, mouse, and hamster cell lines transfected with murine or human CD14 cDNA expression vectors. These transfectants displayed sensitivities to lipid IV_A and RSLA that reflected the sensitivities of macrophages of similar genotype (species) and were independent of the source of CD14 cDNA. For example, hamster macrophages and hamster fibroblasts transfected with either mouse or human-derived CD14 cDNA responded to lipid IV_A and RSLA as LPS mimetics. Similarly, lipid IV_A and RSLA acted as LPS antagonists in human phagocytes and human fibrosarcoma cells transfected with either mouse or human-derived CD14 cDNA. Therefore, the target of these LPS antagonists, which is encoded in the genomes of these cells, is distinct from CD14. Although the expression of CD14 is required for macrophage-like sensitivity to LPS, CD14 cannot discriminate between the lipid A moieties of these agents. We hypothesize that the target of the LPS antagonists is a lipid A recognition protein which functions as a signaling receptor that is triggered after interaction with CD14-bound LPS.**

During the course of serious Gram-negative bacterial infection, lipopolysaccharide (LPS, endotoxin) is shed from bacteria (1, 2). Released forms of LPS are then free to interact with receptors on responsive leukocytes. Binding of the lipid A portion of LPS to its receptors triggers the production and release of potent inflammatory mediators. When these inflammatory mediators are produced in excess, cardiovascular collapse and hemodynamic instability result. The septic shock syndrome remains a major cause of morbidity and mortality in the United States today (3). The search for effective antisepsis therapies to modulate cellular responses to endotoxin has proved elusive due to inadequate knowledge of the molecular events involved in LPS-induced signal transduction.

CD14, a 55-kDa glycosyl-phosphatidylinositol (GPI)-anchored protein present on the surface of phagocytic leukocytes (4), has been shown to bind LPS and subsequently initiate cellular activation (ref. 5; for review, see ref. 6). A soluble form of CD14 in serum can bind LPS and activate vascular endothelia and some other CD14-deficient cells (7–10). LPS-responsiveness has been conferred upon normally hyporesponsive and nonresponsive cells after transfection with CD14 cDNA expression vector (11–13). Furthermore, transfection of murine pre-B lymphocytic cells with chimeric forms of human CD14 in which the C-terminal region of the protein (including the GPI-anchor signal sequence) was replaced with the transmembrane and cytoplasmic domains of heterologous plasma membrane proteins resulted in cell lines with phenotypes identical to a cell line expressing the wild-type GPI-anchored human CD14 cDNA (14). These results demonstrated that the GPI anchor of CD14 is not required for CD14-dependent cellular activation. The finding that CD14 signaled effectively as a soluble molecule, a GPI-anchored molecule, or a transmembrane protein suggested that CD14 may not signal directly but interacts with an additional protein(s) required to effect subsequent signal-transduction events.

Several precursors and analogs of the toxic lipid A moiety from *Escherichia coli* LPS have been shown to inhibit LPS activation of lymphocytes, neutrophils, monocytes, and macrophages. By increasing the concentration of LPS relative to the concentration of antagonist, inhibition by these agents was overcome, suggesting that they competed with LPS for binding to a specific component of the LPS-signaling system. For example, *Rhodobacter sphaeroides* lipid A (RSLA) exhibited LPS antagonist properties in both murine (15–17) and human LPS-responsive cells (15, 18). In contrast to RSLA, the tetraacyldisaccharide lipid A precursor, designated lipid IV_A, inhibited LPS-induced activation of human cells (15, 18, 19) but acted as an LPS mimetic in murine cells (15, 18), demonstrating a species-specific effect of these LPS-receptor antagonists.

Kitchens *et al.* (20, 21) reported that lipid IV_A, when used in nanomolar concentrations under physiologic conditions, effectively blocked LPS-induced activation of human monocytes, whereas micromolar concentrations of lipid IV_A were required to block specific binding of LPS to surface CD14. The difference between the concentration of LPS antagonists required to inhibit signal transduction compared to concentrations required to block specific binding of LPS to CD14 suggested that CD14 was not the cellular target for antagonists such as lipid IV_A. Collectively, these findings are consistent with a model of signal transduction in which LPS-bound CD14 interacts with an as-yet-unidentified protein(s) present in limiting quantities on endotoxin-responsive cells that then induces a signal-transduction event across the plasma membrane. However, a definitive interpretation of these cell-binding studies (20, 21) is complicated by the problems inherent with lipophilic and amphipathic ligands such as LPS. For

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Abbreviations: LPS, lipopolysaccharide; FBS, fetal bovine serum; NF-κB, nuclear factor κB; IFN-γ, interferon γ; RSLA, *Rhodobacter sphaeroides* lipid A; GPI, glycosyl-phosphatidylinositol; EMSA, electrophoretic mobility-shift assay.

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example, binding of LPS to membrane-bound CD14 was not saturable by standard criteria and required the presence of serum cofactors. In addition, the aggregation state of LPS in aqueous suspension could not be defined, and the specific activity of the labeled probe was orders of magnitude less than probes used in typical binding studies using labeled protein ligands. Furthermore, some investigators interpreted the results of these previous studies as consistent with a mechanism whereby the LPS antagonists inhibit signaling by interacting with a domain on CD14 that is distinct from the LPS-binding domain.

To determine if CD14 was, in fact, the target of the LPS-receptor antagonists, as well as to better understand the mechanisms of CD14-mediated cellular activation, we sought to exploit the unusual pharmacology of LPS and its analogs in combination with molecular genetic techniques. We initially hypothesized that CD14 was the molecular target of the LPS antagonists. Mouse macrophages responded similarly to both LPS and lipid IV_A, and these responses were inhibited by RSLA. If CD14 were the pharmacologic target of the LPS antagonists and species-related differences between the primary structure of CD14 accounted for the specific pharmacology of lipid IV_A and RSLA, then Chinese hamster ovary (CHO) cells and human HT1080 fibrosarcoma cells transfected with mouse-derived CD14 cDNA would respond to both LPS and lipid IV_A as agonists. In addition, RSLA would be expected to act as an antagonist of these responses in both cell lines. Furthermore, if CD14 were the pharmacologic target of the LPS antagonists, CHO or HT1080 cell lines transfected with human-derived CD14 cDNA would be expected to respond to LPS and be antagonized by either lipid IV_A or RSLA.

The results of these experiments disproved our hypothesis stated above. CD14 is not the pharmacologic target of RSLA and lipid IV_A. In view of our findings, we propose a model of LPS signal transduction in which a lipid A recognition protein discriminates between the bioactive moieties of CD14-bound LPS, RSLA, or lipid IV_A. We believe that this protein will prove to represent a CD14-associated signal-transducing protein.

MATERIALS AND METHODS

Reagents. Unless otherwise indicated, reagents were obtained from Sigma. Phosphate-buffered saline (PBS) and Ham's F-12 were obtained from BioWhittaker. Heat-inactivated fetal bovine serum (FBS; LPS < 10 pg/ml) was obtained from HyClone. Human serum was prepared from clotted whole blood from healthy volunteers and heat-inactivated at 56°C for 45 min. Ciprofloxacin was from Miles. *Salmonella minnesota* ReLPS (the LPS type) was obtained from Nilofor Qureshi and Kuni Takayama (Middleton Veterans Administration Hospital, Madison, WI). Synthetic lipid IV_A was purchased from ICN. Eisai Research Institute (Andover, MA) provided the synthetic RSLA (compound B287; ref. 22), a synthetic analog that had activity identical to natural RSLA when tested with macrophage cell lines and whole human blood *ex vivo*. Lipids were prepared at 1 mg/ml in PBS and stored at −20°C. Before use, the suspensions were thawed and sonicated in an 80-W water bath sonicator (Laboratory Supplies, Hicksville, NY) for 2 min.

Cell Lines. CHO-K1 (ATCC CCL61), HT1080 (ATCC CCL121), and 70Z/3 (ATCC TIB158) cells were obtained from the American Type Culture Collection. Expression vectors pCDNA1 and pCDNA1/NEO were from Invitrogen and were used to transfect human and murine CD14 cDNA, respectively. The clonal CHO/CD14^{hu} and CHO/NEO cell lines were obtained by stable cotransfection of CHO-K1 cells with a human-derived CD14 cDNA (pcDNA1-CD14^{hu}) and pKONEO or pKONEO alone, respectively, as described in ref. 12. A clonal CHO line transfected with murine CD14 (designated CHO/CD14^{mu}) was derived by stable transfection of CHO-K1 cells with mouse-derived CD14 cDNA (ref. 23; pcDNA1/NEO-CD14^{mu}) by the same methods as described (12), except that polyclonal anti-CD14^{mu} serum was used for flow cytometry analysis. Clonal cell lines from human HT1080 fibrosarcoma cells were similarly obtained by transfection of HT1080 cells with human-derived CD14 cDNA (12) or mouse-derived CD14 cDNA (pcDNA1/NEO-CD14^{mu}). These lines are designated HT1080/CD14^{hu} and HT1080/CD14^{mu}, respectively. Control HT1080 fibrosarcoma cells, designated HT1080/NEO, were transfected with selectable vector pcDNA1/NEO alone. The isolation of murine 70Z/3 pre-B lymphocyte cells expressing human CD14 (RV70Z/3-hCD14) as well as the control cells (RV70Z/3-pCLG), transfected with the retroviral vector lacking the CD14 cDNA, was described (24).

Cell Culture and Stimulation Conditions. The HT1080 cell lines were cultured in α-minimal Eagle's medium (α-MEM)/10% heat-inactivated FBS/ciprofloxacin (10 μg/ml) in a 5% CO_2/95% atmosphere at 37°C. The CHO cell lines were cultured under similar conditions, except that Ham's F-12 medium was substituted for α-MEM. The day before each experiment, 1.0×10^6 cells were plated per well in a six-well tissue culture dish (Costar) in fresh medium. At stimulation, the medium was aspirated and replaced with 1 ml of culture medium/2% human serum containing the appropriate stimulant or vehicle. Culture dishes were returned to a 5% CO_2/95% atmosphere at 37°C for the indicated times. The 70Z/3 cell lines were cultured in suspension at 5% CO_2/95% atmosphere at 37°C in RPMI 1640 medium with glutamine containing 10% FBS, penicillin G (50 units/ml), streptomycin sulfate (50 μg/ml), 2-mercaptoethanol (50 μM), and Geneticin (1 mg/ml). The 70Z/3 cell lines were plated in 24-well tissue culture dishes (Costar) at a density of 1.0×10^6 cells per well in 1 ml of growth medium. Stimulators were added to the cells from ×100 stock solutions in PBS.

Preparation of Peritoneal Exudate Macrophages from Chinese Hamsters. Animal experiments were conducted in strict accordance with protocols approved by the Boston City Hospital Animal Care Committee. Chinese hamsters were purchased from Cytogen (West Roxbury, MA). Peritoneal macrophages were elicited by i.p. injection of 20-week-old Chinese hamsters with 3 ml of sterile 5% (wt/vol) thioglycolate medium (Difco) prepared in pyrogen-free water (Baxter Healthcare, Deerfield, IL). Three days later, the animals were sacrificed by ether anesthesia, and peritoneal exudate cells were harvested by peritoneal lavage using 6 ml of PBS. Cells were pelleted by centrifugation at $200 \times g$ for 8 min at 4°C. The cell pellet was subjected to hypotonic lysis as described (25), washed twice in complete RPMI medium, and resuspended at a density of 1.25×10^6 cells per ml. A 2-ml portion of the cell suspension was added per well in a six-well tissue culture dish, allowed to adhere to the dish for 1 hr in 5% CO_2/95% atmosphere at 37°C, and washed again with complete medium. After an additional 1-hr incubation, the appropriate stimulant or vehicle was added. The plates were incubated at 5% CO_2/95% atmosphere at 37°C for 1 hr before extraction of crude nuclear proteins.

Nuclear Extract Preparation. The procedure used was a modification of a published procedure (11). After stimulation, cells were washed with ice-cold PBS/2% FBS (PBS/FBS) and then harvested mechanically with a rubber policeman and 1 ml of PBS/FBS. Cells were collected by centrifugation for 5 sec at ≈11,600 × *g* (speed 12) in an Eppendorf Microfuge (model 5415C). The cell pellet was resuspended in 400 μl of buffer I (11) and incubated for 15 min on ice. The solution was adjusted to 0.5% Nonidet-P40 [25 μl of a 10% (vol/vol) solution] and mixed (vortex at full speed) for 10 sec. Nuclei were collected by centrifugation for 5 min at ≈2000 × *g* (speed 2) and gently resuspended in 50 μl of buffer II (11); the tubes were then incubated on ice for 15 min. Extracts were cleared by centrif-

ugation at full speed (speed 14; ≈13,500 × *g*) for 15 min; the supernatants were transferred to fresh microcentrifuge tubes and were then stored at −80°C. Extracts (10 μl) were assayed for protein concentration using the Bio-Rad protein concentration reagent according to the manufacturer's instructions.

Electrophoretic Mobility-Shift Assay (EMSA). Crude nuclear extracts were assayed for nuclear factor κB (NF-κB) DNA binding as described (11), except that only 7 μl of the 20-μl DNA-binding reactions were analyzed by nondenaturing gel electrophoresis.

Analysis of Surface IgM Expression in the 70Z/3 Cell Lines. Expression of surface IgM was assayed by flow cytometric analysis on a FACScan-Consort VAX flow microfluorimeter (Becton Dickinson) using fluorescein isothiocyanate-conjugated rat anti-mouse IgM (Becton Dickinson) as described (25).

RESULTS

We have previously reported that transfection of CHO-K1 cells with a human-CD14 cDNA conferred at least two macrophage-like responses to LPS, including the release of arachidonic acid (12) and the translocation of NF-κB from the cytosol to the nucleus (11). Nuclear translocation of NF-κB and related *c-rel* transcription factors allows them to bind specific cis-acting regulatory elements important for transcriptional regulation of LPS-inducible proinflammatory cytokine genes (26, 27). Nuclear localization of NF-κB in response to endotoxin occurs independently of ongoing transcription and translation (11) and can be used as a simple criterion for LPS mimetic activity after exposure to various lipid A-like molecules.

To test our hypothesis that CD14 may be the target of LPS antagonists, cell lines transfected with either human or mouse-derived CD14 were exposed to LPS alone or in combination with synthetic RSLA or lipid IV_A for 60 min. Nuclear NF-κB levels were detected by the ability of proteins in nuclear extracts to retard the mobility of a radiolabeled DNA probe containing the murine immunoglobulin κ light-chain gene enhancer sequence in the EMSA. Mock-transfected CHO/NEO or HT1080/NEO cells did not translocate NF-κB after exposure to LPS, lipid IV_A, or RSLA, although both lines responded to an alternative, non-CD14-mediated ligand, tumor necrosis factor α (Fig. 1*A*). In contrast to these control cell lines, nuclear levels of NF-κB increased in the CHO/$CD14^{hu}$ and CHO/$CD14^{mu}$ cell lines after exposure to 10 ng of LPS/ml (Fig. 1*B*, lane 2). Inclusion of RSLA or lipid IV_A at 1000 ng/ml did not inhibit this response in either the CHO/$CD14^{hu}$ or CHO/$CD14^{mu}$ cell lines. Instead, both compounds exhibited LPS mimetic activity in the absence of added LPS in both CD14-transfected Chinese hamster cell lines (Fig. 1*B*, lanes 3 and 5, respectively). Treatment of HT1080/$CD14^{hu}$ and HT1080/$CD14^{mu}$ cells with 10 ng of LPS/ml similarly resulted in nuclear localization of NF-κB (Fig. 1*B*). Neither RSLA nor lipid IV_A induced NF-κB translocation in the CD14-transfected HT1080 cell lines (Fig. 1*B*, lanes 3 and 5, respectively). Most importantly, the observed response to LPS was inhibited by incubation with 1000 ng of RSLA or lipid IV_A/ml (Fig. 1*B*, lanes 4 and 6, respectively).

The results of these experiments done with a human or mouse-derived CD14 cDNA expressed in a human genetic background were virtually identical to those obtained using human monocytes/macrophages (15) and the human THP-1 promonomyelocytic leukemia cell line (unpublished results), which were also antagonized by both of these agents. The specificity of the response observed in CHO/$CD14^{hu}$ and CHO/$CD14^{mu}$ cells to these lipid A-like molecules was independent of the species from which the transfected CD14 cDNA was derived (i.e., human or mouse). The lack of correlation between the species-specific effects of these lipid A analogs

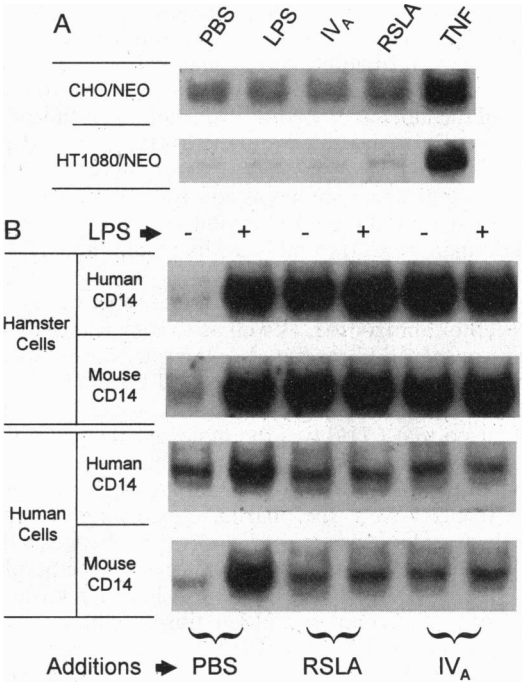

FIG. 1. CD14 expression confers sensitivity to endotoxin but not species specificity. (*A*) Mock-transfected CHO/NEO and HT1080/NEO cells were treated in 1 ml of Ham's F-12 medium/2% heat-inactivated human serum by adding 10 μl of PBS containing no additions or an equal volume of PBS containing LPS, RSLA, or lipid IV_A (final concentration, 100 ng/ml). As a control, CHO/NEO and HT1080/NEO cells were stimulated with either mouse or human tumor necrosis factor (TNF, final concentration, 100 ng/ml; Genzyme), respectively. Nuclear proteins were prepared, and nuclear levels of NF-κB were measured by using the EMSA. The figure shows the portion of the gel containing nuclear NF-κB bound to probe DNA. (*B*) The CD14-transfected cell lines indicated were incubated in 1 ml of Ham's F-12 medium/2% human serum with or without 10 ng of LPS/ml at 37°C for 60 min. In some wells, cells were exposed simultaneously to LPS plus either RSLA or lipid IV_A (final concentration, 1000 ng/ml). Nuclear extracts were prepared and analyzed as described.

and the species from which CD14 was derived indicated that the pharmacologic target for lipid IV_A is not CD14. Rather, these data are most consistent with the existence of a factor encoded in the genome of the target cell, distinct from CD14, which possesses the ability to discriminate between the lipid A moieties of LPS, RSLA, and lipid IV_A. We hypothesize that this factor most likely functions as the signal-transducing component of the endotoxin signaling system in leukocytic phagocytes.

Native Hamster Phagocytes Are Responsive to RSLA and Lipid IV_A. This model of CD14-dependent LPS-inducible signal transduction suggested that the species-specific pharmacology of lipid IV_A and RSLA depended upon the particular species from which the proposed CD14-dependent lipid A recognition protein was derived. We predicted that native hamster phagocytic leukocytes should also respond to RSLA and lipid IV_A because they would be expected to express the same genetic element encoding the RSLA/lipid IV_A-sensitive protein expressed in the CD14-transfected CHO cell lines. Peritoneal exudate macrophages were elicited in Chinese hamsters with thioglycolate and then harvested by peritoneal lavage. Adherent exudate cells were plated in tissue culture dishes and exposed for 60 min to either LPS, RSLA, or lipid IV_A at a concentration of 10 or 100 ng/ml in complete RPMI medium. Nuclear extracts were prepared from these cells, and NF-κB levels were measured by EMSA. As predicted, LPS, RSLA, and lipid IV_A were effective at inducing nuclear

localization of NF-κB compared to control hamster macrophages exposed to PBS alone (Fig. 2). These effects of RSLA and lipid IV_A were identical to those seen using hamster fibroblasts transfected with either mouse or human-derived CD14 cDNAs (Fig. 1*B*).

Human CD14 Enhances Sensitivity of a Mouse pre-B Cell Line to Lipid IV_A. We extended this analysis to a mouse pre-B cell line, 70Z/3, to determine whether expression of the human-derived CD14 cDNA in a mouse genetic background would confer increased responsiveness to lipid IV_A, or whether, as a result of the expression of human CD14, lipid IV_A would antagonize the effects of LPS. Treatment of wild-type 70Z/3 lymphocytes with endotoxin or lipid IV_A induces expression of surface IgM molecules associated with the differentiated immature B-cell phenotype (28). Expression of human CD14 in 70Z/3 cells has been shown to increase sensitivity of these cells to LPS (14, 24, 29). 70Z/3 pre-B lymphocytic cells expressing human CD14 (RV70Z/3-hCD14) and control cells (RV70Z/3-pCLG), transfected with the retroviral vector lacking the *CD14* gene (24) were incubated for 20 hr with increased concentrations of LPS or synthetic lipid IV_A. Surface IgM was measured using flow cytometric analysis using fluorescein isothiocyanate-conjugated rat anti-mouse IgM. As expected, expression of human CD14 in RV70Z/3-hCD14 cells conferred increased sensitivity to LPS compared to control RV70Z/3-pCLG cells (Fig. 3*A*). Similarly, the 70Z/3-hCD14 cell line demonstrated increased sensitivity to synthetic lipid IV_A (Fig. 3*B*). Thus, the murine sensitivity to lipid IV_A (i.e., LPS mimetic) was enhanced by the presence of human CD14. These results showed that human CD14 enhances the ability of lipid IV_A to initiate signal transduction when expressed in a mouse genetic background.

DISCUSSION

The results of experiments done to date characterizing the effects of RSLA and lipid IV_A on a variety of cell types expressing either human, mouse, or hamster CD14 are summarized in Table 1. Data are divided into three groups representing the genetic background of each cell type—i.e., human, mouse, or hamster. When expressed heterologously in nonresponder fibroblasts (CHO, HT1080) or hyporesponsive pre-B cells (70Z/3), CD14 greatly enhanced cellular sensitivity to LPS. The type of response to RSLA and lipid IV_A depended on the genetic background of the cell line and was independent of the species from which the expressed CD14 was derived. These observations are best explained by the existence of a genetic element unique from CD14 that encodes the critical pharmacologic target of these agents. This molecule is most likely a protein that functions to bind and discriminate between LPS, RSLA, and lipid IV_A. We propose that recognition of CD14-bound LPS, RSLA, and lipid IV_A by the hamster

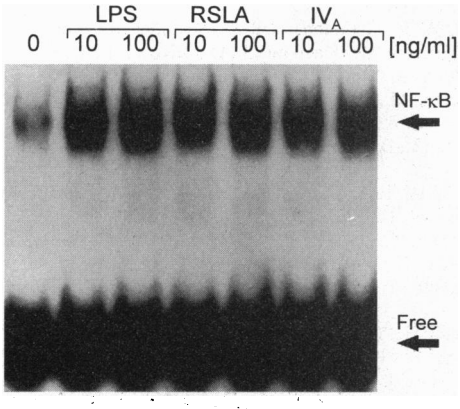

FIG. 2. Chinese hamster macrophages respond to LPS, lipid IV_A, and RSLA. Peritoneal exudate macrophages were prepared from Chinese hamsters, plated in Ham's F-12 medium/10% FBS, and left untreated or exposed to the indicated concentration (ng/ml) of LPS, RSLA, or lipid IV_A. Cells were incubated for 1 hr at 37°C, nuclear extracts were prepared, and NF-κB levels were quantified by EMSA and autoradiography. The arrow labeled NF-κB points to the band that represents retarded NF-κB/DNA complex; the arrow labeled Free points to unbound DNA probe.

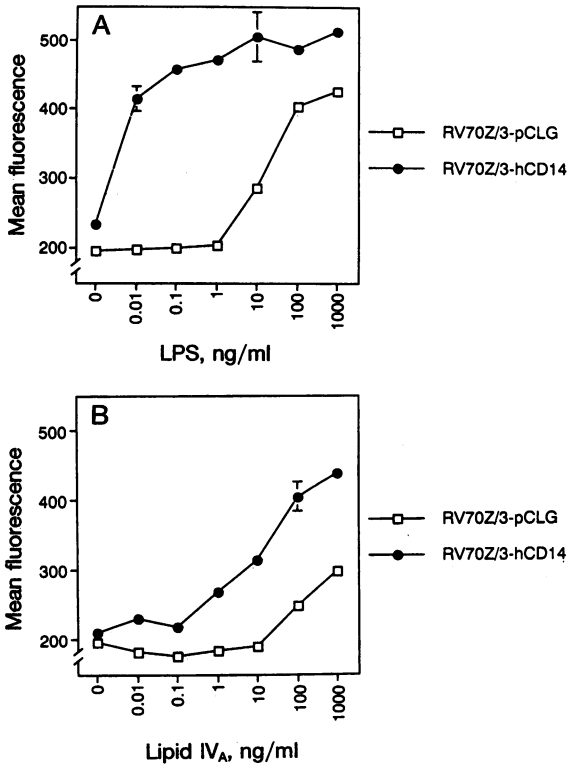

FIG. 3. Effect of LPS and synthetic lipid IV_A on surface IgM expression in 70Z/3 cells. RV70Z/3-hCD14 and RV70Z/3-pCLG cells were plated in 24-well tissue-culture dishes and then incubated with the indicated concentrations of LPS (*A*) and synthetic lipid IV_A (*B*). After 20 hr, surface expression of surface IgM was quantified by immunofluorescence analysis on a FACScan-Consort VAX flow cytometer (Becton Dickinson).

Table 1. Species-specific effects of lipid A-like molecules are determined by the cell species

Cell species	Cell line	LPS	Lipid IV_A	RSLA
Human	Phagocytes*	+	−	−†
	HT1080/CD14^{hu}	+	−	−
	HT1080/CD14^{mu}	+	−	−
Mouse	Phagocytes‡	+	+	−†
	70Z/3	+	+	−§
	70Z/3-CD14^{hu}	++¶	++	−§
Hamster	Peritoneal macrophages	+	+	+
	CHO/CD14^{hu}	+	+	+
	CHO/CD14^{mu}	+	+	+

A plus (+) sign indicates that the agent acted as an agonist in the indicated cells; a minus (−) sign indicates that the agent acted as an LPS antagonist.

*Includes monocytes/macrophages, polymorphonuclear phagocytes, THP-1, and Mono Mac 6 cell lines.

†Both natural and synthetic RSLA antagonized LPS responses in these cell types (ref. 30; data not shown).

‡Includes peritoneal macrophages and RAW 264.7 and J774 macrophage-like cell lines.

§Natural bacterial RSLA was used (23).

¶Double plus sign (++) indicates that expression of human CD14 enhanced the responsiveness of these cells to the indicated agent.

homologue of this LPS-recognition protein results in productive signal transduction. Similarly, we hypothesize that the mouse homologue of the putative LPS-recognition protein binds LPS and lipid IV_A in a productive manner, culminating in signal transduction, but binds RSLA in a nonproductive manner. Finally, the human homologue of this protein responds to LPS only but binds both RSLA and lipid IV_A in a nonproductive manner. Thus, when these compounds are present in excess concentrations relative to LPS, they compete with LPS for binding sites on the surface of this recognition protein and antagonize subsequent signal transduction. These results are consistent with the observation that these LPS antagonists apparently function competitively (15). Although these experiments only examined LPS-induced translocation of NF-κB, we believe the conclusions may be generalized to include all LPS-induced responses seen in leukocytes. This generalization rests on the fact that these antagonists have been shown to block all LPS-inducible responses tested to date in leukocytes, including the translocation of NF-κB.

Although these experiments provide strong evidence for a lipid A-recognition protein to discriminate between the various lipid A moieties, they do not elucidate the mechanism whereby LPS activates responsive cells. Several possibilities exist. A protein that is functionally associated with CD14 may interact directly with CD14-bound LPS. Alternatively, such a receptor might serve as an acceptor for LPS delivered by CD14. The target of these LPS antagonists may even reside in an intracellular location, although there is little evidence supporting a requirement for LPS internalization for signal transduction (see ref. 6 and the references therein).

CD14 has recently been recognized to play a role in potentiating cellular responses to a variety of bacterial and fungal products other than LPS (31). The recognition that a critical LPS-signaling molecule exists downstream from CD14 and confers ligand specificity explains, in part, the apparently promiscuous nature of the CD14 receptor. The downstream LPS-signaling receptor hypothesized above might not be expected to recognize these other ligands. In fact, we have observed that CHO/$CD14^{hu}$ cells failed to respond to lipoarabinomannan from *Mycobacteria* (data not shown), a known CD14 ligand capable of stimulating cytokine release from human phagocytes (31, 32). Thus, an additional conclusion that is inferred from these studies is that different bacterial products share CD14 as a binding receptor but differ with respect to downstream elements necessary for specific cellular activation.

The putative lipid A-recognition molecule implied by these results is expressed in both wild-type hamster CHO-K1 fibroblasts and human HT1080 fibrosarcoma cells before transfection with CD14. Such findings suggest that LPS-recognition molecules are present on a wide variety of cell types and are not limited to cells of known immune function. This remarkably ubiquitous expression of LPS-recognition elements underscores the fundamental physiological requirement for recognition of endotoxin to respond to bacterial invasion in an appropriate and specific manner.

The interferon γ (IFN-γ) signal-transducing system provides a useful paradigm with which to contrast our current knowledge of CD14-dependent LPS-inducible signal transduction. Both human and simian cells, when transfected with the murine IFN-γ-binding receptor (α chain), failed to be activated after exposure to murine IFN-γ and yet were capable of responding normally to human IFN-γ (33). Studies using hybrid cells established that responsiveness to murine IFN-γ could be conferred by introduction of mouse chromosome 6, implying that a second protein, in addition to the IFN-γ receptor α chain, was necessary for species-specific signaling. Indeed, by taking advantage of the species-specific responses to murine IFN-γ, the β chain of the IFN-γ receptor was identified (32). Our observations suggest that a similar strategy might successfully identify the putative CD14-associated LPS-signal transducer. Once identified, it should be possible to rationally approach the study of LPS signaling and design potent therapeutic agents for endotoxin-mediated diseases such as septic shock.

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