Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin

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ABSTRACT Endotoxin stimulates leukocytes to release cytokines that initiate septic shock in humans and animals. CD14, a glycosyl-phosphatidylinositol-anchored membrane glycoprotein, is an endotoxin receptor on leukocytes, and endotoxin binding to CD14 induces cytokine production. Here we show that glycosyl-phosphatidylinositol-anchored or integral membrane CD14 mediates identical cellular responses to endotoxin, including NF- κ B activation and protein tyrosine phosphorylation. We also show that an anti-CD14 monoclonal antibody that does not block endotoxin binding to CD14 nonetheless inhibits cell activation by endotoxin. These findings suggest that binding of endotoxin to cell-surface CD14 is followed by subsequent interactions of the endotoxin-CD14 complex with additional membrane component(s) that enable transmembrane signaling. This function of CD14 may be prototypic for other members of the glycosyl-phosphatidylinositol-anchored family of proteins that do not play a primary role in signal transduction but rather are the principal ligand-binding units of membrane-bound receptor complexes.

Bacterial endotoxin (lipopolysaccharide; LPS), a glycolipid of the outer membrane of all Gram-negative bacteria, acts at picomolar/nanomolar concentrations to induce cytokines and other inflammatory mediators (1, 2). Exposure to LPS often results in a syndrome known as septic shock (3). Considerable evidence has provided support for the contention that pathophysiologic changes leading to septic shock are initiated by cytokines principally released from LPS-stimulated macrophages (4).

Until recently little was known about the identity of receptors and signaling mechanisms for LPS (5–8). CD14, a 55-kDa glycosyl-phosphatidylinositol (GPI)-anchored glycoprotein of leukocytes (9), is a receptor for LPS or complexes of LPS and LPS-binding protein (10–12). A substantial body of data shows that LPS-CD14 interactions control many, if not all, responses to LPS under physiologic conditions (5, 13).

The murine pre-B-cell line 70Z/3 does not express CD14 (11), but LPS stimulates NF- κ B-dependent synthesis and expression of membrane IgM (14). However, in contrast to the subnanomolar LPS concentrations that stimulate leukocytes, micromolar LPS concentrations are needed for activation of 70Z/3 cells. We have shown that expression of GPI-anchored, human CD14 (hCD14) in 70Z/3 cells (70Z/3-hCD14 cells) results in a 1000-fold reduction in the amount of LPS required for NF- κ B activation and surface IgM expression (11). Thus, 70Z/3 cells provide a system to study the function of CD14 as an LPS receptor and to explore, in general, the mechanisms by which GPI-anchored receptors mediate cell activation.

Here we demonstrate that LPS induces identical cellular responses in stably transfected 70Z/3 cells expressing GPI-

anchored or integral membrane forms of CD14. Moreover, we show that it is possible to block CD14-dependent cell activation by addition of an anti-CD14 monoclonal antibody (mAb) that does not block LPS binding to CD14. These findings suggest that CD14 functions solely as an LPS-binding protein on the cell membrane and that binding of LPS to CD14 enables efficient interactions of LPS or the LPS-CD14 complex with additional membrane components involved in signaling. These studies define a function for CD14 that may be prototypic for some other members of the family of GPI-anchored receptors.

MATERIALS AND METHODS

Cells and Reagents. Published methods were used to culture 70Z/3 cells (11), to prepare Re595 LPS and a fluorescein derivative of Re595 LPS [fluorescein isothiocyanate (FITC)-LPS] (11), and to prepare recombinant soluble hCD14 (6). Murine mAbs to hCD14, MY4, FITC-MY4 (Coulter) and 18E12, a mAb that was derived from a panel of mAbs derived from immunization with recombinant soluble hCD14 (D.L., unpublished work), were used as purified IgG preparations. Taxol (Calbiochem) was dissolved in dimethyl sulfoxide and stored in the dark at 4°C. Phosphatidylinositol-specific phospholipase C (PIPLC) was provided by Martin Low (Columbia University, New York).

Vector Construction. DNA encoding hCD14 was inserted into the pRc/RSV vector as described (11). To construct the plasmid encoding hCD14 and the GPI-attachment site from decay-accelerating factor (DAF) (pRc/RSV-hCD14DAF) a *Pvu* II-*Taq* I 144-bp PCR fragment encoding 48 amino acids (Cys-288 to Arg-335) of hCD14 (15), a *Taq* I-*Apa* I 125-bp synthetic DNA fragment encoding the final 37 amino acid residues of DAF (16, 17), and a stop codon were inserted between the *Pvu* II and *Apa* I sites of pRc/RSV-hCD14. The sequence of the synthetic DNA used for this is shown in Fig. 1.

To construct a plasmid containing an insert encoding hCD14 with the transmembrane domain and cytoplasmic tail of tissue factor (TF) (pRc/RSV-hCD14TF) a *Taq* I-*Apa* I 152-bp PCR fragment encoding the final 46 amino acids of TF (18) and a stop codon were inserted into pRc/RSV-hCD14 as above. To construct the plasmid encoding hCD14 with the transmembrane domain and cytoplasmic tail of the murine class I (CI) molecule, H-2K^b (19) (pRc/RSV-hCD14CI), a 225-bp PCR fragment encoding the final 74 amino acids of H-2K^b and a stop codon were inserted into pRc/RSV-hCD14 as above. The nucleotide sequences inserted into plasmids were confirmed by sequencing. Transfection of 70Z/3 cells

Abbreviations: LPS, lipopolysaccharide or endotoxin; hCD14, human CD14; DAF, decay-accelerating factor; GPI, glycosyl-phosphatidylinositol; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; CI, class I; TF, tissue factor; PIPLC, phosphatidylinositol-specific phospholipase C; CNTFR, ciliary neurotrophic factor-binding protein; sCD14, soluble CD14.

1 25 50
 5'-CGATTGCCAATAAAGGATCCGGAACCACTTCAGGTACCAACAGCTCTTCTATCTGGACACAG
 3'-TAACGGTTTATTTCCTAGGCTTGGTGAAGTCCATGGTGTGCAGAAGATAGACACTGTGTGC
 75 100 125
 TGTTCACGTTGACAGGCTTCTTGGACACTAGTAACATATGGGATGTGCTGACTTAAGGGCC-3'
 ACAAAGTGCAACTGTCCAGACGAACCGTGTGATCATTTGATACCCCTAACGACTGAATTC-5'

FIG. 1. Sequence of synthetic DNA.

and analysis of CD14 expression by cell cytometry were done as described (11).

Cell Activation. All cell-activation studies have been done with 10% fetal calf serum because we had shown that 10% fetal calf serum substitutes for purified LPS-binding protein (11). LPS-induced expression of surface IgM was measured as described (11). NF- κ B activation was measured in nuclear extracts of LPS-treated 70Z/3 cells as described (20). A double-stranded oligonucleotide containing the NF- κ B site from the immunoglobulin enhancer (5'-CAGAGGGGACTT-TCCGAGA-3') was radiolabeled with [γ -³²P]ATP by incubation with nuclear extracts at room temperature for 20 min before analyzing samples on a 4% nondenaturing polyacrylamide gel (21). Addition of excess unlabeled NF- κ B oligonucleotide but not addition of an oligonucleotide with a mutated NF- κ B site blocked appearance of the radiolabeled band in the gel-shift assay (data not shown). Protein tyrosine phosphorylation was determined as described (37) by enhanced chemiluminescence with the antiphosphotyrosine mAb, FB2 (American Type Culture Collection CRL1891).

RESULTS

Expression of GPI-Anchored or Integral Membrane CD14. We constructed plasmids encoding hCD14 for expression as an integral membrane or GPI-anchored protein (Fig. 2). Sequences encoding the transmembrane domains and cytoplasmic tails of the integral membrane proteins, TF or a murine CI gene product, H-2K^b, were chosen because they do not encode any obvious signaling motifs (22, 23). The constructs for integral membrane CD14 required modification of sequences encoding the COOH-terminal portion of the CD14 polypeptide. To insure that changes in this region do not alter CD14 function we prepared a construct that incor-

porated the sequences encoding 37 COOH-terminal amino acids of the GPI-anchored protein DAF (pRc/RSV-hCD14DAF) (16, 17), resulting in hCD14 with a GPI-attachment site distinct from that of natural hCD14. Five different stably transfected 70Z/3 cell lines were established after transfection with empty vector (70Z/3-RSV) or the constructs shown in Fig. 2.

CD14 expression was measured by flow cytometry after staining with the anti-hCD14 mAb MY4. Whether the expressed CD14 was GPI-anchored or an integral membrane protein was evaluated by comparing mAb MY4 staining before and after PIPLC (24) treatment (Fig. 3). mAb MY4 stained the 70Z/3-hCD14, -hCD14DAF, -hCD14TF, and -hCD14CI cells, demonstrating that transfection resulted in surface expression of the proteins encoded by the four constructs. PIPLC treatment of 70Z/3-hCD14 and -hCD14DAF cells resulted in loss of MY4 staining, indicating that hCD14 and the hCD14DAF are attached to the membrane via a GPI anchor. In contrast, PIPLC treatment of 70Z/3-hCD14TF or -hCD14CI cells did not reduce the cell fluorescence after mAb MY4 staining, as expected, if the CD14 is expressed as an integral membrane protein.

LPS Activation of Transfected 70Z/3 Cells. The effects of LPS on 70Z/3 cells include expression of IgM, protein tyrosine phosphorylation, and NF- κ B activation (11, 25, 37). To compare the response of 70Z/3 cells expressing GPI-anchored or integral membrane forms of CD14 we measured the LPS-concentration dependency for surface IgM expression as well as the kinetics of LPS-induced NF- κ B activation and protein tyrosine phosphorylation (Fig. 4 a-c).

When we added Re595 LPS at 1 pg-1 μ g/ml to the four CD14-positive cell lines as well as to 70Z/3-RSV cells and measured expression of IgM 24 hr after LPS addition, we observed that each CD14-positive cell line had nearly identical LPS dose-response curves (Fig. 4a) and responded to $\approx$ 1000-fold less LPS than the 70Z/3-RSV cells (11). Because there is a substantial latent period before surface IgM is expressed (14), we considered that early activation events might have different kinetics in cells with GPI-anchored or integral membrane CD14 and that these differences might not be revealed by measurements of surface IgM.

FIG. 2. Plasmids for GPI-anchored or transmembrane CD14. (a) Schematics of pRc/RSV constructs used for expression of hCD14 in 70Z/3 cells. RSV LTR, Rous sarcoma virus long terminal repeat; UTR, untranslated region. (b) Amino acid sequences of additional residues incorporated into hCD14 constructs encoding GPI-anchored or integral membrane CD14; $\downarrow$ denotes GPI-anchoring site of DAF (16, 17).

FIG. 3. Flow cytometry analysis of transfected 70Z/3 cells. Expression of CD14 in transfected cells was measured by flow cytometry after staining with FITC-MY4 as described (11). Cells were also pretreated with PIPLC (+) before staining with FITC-MY4 as described (11). 70Z/3-hCD14, -hCD14DAF, -hCD14TF, and -hCD14CI cells express 1×10^4 , 5.5×10^4 , 1.3×10^4 , and $1-2 \times 10^4$ molecules of CD14 per cell, respectively (P.S.T., unpublished results). RSV, Rous sarcoma virus.

To address this consideration we measured the kinetics of two more rapid responses to Re595 LPS at 1 ng/ml—namely, NF- κ B activation and protein tyrosine phosphorylation. Rapid NF- κ B activation was seen in all CD14-bearing 70Z/3 cells within 15 min after LPS addition. These measurements failed to reveal any differences in responses to LPS when cell lines with GPI-anchored or integral membrane CD14 were compared (Fig. 4b). We have shown that LPS induces the rapid protein tyrosine phosphorylation of a 38-kDa protein (p38) in 70Z/3-hCD14 cells, Raw 264.7 cells, and in peritoneal exudate macrophages that is distinct from the 44- or 41-kDa isoforms of the mitogen-activated protein kinases (37). The kinetics of p38 tyrosine phosphorylation in the five sets of 70Z/3 cells were evaluated after LPS addition. Nearly identical patterns of LPS-induced p38 tyrosine phosphorylation were observed in all four sets of CD14-bearing cells; maximum phosphorylation occurred 15 min after LPS addition (Fig. 4c). These data show that conversion of CD14 to an integral membrane protein does not uncouple LPS-induced transmembrane signaling. Addition of mAb MY4 to the cells before LPS markedly inhibited NF- κ B activation (Fig. 5c) and protein tyrosine phosphorylation (data not shown), demonstrating the dependence on CD14. In contrast to results shown here for CD14-bearing 70Z/3 cell lines, 70Z/3-RSV cells displayed reduced sensitivity to LPS for both NF- κ B activation and p38 phosphorylation. Increasing LPS concentrations 100- to 1000-fold results in p38 phosphorylation and marked NF- κ B activation in 70Z/3-RSV cells (11, 37).

Soluble CD14 (sCD14) Does Not Replace Membrane-Bound CD14. LPS binds to sCD14 lacking a GPI anchor, and LPS-sCD14 complexes are agonists for endothelial cells under conditions where LPS or sCD14 alone have no stimulatory effect (6, 8). Thus, we wondered whether LPS-sCD14 complexes would stimulate 70Z/3-RSV cells under conditions where LPS or sCD14 alone were inactive. If so, membrane attachment of CD14 would not seem essential for enhanced responsiveness of 70Z/3 cells to LPS and would explain the lack of effect of changing CD14 from a GPI-anchored to an integral membrane protein. We added sCD14 to 70Z/3-RSV cells and asked whether LPS-induced NF- κ B activation was observable (Fig. 5a). The presence of sCD14 failed to enhance the sensitivity of 70Z/3-RSV to LPS. Thus,

FIG. 4. LPS stimulation of transfected 70Z/3 cells. 70Z/3 cells were cultured and stimulated with Re595 LPS as described (11). (a) LPS-induced surface expression of IgM. (b) LPS-induced NF- κ B activation was measured in 70Z/3 cells after addition of Re595 LPS at 1 ng/ml for the designated times (numbers indicate min) using described procedures. (c) Protein tyrosine phosphorylation was determined as described (7, 37) in samples prepared from 70Z/3 cells exposed to Re595 LPS at 1 ng/ml for the designated times (numbers indicate time in minutes).

CD14 must be anchored to the cell membrane of 70Z/3 cells to increase the sensitivity of these cells to LPS.

Anti-CD14 Antibody Inhibits LPS-Induced Activation Without Blocking LPS Binding to CD14. After some GPI-anchored receptors bind ligand, the resultant complex undergoes additional interactions that lead to cell activation or ligand internalization (26, 27). To investigate whether additional steps are required for LPS-induced cell activation after LPS binds to CD14, we compared the effects of two anti-hCD14 mAbs, MY4 or 18E12, on binding of FITC-LPS to CD14-bearing 70Z/3 cells and on LPS-induced NF- κ B activation (Fig. 5b and c, respectively). Both of these mAbs inhibit LPS-induced cytokine release by monocytes/macrophages (R.J.U., unpublished data). Addition of mAb MY4 to each CD14-bearing cell line markedly inhibited LPS binding as well as NF- κ B activation. In contrast, mAb 18E12 had little or no effect on LPS binding but inhibited NF- κ B activation.

FIG. 5. Effect of sCD14 and anti-hCD14 on LPS stimulation of transfected 70Z/3 cells. (a) Re595 LPS (1 ng/ml) was added to 70Z/3-RSV or 70Z/3-hCD14 cells in the absence or presence of increased amounts of soluble CD14, and NF- κ B activation was performed as noted. (b) Effects of mAb MY4 and 18E12 on binding of FITC-LPS to transfected 70Z/3 cells. Cells suspended in RPMI 1640/10% fetal calf serum were preincubated with or without mAb at 10 μ g/ml at 37°C for 30 min followed by the addition of FITC-Re595-LPS at 1 ng/ml (11) for 15 min at 37°C. Binding was stopped by adding ice-cold RPMI 1640 medium and chilling on ice until flow cytometry analysis. Cell-associated fluorescence was measured as described (11). The different levels of FITC-LPS binding are related to differences in expression of hCD14 in the transfected cell lines (see legend for Fig. 2). Results shown are mean $\pm$ SD ($n = 3$). (c) Transfected 70Z/3 cells were maintained in the presence or absence of mAbs to hCD14 as noted above. Cells were treated with Re595 LPS at 1 ng/ml or with 100 μ M taxol for 15 min at 37°C, and NF- κ B activation was measured in nuclear extracts.

Other anti-CD14 mAbs typified by 63D3 (28) that bind to CD14 on leukocytes and CD14-bearing 70Z/3 cells do not inhibit LPS binding or cell activation (data not shown).

To insure that the inhibitory effects of mAbs 18E12 and MY4 are specific for LPS we investigated whether these mAbs would block NF- κ B activation induced by taxol. The characteristics of taxol-induced NF- κ B activation are identical in 70Z/3-RSV and 70Z/3-hCD14 cells (J.-D.L., unpublished data). Taxol induced identical levels of NF- κ B in each of the five sets of transfected cells; neither mAb MY4 nor mAb 18E12 inhibited taxol (Fig. 5c). Thus, the effects of mAb MY4 or 18E12 on LPS-induced NF- κ B activation do not result from nonspecific effects of these antibodies.

DISCUSSION

Conversion of CD14 to an integral membrane protein does not uncouple LPS-driven cell events associated with cell activation. These findings show that the signal-transduction capacity of GPI-anchored proteins is not always dependent on the GPI anchor itself. Our findings with CD14 were unexpected because previous studies have shown that conversion of three different GPI-anchored T-cell proteins to integral membrane proteins abrogated their function as signaling receptors (29–31). In these cases cell activation occurs after antibody-induced receptor cross-linking. The existence of a transmembrane protein with affinities for the GPI anchor of the receptor and for intracellular proteins such as tyrosine kinases attached to the inner face of the plasma membrane has been proposed to account for signaling by this mechanism (31–33). This model provides an explanation for the importance of the GPI anchor when receptor cross-linking initiates signaling, as observed with T-cell antigens, but clearly our findings suggest that this model does not apply to CD14 in its function as an LPS receptor. The data presented here support a model where CD14 is the ligand-binding subunit of a membrane-receptor complex for LPS and is not actively involved in signal transduction. Whether additional membrane components that participate in signaling also bind LPS remains to be determined.

There are remarkable similarities between the properties of CD14 and another GPI-anchored receptor, the ciliary neurotrophic factor-binding protein (here termed CNTFr) (34). After soluble forms of CD14 or CNTFr bind their respective ligands, the resultant heteromeric complex can activate some cell types that do not normally express the GPI-anchored receptor (6, 34, 35). Comparative studies of the function of GPI-anchored or transmembrane forms of CNTFr have not been reported, so parallels between our findings and those with the CNTFr cannot yet be drawn. The receptor complex for CNTF includes signaling components common to other cytokines (34). Whether CD14 is part of a receptor complex that includes signaling molecules used by other agonists is unknown, but it is likely that the additional components will include either a transmembrane protein tyrosine kinase or a protein that once engaged by LPS or LPS-CD14 enables activation of an intracellular protein tyrosine kinase (7, 36, 37). Even in the absence of information about the identity of additional components of this postulated receptor complex for LPS, therapeutics that target LPS-CD14 interactions may be very effective in septic shock because the importance of these interactions has been demonstrated experimentally under physiologic conditions.

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