Transfection of CD14 into 70Z/3 Cells Dramatically Enhances the Sensitivity to Complexes of Lipopolysaccharide (LPS) and LPS Binding Protein

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Summary

Bacterial endotoxin (lipopolysaccharide [LPS]) causes fatal shock in humans and experimental animals. The shock is mediated by cytokines released by direct LPS stimulation of cells of monocytic origin (monocyte/macrophage [MO]). Recent studies have supported the concept that the plasma protein, LPS binding protein (LBP), plays an important role in controlling MO responses to LPS. Specifically, evidence has been presented to suggest that CD14, a membrane protein present in MO, serves as a receptor for complexes of LPS and the plasma protein LPS binding protein (LBP). In this function CD14 mediates attachment of LPS-bearing particles opsonized with LBP and appears to play an important role in regulating cytokine production induced by complexes of LPS and LBP. The $CD14^-$, murine pre-B cell line 70Z/3 responds to LPS by synthesis of κ light chains and consequent expression of surface IgM. To better understand the role of CD14 in controlling cellular responses to LPS, we investigated the effect of transfection of CD14 into 70Z/3 cells on LPS responsiveness. We report here that transfection of human or rabbit CD14 cDNA into 70Z/3 cells results in membrane expression of a glycosyl-phosphatidylinositol-anchored CD14. When LPS is complexed with LBP, CD14-bearing 70Z/3 cells bind more LPS than do the parental or 70Z/3 cells transfected with vector only. Remarkably, the expression of CD14 lowers the amount of LPS required to stimulate surface IgM expression by up to 10,000-fold when LPS dose-response curves in the $CD14^-$, parental and CD14-bearing, transfected 70Z/3 cells are compared. In contrast, the response of CD14-bearing 70Z/3 cells and the parental 70Z/3 cell line ($CD14^-$) to interferon γ is indistinguishable. LPS stimulation of the parental and CD14-bearing 70Z/3 cells results in activation of NF-kB. These data provide evidence to support the concept that the LPS receptor in cells that constitutively express CD14 may be a multiprotein complex containing CD14 and membrane protein(s) common to a diverse group of LPS-responsive cells.

The endotoxin, or LPS, of Gram-negative bacteria is one of the most potent bacterial toxins known. Picomolar concentrations of LPS induce pathophysiologic changes in humans and experimental animals (1–6) that include enhanced leucocyte adherence to endothelial cells, fibrin deposition in a variety of organs, fever, hypotension, and alterations in cellular energy metabolism. These changes result in the often fatal syndrome known as septic shock or multi-organ failure (7, 8). In humans and experimental animals, it is well established that cytokines released from LPS-stimulated monocytes/macrophages (MO)[1] mediate many of the cellular changes that cause septic shock (1–5).

There is ample, albeit circumstantial, experimental data to support the contention that LPS acts via a specific plasma membrane receptor (9–16). Despite the remarkable progress during the past decade in identifying and characterizing the properties of membrane receptors for nearly every type of biologically active substance, there is little information about the molecular identity of LPS receptors in MO. Given the important role for LPS in human disease, it is important to bridge this gap in our knowledge.

The biological activity of LPS is substantially modified by plasma proteins, and recent studies from our laboratory have identified a novel, receptor dependent pathway of MO stimulation that involves complexes of LPS and the plasma protein LPS binding protein (LBP) (12, 17, 18). LPS-LBP complexes interact with MO via a membrane receptor identified as CD14 (18). CD14 is a 55-kD glycosyl-phosphatidylinositol

[1] *Abbreviations used in this paper:* GPI, glycosyl-phosphatidylinositol; LBP, LPS binding protein; MO, monocytes/macrophages; PI-PLC, phosphatidylinositol-specific phospholipase C; RSV, Rous Sarcoma Virus.

(GPI)-anchored membrane protein that is present on the surface of all cells of monocytic origin (19–21). In this function CD14 serves as a receptor for LPS-bearing particles opsonized with LBP and appears to play an important role in regulating LPS-induced cytokine responses (12, 18, 19).

Because LPS also stimulates cells that do not express CD14, there seems to be both CD14-dependent and CD14-independent pathways for LPS-induced cell activation. In this regard several different membrane proteins distinct from CD14 have been suggested to serve as LPS receptors in both MO and other LPS-responsive cell types (9–16). However, relationships between CD14-dependent and -independent pathways for LPS stimulation have not been elucidated.

Herein we describe experiments with a murine pre-B cell line, 70Z/3, that is induced by LPS or cytokines such as IFN-γ to synthesize κ light chains, and as a consequence expresses surface IgM (12). We have transfected this $CD14^-$ cell line with human or rabbit CD14 and the resulting transfected cells constitutively express surface-bound CD14. The $CD14^+$ 70Z/3 cells bind LPS by LBP/CD14-dependent mechanisms; surface IgM expression can be induced by 10,000-fold less LPS than that required in parental or vector-transfected 70Z/3 cells. In contrast, IgM induction by IFN-γ is identical in parental, control transfectants or $CD14^+$ transfectants. These data provide support for the contention that the CD14 plays a crucial role in recognition of LPS and that the LPS receptor on MO may be multimeric containing CD14 and an as yet unidentified additional membrane protein(s).

Materials and Methods

LPS. Re595 LPS was isolated from lyophilized *Salmonella minnesota* Re595 bacteria as described (1). LPS from *Escherichia coli* 0111:B4 was purchased from List Biological Laboratories (Campbell, CA) and synthetic lipid A was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Stock solutions of LPS or synthetic lipid A were prepared, stored, and utilized as described (22). LBP was isolated from acute phase rabbit serum as described (23).

mAbs. The anti-human CD14, MY4 (IgG2b), was purchased from Coulter Immunology (Hialeah, FL), and the anti-human CD14, 63D3, was isolated from culture supernatants of the hybridoma cell line (HB44; American Type Culture Collection, Rockville, MD); the rat anti-murine CD45 (B220) mAb 14.8 (24) was a gift from P. Linton (The Scripps Research Institute, La Jolla, CA); IgG2b mAbs against the α subunit of human chorionic gonadotropin (Biodesign, Pasadena, CA) or human α-1-antitrypsin (Zymed Laboratories, San Francisco, CA) were used as controls for MY4; FITC-conjugated goat anti-murine IgG was purchased from Gibco Laboratories (Grand Island, NY). Sodium azide was removed from mAb solutions by dialysis against sterile, pyrogen-free PBS.

Cells. Parental or transfected 70Z/3 cells were cultured as described (13).

Other Reagents. $F(ab')_2$ preparations of FITC-conjugated rabbit anti-murine IgM or FITC-conjugated rabbit anti-rat IgG were obtained from Zymed Laboratories. Phosphatidylinositol-specific phospholipase C (PI-PLC) was a gift from M. Low (Columbia University, New York) and used as described (25). Recombinant murine IFN-γ was a gift of R. D. Schreiber (Washington University, St. Louis, MO).

Rabbit and Human CD14 cDNA. The details of cloning rabbit CD14 from an EMBL3 rabbit genomic library (Clontech) and isolation of rabbit CD14 cDNA will be described elsewhere (Lee, J.-D., manuscript in preparation). The nucleotide sequence of rabbit CD14 cDNA has been entered in GenBank (accession no. M85233). Amino acid sequence comparisons from deduced amino acid sequences revealed that rabbit CD14 gene shows 73% and 64% identity with human and murine CD14, respectively. A full-length cDNA clone of human CD14 was a generous gift from B. Seed, (Massachusetts Institute of Technology, Boston, MA) (21).

Expression of Rabbit and Human CD14 in 70Z/3 Cells. The coding region of human or rabbit CD14 was cloned into the XbaI site of a eucaryotic expression vector, pRc/RSV (Invitrogen, San Diego, CA) containing a neomycin resistance gene for the selection of stable transformants and a Rous Sarcoma Virus (RSV) LTR promoter for transcription initiation of inserted DNA. Electroporation was used to transfer the pRc/RSV constructs containing rabbit or human CD14 or the pRc/RSV vector only into 70Z/3 cells as described (26), except that a 400-V/cm, 20-ms pulse was used. G418 (1 mg/ml) was used for stable transformant selection as described (26). Identification of the presence of surface CD14 in transfected 70Z/3 cells was performed with mAbs to CD14, MY4, or 63D3, by standard procedures using FITC goat anti-murine IgG as a second antibody.

PCR Analysis of 70Z/3 Cells. $Poly(A)^+$, RNA was isolated from RAW 264.7 or 70Z/3 cells with a Micro Fast Track Kit (Invitrogen) and first-strand cDNA was synthesized using oligo(dT) as a primer as described (27). PCR were performed as described (27) annealing at 56°C. These reactions used 1 U/reaction of Perfect Match DNA polymerase enhancer (Stratagene, La Jolla, CA). Synthetic oligonucleotides specific for murine CD14 were prepared from the published sequence (28): 5′-CGTCTAGAAGAACACCATCGCTGTAAAG-3′; 5′-GCTCTAGAATTCGTTGACGAGGACCCGC-3′; and for rabbit CD14 as recorded in GenBank (accession no. M85233): 5′-CGTCTAGAACCATGGAGCCCGTGCCCTGCT-3′; 5′-CCTCTAGATTAGATAAGCCCCTGC-3′.

Stimulation of Surface IgM Expression in 70Z/3 Cells. Parental or transfected 70Z/3 cells were cultured in T-75 flasks with RPMI 1640 containing 10 mM Hepes, 10% FCS, 2 mM L-glutamine, 0.05 mM 2-ME, and antibiotics as described (13). Cells used to measure IgM expression were removed from T-75 cultures and washed three times with serum-free RPMI 1640 containing Hepes, L-glutamine, 2-ME, and antibiotics as described (13). The washed cells were then placed into 48-well plates (Costar, Cambridge, MA) in 0.5 ml of RPMI 1640 at a cell density of 2×10^6 cells/ml under serum-free conditions; in some experiments, FCS was added as noted in the text or figure legends. In experiments performed under serum-free conditions, purified LBP was added to the cells immediately followed by the addition of LPS. Surface IgM expression in 70Z/3 cells stimulated with LPS or other agonists was quantitated in flow cytometry studies with an $F(ab')_2$, FITC rabbit anti-murine IgM (Zymed Laboratories) as described (13, 26); data are expressed as the percent of cells expressing surface IgM. The specificity of the fluorescence changes detected was confirmed using a control $F(ab')_2$, FITC rabbit anti-rat IgG (Zymed Laboratories). Flow cytometry analysis was performed using a FACScan® instrument, and data were analyzed with the LYSIS II computer program (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Data for IgM expression are reported for an individual experiment; all experiments have been replicated at least twice yielding similar results.

Measurement of NF-kB Activation. NF-kB activity was measured in nuclear extracts exactly as previously described (29, 30).

Results

Expression of CD14 in 70Z/3 Cells. We made pRc/RSV constructs containing full-length cDNA for either rabbit or human CD14 (21) and transfected 70Z/3 cells with the complete construct (70Z/3-rCD14 or 70Z/3-hCD14) or the vector alone (70Z/3-RSV). Identification of stable clones expressing CD14 was accomplished using flow cytometry of cells stained with the anti-human CD14 mAb MY4 (31) that also cross-reacts with native rabbit CD14 (R. J. Ulevitch, unpublished data). Surface expression of CD14 in the transfectants was established by treatment of cells with PI-PLC, before antibody staining. Flow cytometry data from studies of 70Z/3 cells transfected with human CD14 cDNA (70Z/3-hCD14) analyzed with MY4 before and after PI-PLC treatment are shown in Fig. 1. As a control we stained the 70Z/3-hCD14 with an anti-murine CD45 mAb since this membrane protein is expressed on the surface of 70Z/3 cells, but is not GPI linked (32). Staining of 70Z/3-hCD14 cells with MY4 revealed a homogeneous population of fluorescent cells not detected when MY4 was replaced by an isotype control mAb. Identical staining was observed with another anti-human CD14 antibody, 63D3 (data not shown). Importantly, staining with MY4 was lost in cells treated with PI-PLC. In contrast, PI-PLC pretreatment did not change the reactivity with the anti-CD45 mAb. An identical set of observations were recorded in studies with 70Z/3 cells transfected with rabbit CD14 cDNA (70Z/3-rCD14) (data not shown). There are no mAbs to murine CD14 presently available that would permit establishing the absence of CD14 expression on parental or 70Z/3-RSV cells by means of flow cytometry or surface labeling/immunoprecipitation studies. Since the cDNA sequence of murine CD14 is known (20, 28), we were able to use PCR to verify the absence of murine CD14 mRNA in 70Z/3 cells. Primers specific for murine and rabbit CD14 were synthesized and used to quantify expression of murine or rabbit CD14 mRNA in 70Z/3-RSV or 70Z/3-rCD14 cells. The murine macrophage–like cell line RAW 264.7 was used as a source of murine CD14 mRNA to confirm the specificity of the primers and PCR reaction. The results of this experiment shown in Fig. 2 demonstrate that a strong mRNA (1.3-kb) band was detected in RAW264.7 cells with the murine CD14–specific primers. PCR analysis of RAW264.7 cells with rabbit CD14–specific primers also revealed a much weaker signal at 1.3 kb. This is not surprising considering the highly conserved sequences of murine and rabbit CD14. In contrast, no PCR product was observed in either 70Z/3-RSV or 70Z/3-rCD14 cells using murine CD14–specific primers. Importantly, however, the rabbit CD14–specific primers revealed a strong signal of appropriate size (1.3 kb) in the 70Z/3-rCD14 cells. In control experiments we ascertained that if the 70Z/3 cells contained 1/1,000 of the murine CD14 mRNA present in RAW264.7 cells our PCR strategy would detect this.

The 1.3-kb PCR product detected in RAW 264.7 cells and in 70Z/3-rCD14 cells was isolated, subcloned into a pRc/CMV vector, and sequenced. We obtained sequences identical to the known nucleotide sequences of murine (20, 28) or rabbit CD14 cDNA, respectively (GenBank accession no. M85233).

LBP/CD14-dependent Binding of FITC-LPS to 70Z/3-hCD14 Cells. We next performed experiments to compare binding of LPS to 70Z/3-RSV or 70Z/3-hCD14 cells. To do this we prepared a fluorescent derivative of Re595 LPS (FITC-LPS) and used flow cytometry to examine binding at 22°C of FITC-Re595 LPS to 70Z/3-RSV or 70Z/3-hCD14 cells. Binding of FITC-LPS was evaluated in the presence or absence of LBP.

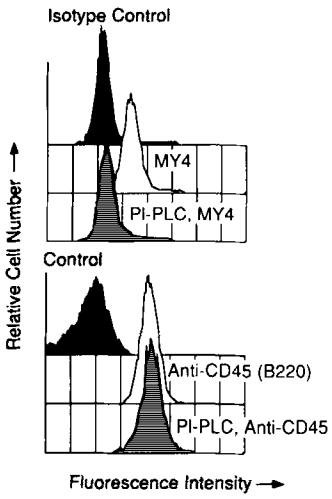

Figure 1. FACS® analysis of 70Z/3-hCD14 cells. 70Z/3 cells were stained with the mAb MY4 (anti-human CD14) or 14.8 (anti-murine CD45) before or after treatment with PI-PLC. PI-PLC treatment was performed as noted in the legend to Table 1. An IgG2b mAb (anti-human α-1-antitrypsin) and normal rat serum served as specificity controls.

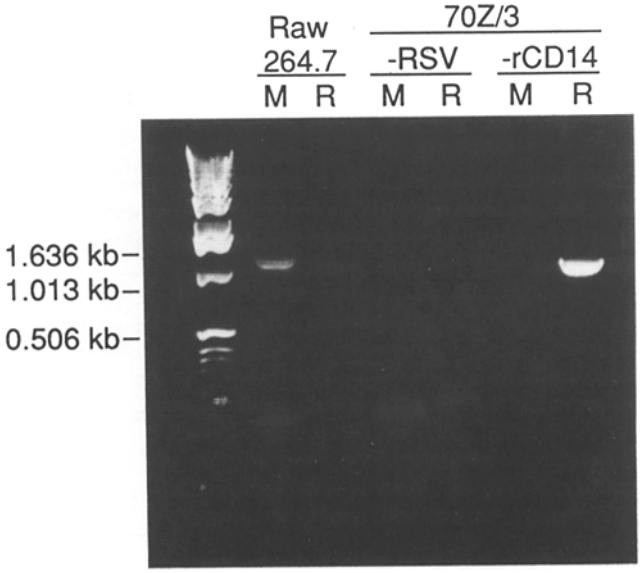

Figure 2. PCR analysis of RAW 264.7 cells and 70Z/3-RSV or 70Z/3-rCD14 cells for CD14 mRNA. Procedures and composition of murine and rabbit specific primers are noted in Materials and Methods. *M*, PCR performed with murine CD14-specific primers; and *R*, PCR performed with rabbit-specific primers.

A group of cells for each condition was also treated with PI-PLC. These data are expressed as median channel number, a measurement that reflects the amount of FITC-LPS bound per cell. Results of a single, representative experiment are shown in Table 1. In the absence of LBP, the median channel number was nearly identical in 70Z/3-RSV and 70Z/3-hCD14 cells, and PI-PLC pretreatment did not reduce the median channel number. In contrast, an increase in median channel number in the presence of LBP was observed with 70Z/3-hCD14 cells. This increase was completely inhibited by pretreatment with PI-PLC. Under the conditions of this experiment PI-PLC treatment removes >90% of the CD14 expressed in the 70Z/3-hCD14 cells as measured by staining with MY4.

Two additional experiments to be described elsewhere (K. Kato, manuscript in preparation) provide evidence for the importance of CD14 expression in the binding of LPS to CD14-bearing 70Z/3 cells. Inclusion of the anti-human CD14 mAb MY4 or a 100-fold excess of nonfluoresceinated Re595 LPS reduced the median channel number to the values observed with 70Z/3-RSV cells or PI-PLC-pretreated 70Z/3-hCD14 cells (data not shown).

Functional Consequences of CD14 Expression in 70Z/3 Cells. 70Z/3 cells respond to LPS or cytokines such as IFN-γ by synthesizing κ light chains with subsequent expression of surface IgM (12, 13, 26, 33). To determine if CD14 expression changes the response of 70Z/3 cells to these agonists, we quantitated surface IgM expression 24 h after stimulation with varying doses (1 pg/ml to 1 μg/ml) of LPS or IFN-γ. To study LPS responses we used LPS isolated from rough (Re595 LPS) or smooth (0111:B4 LPS) form bacteria or synthetic lipid A and compared cellular responses in the presence or absence of added LBP.

70Z/3 cells transfected with vector alone (70Z/3-RSV) or 70Z/3-rCD14 cells were placed in 48-well plates in serum-free RPMI 1640 and stimulated with the two different LPS preparations or synthetic lipid A in the presence and absence of added LBP. Results from this experiment are shown in Fig. 3. Dose-response curves observed for the two different LPS preparations or synthetic lipid A using CD14⁻, 70Z/3-RSV cells were essentially the same in the presence and absence of added LBP. Moreover, identical dose-response curves were obtained using the parental 70Z/3 cells (data not shown). These studies also revealed that concentrations of LPS or synthetic lipid A >0.1 μg/ml are required to produce measurable changes in surface IgM expression during the 24-h stimulation period. In contrast to the LPS dose-response data obtained with CD14⁻, 70Z/3 cells, stimulation of 70Z/3-rCD14 with as little as 10 pg LPS/ml in the presence of LBP resulted in induction of surface IgM. In the presence of LBP, Re595 or 0111:B4 LPS produced maximum responses with 0.1–1 ng LPS/ml, and synthetic lipid A was maximally active between 1 and 10 ng/ml. Thus, expression of CD14

Table 1. *PI-PLC Pretreatment Inhibits LBP-dependent Binding of FITC-LPS to 70Z/3-hCD14 Cells*

Cell	LPS	LBP	PI-PLC	Median channel number
70Z/3-RSV	–	–	–	2.16
	+	–	–	4.66
	+	–	+	4.63
	+	+	–	5.56
	+	+	+	5.29
70Z/3-hCD14	–	–	–	2.20
	+	–	–	4.31
	+	–	+	4.25
	+	+	–	8.27
	+	+	+	5.35

PI-PLC treatment (2.5 U/ml) of 70Z/3-RSV or 70Z/3-hCD14 cells (10^6 cells/ml) in HBSS was performed at 37°C for 60 min. After centrifugation, the cells were resuspended in HBSS containing 0.1% BSA and 0.05% sodium azide; FITC-Re595 LPS (20 ng/ml) ± 300 ng/ml rabbit LPB were added the cells maintained for 30 min at 22°C and subjected to FACS® analysis.

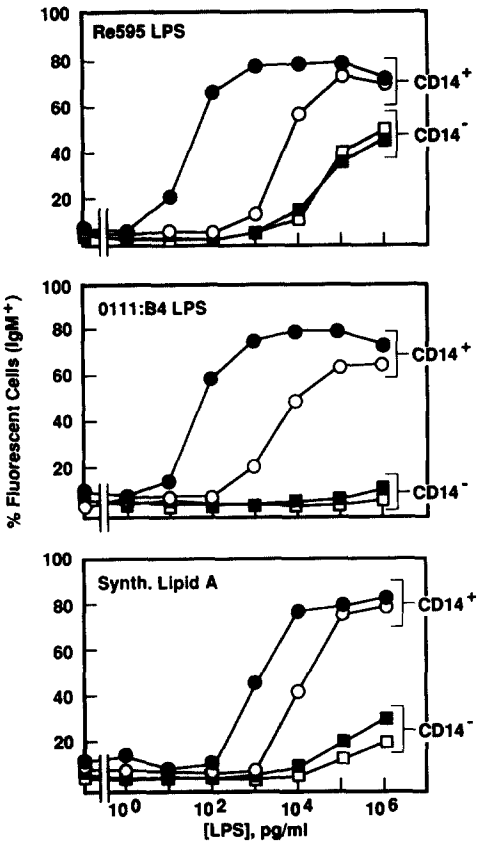

Figure 3. Stimulation of 70Z/3-RSV cells (CD14⁻) or 70Z/3-rCD14 cells (CD14⁺) with varying doses of LPS (Re595 or 0111:B4 LPS) or synthetic lipid A. Stimulation in the presence of 100 ng/ml rabbit LBP (● and ■) and in the absence of LBP (○ and □). Surface IgM expression measured 24 h after addition of LPS or LPS-LBP is described in Materials and Methods.

results in up to a 10,000-fold decrease in the concentration of LPS required to stimulate surface IgM expression. Even in the absence of LBP, the 70Z/3-rCD14 cells displayed substantially increased sensitivity to LPS, suggesting that LPS can directly interact with CD14. In the studies not shown here we observed the same effects using 70Z/3-hCD14 cells stimulated with Re595 LPS or Re595 LPS-LBP complexes.

In studies with 70Z/3-hCD14 or 70Z/3-rCD14 we observed that we could replace LBP with 10% FCS, but not with albumin-containing medium (AIM-V medium; Gibco Laboratories) (data not shown). Subsequent experiments were therefore performed with either complexes of LPS and LBP in serum-free medium (RPMI 1640) or with medium containing FCS as noted in figure legends.

To exclude the possibility that transfection itself and/or subsequent expression of CD14 nonspecifically changes the response of 70Z/3 cells to agonists unrelated to LPS, we evaluated the response of the parental or transfected 70Z/3 cells to IFN-γ. Unlike the marked effects of CD14 expression on LPS responses, data shown in Fig. 4 demonstrate that induction of surface IgM by IFN-γ in 70Z/3, 70Z/3-RSV, or 70Z/3-rCD14 cells is identical. Comparable results were noted when IFN-γ was used to stimulate 70Z/3-hCD14 cells (data not shown).

LPS-induced NF-kB Activation in 70Z/3 Cells. In 70Z/3 cells induction of κ light chain synthesis by LPS or cytokines involves a marked increase in transcription of κ light chain mRNA. LPS stimulation has been shown to activate two nuclear factors, NF-kB and OTF-2 (12). In contrast, IFN-γ induces surface IgM expression without activation of NF-kB (34, 35). Since both NF-kB-dependent and -independent pathways for κ light chain mRNA induction are available in 70Z/3 cells, we next asked whether the very low stimulatory doses of LPS that stimulate CD14-bearing 70Z/3 cells also activate NF-kB. To do this we prepared nuclear extracts from 70Z/3-RSV or 70Z/3-rCD14 cells stimulated with LPS for 4 h and examined these extracts for NF-kB activity in gel shift assays. Results shown in Fig. 5 indicate that stimulation of 70Z/3-rCD14 cells with 100 pg/ml Re595 LPS resulted in marked increase in NF-kB, indicating that the low concentrations of LPS that stimulate surface IgM expression also activate NF-kB. In contrast, while 100 ng/ml Re595 LPS stimulated 70Z/3-RSV to activate NF-kB, an LPS dose of 100 pg/ml was without effect. Northern blot analysis of κ light chain mRNA performed with total RNA isolated from LPS-stimulated 70Z/3-RSV or 70Z/3-rCD14 cells revealed that in the absence of added LPS, κ light chain mRNA was not detectable in either cell type. Addition of as little as 100 pg Re595/ml to 70Z/3-rCD14 cells produced a strong κ light chain mRNA signal at 12 h in the Northern blots, while in contrast, 10 ng/ml Re595 LPS was required to achieve a comparable signal in 70Z/3-RSV cells (data not shown).

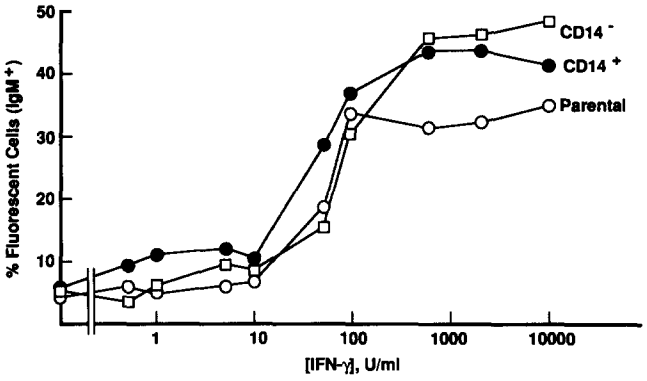

Figure 4. Stimulation of 70Z/3 (parental cells, CD14⁻), 70Z/3-RSV (CD14⁻), or 70Z/3-rCD14 (CD14⁺) cells by recombinant murine IFN-γ. Cell culture performed in RPMI 1640 containing 10% FCS FACS® analysis of surface IgM expression performed after 48 h are described in Materials and Methods.

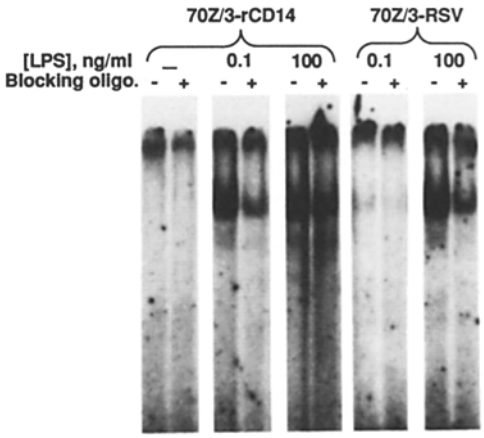

Figure 5. LPS-induced NF-kB activation in 70Z/3-RSV or 70Z/3-rCD14 cells. Cells were maintained at 37°C for 4 h with Re595 LPS in RPMI 1640 containing 5% FCS and nuclear extracts prepared as described (30). Nuclear extracts (2 μg) were mixed with 0.1 μg of the 32P-NF-kB probe, and as noted in the figure, in alternating lanes a 10-fold excess (+) of unlabeled NF-kB oligonucleotide was added. This mixture was subjected to electrophoresis in a 6% acrylamide gel, and after gel drying overnight, autoradiography was performed. The details of methods are as described (29).

Inhibition of LPS Stimulation of 70Z/3 Cells. We next sought to show that CD14 plays a crucial role in mediating enhanced responsiveness to LPS by using a mAb against human CD14, MY4, to block LPS stimulation of CD14-bearing cells. The result of pretreatment of 70Z/3-RSV or 70Z/3-hCD14 cells with MY4 or an isotype control mAb on LPS or LPS-LBP stimulation is shown in Table 2. MY4 blocked effects of both LPS and LPS-LBP complexes on 70Z/3-hCD14 cells. Raising the LPS concentration overcomes the inhibition, but required 10–100-fold more LPS to achieve comparable levels of IgM expression observed in 70Z/3 cells treated with an isotype control. In contrast, MY4 has no effect on the LPS dose-response curve observed with 70Z/3-RSV cells. In experiments not shown here we have observed similar inhibition with several other murine mAbs (28C5 and 18E12) produced by immunization with recombinant human CD14 (R. J. Ulevitch, unpublished data). The IFN-γ dose-response

Table 2. *Effect of Anti-CD14 (MY4) on LPS-induced Stimulation of 70Z/3-RSV or 70Z/3-hCD14 Cells*

	70Z/3-RSV (−LBP)			70Z/3-hCD14 (−LBP)			70Z/3-hCD14 (+LBP)		
LPS	−*	Isotype control‡	MY4§	−	Isotype control	MY4	−	Isotype control	MY4
ng/ml		%			%			%	
0.001	−‖	−	−	−	−	−	5	5	1
0.01	−	−	−	−	−	−	24	27	2
0.1	−	−	−	9	9	1	45	51	12
1	1.5	−	−	30	29	5	64	66	36
10	15	7	16	51	50	24	66	66	49
100	36	34	37	61	59	50	64	70	60
1000	58	55	54	71	68	60	74	72	62

* No pretreatment.
‡ IgG2b mAb, anti-human alpha-1-anti trypsin; 10 μg/ml.
§ [MY4], 10 μg/ml.
‖ No induction.

for stimulation of 70Z/3-RSV or 70Z/3-hCD14 cells was not changed by the presence of MY4 (data not shown).

Discussion

We have performed experiments with 70Z/3 cells, a murine pre-B cell line that responds to LPS by synthesis of κ light chains and consequent surface expression of IgM (12, 26). 70Z/3 cells do not express CD14, and LPS dose-response curves are identical in the presence and absence of LPS binding protein. Transfection of 70Z/3 cells with cDNA coding for human or rabbit CD14 resulted in a stable line expressing a GPI-linked CD14. LPS dose-response curves show that the presence of CD14 reduces the amount of LPS required to induce surface IgM up to 10,000-fold without changing the dose dependency of a cytokine agonist, IFN-γ. These data unequivocally show the importance of CD14 as a receptor for LPS and/or LPS-LBP complexes. Moreover, results in this report provide support for the contention that cells such as MO that constitutively express CD14 recognize LPS by a receptor that includes CD14 and an as yet unidentified membrane protein(s) that may be similar in all LPS-responsive cells.

Although several reports have suggested that human B cells and B cell lines express a CD14-like protein, proof of the presence of authentic CD14 mRNA and/or protein is lacking (36–38). Here we show in several different ways that 70Z/3 cells do not express CD14. The parental (data not shown) or 70Z/3-RSV cells displayed identical dose-response curves using LPS or LPS-LBP complexes with two different types of LPS as well as with synthetic lipid A. In contrast, all studies from our own and other laboratories using LPS-responsive cells that constitutively express CD14 consistently demonstrate marked enhancement of LPS responses by LBP (17, 18, 39). Moreover, in studies to be described elsewhere we show that the human monocyte–like cell line THP-1 fails to demonstrate LBP-dependent LPS responses until CD14 expression is induced after treatment with 1,25-dihydroxy vitamin D3 (R. J. Ulevitch, unpublished data). Finally, recent studies with human MO genetically deficient in CD14 demonstrate reduced LPS binding (40).

More direct evidence for the absence of CD14 in the parental 70Z/3 cells was obtained by PCR analyses with primers specific for murine CD14 in mRNA. PCR failed to reveal the presence of murine CD14 mRNA transcripts in the parental 70Z/3 (data not shown) or 70Z/3-RSV cells. However, the same primers detected authentic murine CD14 mRNA in the murine macrophage–like cell line RAW 264.7. The sensitivity of the PCR method allows for the detection of levels of CD14 mRNA that are 1,000-fold less than expressed in the RAW 264.7 cells. PCR analysis with primers specific for rabbit CD14 clearly demonstrated rabbit CD14 mRNA in 70Z/3-rCD14 transfectants, but not in 70Z/3-RSV cells.

Evidence derived from several different experimental approaches shows that transfection of 70Z/3 cells with either rabbit or human CD14 cDNA results in surface expression of GPI-linked CD14. Using flow cytometry we demonstrated PI-PLC-sensitive fluorescent labeling of CD14 transfected cells by mAbs known to detect CD14 (MY-4 and 63D3) (31). In studies not shown here, detergent-solubilized proteins from surface-radioiodinated 70Z/3-hCD14 cells analyzed by SDS-PAGE after immunoprecipitation with the anti–human CD14 mAbs MY4 and 63D3 revealed protein bands identical to native and recombinant human CD14 (R. J. Ulevitch, unpublished data). Thus, the totality of evidence is consistent with the conclusion that 70Z/3 cells do not express CD14, but that CD14 expression can be achieved by transfection.

Inclusion of as little as 10 ng/ml LBP was sufficient to provide a maximum enhancement of the response of $CD14^+$ transfectants to LPS; increasing the concentration of LBP up to 300 μg/ml provided no additional enhancement and did

not inhibit (data not shown). Our recent studies of LBP enhancement of LPS-induced cytokine production (40a) indicate that other LPS binding proteins, including bactericidal-permeability increasing protein (BPI), which shares amino acid sequence identity with LBP, cannot substitute for LBP. Despite the identification of other LPS binding proteins in serum (41–45), to date, LBP is unique in its effects of LPS responses. Although we have reported here that FCS can substitute for purified LBP, the identity of the protein(s) in FCS responsible for this is not known and requires further study.

Despite the remarkable effect of CD14 expression on LPS dose-response curves, the mechanism of action of LPS in the transfected 70Z/3 cells appears unchanged. Previous studies have demonstrated that stimulation of 70Z/3 cells by LPS is lipid A dependent (33), as are the effects described here with CD14-bearing 70Z/3 cells. LPS stimulation of 70Z/3 cells by LPS has been shown to involve new transcription of κ light chain mRNA and depends on activation of several DNA binding proteins, including NF-κB (12). Here we show that addition of 100 pg/ml LPS to 70Z/3-rCD14 cells activates NF-kB while 70Z/3-RSV requires substantially more LPS to achieve comparable levels of NF-kB activation. Similarly, Northern blot analysis has revealed marked increases in steady-state levels of κ light chain mRNA occurring with 100 pg/ml LPS in CD14-bearing transfectants, while the parental or 70Z3-RSV cells required 100–1,000-fold more LPS to observe comparable changes in mRNA (data not shown).

The data reported herein raise the possibility that the LPS receptor on CD14-bearing cells such as MO may consist of several proteins, including CD14, and other as yet unidentified membrane protein(s) that are common in all LPS-responsive cell types. We suggest that both CD14 as well as additional receptor component(s) bind LPS (lipid A), and binding to both proteins is required to achieve the marked sensitivity to LPS observed with the CD14$^+$, 70Z/3 cells. Previous studies from our laboratory suggested that CD14 is a receptor for complexes of LPS and LBP (18, 39). Observations reported here suggest this view may require modification since LPS dose-response studies and blocking studies with anti-CD14 mAb suggest that CD14 may recognize LPS directly. Biochemical studies underway in our laboratory may also help to clarify this issue.

The LPS receptor on MO may be similar to the high affinity receptor for IL-2, a heterodimer where the presence of both subunits is necessary for expression of the high affinity, functional IL-2 receptor complex, but independent expression of either the α or β subunits of the IL-2 receptor is sufficient to support low affinity IL-2 binding (46, 47). Parallels exist for LPS, since cells devoid of CD14 appear to bind LPS by receptor-dependent mechanisms and cells that do not bind LPS can be engineered to express CD14 and subsequently bind LPS (R. J. Ulevitch, unpublished data). Furthermore, MO genetically deficient in CD14 display a marked reduction in serum-dependent LPS binding (39). Alternatively, the LPS receptor may resemble the IL-6 receptor, which is a heterodimer consisting of a ligand binding subunit and a non-ligand binding membrane glycoprotein (48). Whether binding to CD14 is sufficient to generate a transmembrane signal that causes cell stimulation is not yet known. Preliminary data suggest that complexes of LBP formed with LPS antagonists or partial lipid A structures that bind to CD14 do not stimulate either 70Z/3-CD14 cells (R. J. Ulevitch, unpublished data). However, complete understanding of the role of CD14 in transmembrane signaling requires extensive study.

Derivatives of LPS containing photochemically activated crosslinkers (41) that incorporate a radioactive label have been used to identify membrane proteins that function as LPS receptors (13–15). Membrane proteins distinct from CD14 have been radiolabeled by crosslinking, but in no case has a single protein been isolated, characterized, and shown definitively to be involved in transmembrane signaling. Moreover, the published crosslinking studies have used LPS concentrations that are several orders of magnitude higher than required for cell stimulation, thereby leading to questions about the specificity of crosslinking. The present studies point to new opportunities for crosslinking strategies to identify membrane proteins, in addition to CD14, that are involved in LPS-induced cell stimulation, under conditions where nonspecific interactions of LPS will be minimal. As these studies progress we should be able to better define the function of CD14 in relaying signals from LPS in the extracellular environment to the nucleus and the relationship of CD14 to other membrane proteins involved in LPS recognition.

This work was supported by U.S. Public Health Service grants AI-15136, AI-25563, GM-28485, GM-36854, American Heart Association Fellowship no. 91-60 (J.-D. Lee), and by the Department of Veterans Affairs (T. N. Kirkland). This is publication no. 7192-IMM from The Scripps Research Institutes.

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Received for publication 5 March 1992.

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