Cellular Binding of Soluble CD14 Requires Lipopolysaccharide (LPS) and LPS-binding Protein*

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The stimulation of nonmyeloid cells by lipopolysaccharide (LPS) is mediated by the serum protein, soluble CD14 (sCD14). We have examined the interaction of sCD14 with whole cells using a biologically active radio-labeled sCD14 molecule as a ligand. Specific binding of sCD14 to nonmyeloid cells is detected only when it is first incubated with both LPS and the serum LPS-binding protein (LBP). Through the use of an anti-CD14 monoclonal antibody, we demonstrate that sCD14 must interact with LPS in order for cellular binding to occur. Although LBP is traditionally known to function as a catalyst in the transfer of LPS to sCD14, our results reveal that LBP is actually a physical part of sCD14-containing, cell-associating complexes. The LPS-and-LBP-dependent cell surface binding of sCD14 appears to be distinct from events leading to cell stimulation, since certain anti-CD14 and anti-LBP monoclonal antibodies have different effects on cellular binding versus cellular activation. Bound sCD14 is internalized, indicating that the LBP- and LPS-dependent binding of sCD14 may represent a novel general mechanism by which nonmyeloid cells clear LPS.

In addition to macrophages and neutrophils, a variety of nonmyeloid cells have been shown to be responsive to the presence of LPS. Specifically, epithelial, endothelial, astrocytoma, dendritic, and smooth muscle cells become stimulated by LPS in a serum-dependent fashion. The serum component responsible for the activation of these cells has subsequently been shown to be sCD14 (8–10). Thus, the apparent function of sCD14 is that it enables certain cells that do not possess mCD14 to respond to low doses of LPS. sCD14 can bind LPS directly to form sCD14-LPS complexes; however, the kinetics of this reaction are slow. In this regard, LBP has been shown to catalyze the transfer of LPS to sCD14 (11, 12).

In addition to the proinflammatory responses mediated by LPS, a number of pathways have been identified by which cells clear and neutralize LPS, thereby removing it as a toxin and dampening these responses (13, 14). The scavenger receptor bind and internalize the LPS precursor lipidIVA as well as other negatively charged ligands (15). Moreover, it has been shown that LBP participates in the binding and uptake of LPS in a mCD14-dependent manner that is distinct and separate from events leading to signal transduction (13, 14, 16). Furthermore, it has been recently shown that whole bacteria opsonized with LBP bind to cell surface mCD14, leading to phagocytosis (17).

Here, we examine LPS-dependent binding events on the surface of nonmyeloid cells utilizing a biologically active radio-labeled sCD14 molecule. Through this approach we have characterized a novel sCD14 binding phenomenon, exhibited by a variety of non-mCD14-bearing cells, that is dependent on both LPS and LBP. We also demonstrate that the observed binding does not directly lead to cellular activation and that it exhibits similar characteristics to the binding of LBP-LPS complexes to mCD14-bearing cells (16). The LPS- and LBP-dependent binding of sCD14 results in cellular internalization. Taken together, these results suggest that this binding phenomenon represents a general cellular mechanism for the clearance of LPS.

EXPERIMENTAL PROCEDURES

Construction of CD14AH—The XbaI fragment of full-length human CD14 was subcloned from pUC19 into the XhoI site of pUC19 to create pUC19hCD14. A double-stranded oligonucleotide, encoding a protein kinase A site and a six-histidine tag, 5'-GG GCC GCC AGC CAC GCT AGC ACG CAA GCC-3' (coding strand) and 5'-TG CGT CGT GCT AGC-3' (anti-coding strand), was included in a ligation reaction containing the XhoI overhang, was included in a ligation reaction containing the 790-base pair SacII to BanII fragment of hCD14 and the large fragment of pUC19 hCD14 digested with SacII and NcoI. This resulted in pUC19hCD14AH, which encodes the human CD14 protein with the last 35 amino acids and the putative GPI-anchoring site, being replaced by the amino acid sequence LRRSVAGAGHHHHHHH. The hCD14AH sequence was subsequently subcloned from pUC19hCD14AH as an NcoI/BamHI fragment into the multiple cloning site of the baculovirus vector pACSG2 (Pharmingen, San Diego, CA). The final construct was se-
Expression of CD14AH in Baculovirus—SF-9 insect cells derived from Spodoptera frugiperda were grown in SF-900II media (Life Technologies, Inc.) containing 5% fetal calf serum. Recombinant baculovirus was produced by cotransfecting the pAcCG2-hCD14AH vector with BaculoGold DNA (Promega Corp., Madison, WI) into SF-9 cells using LipofectAMINE (Life Technologies). After 3 days, the medium was removed, centrifuged to remove loose cells, and subsequently used to infuse a new flask of SF-9 cells. The procedure was repeated to amplify the recombinant virus. The viral titer was determined using the end point dilution assay (19). sCD14AH protein was produced by infecting BTI-TN-5B1-4 (high five) cells derived from Trichoplusia ni (Invitrogen, San Diego, CA) with the amplified recombinant virus. High five cells were grown in spinner flasks using SF-900II medium containing 5% fetal calf serum and infected at a multiplicity of infection of 5. After 3 days, the cells were removed by centrifugation. The expression of sCD14AH in SF-9 and high five cells was confirmed by Western blot of infected culture supernatants using polyclonal anti-goat hCD14 as the primary antibody.

Purification of CD14AH—Culture supernatant from infected high five cells was dialyzed against buffer A (100 mM sodium phosphate, 300 mM sodium chloride, pH 8.0) and filtered through a 0.22 μm filter. After adding nickel-nitrirotiacetic acid beads (Qiagen, Santa Clarita, CA) the filtrate was gently mixed for 3 h. A column was prepared and washed sequentially with 5 column volumes of buffer A, then buffer A containing 1 M imidazole, and finally buffer A containing 25 mM imidazole. The sCD14AH protein was eluted with 2 column volumes of buffer A containing 100 mM imidazole. The eluate was dialyzed against phosphate-buffered saline. The final protein was analyzed by SDS-PAGE using a 10% polyacrylamide gel and was quantified by protein assay and by enzyme-linked immunosorbent assay. Typically, this system yielded approximately 5 mg of sCD14AH protein per liter of medium.

Labeling of sCD14AH—Purified sCD14AH was radiolabeled at the protein kinase A site as described (20). Briefly, 20 μg of purified sCD14AH was added to 20 μl Tris-HCl (pH 7.4), 100 mM NaCl, 12 mM MgCl2, 1 μCi of [γ-33P]ATP (2000 Ci/mmol) and 10 units of bovine heart muscle kinase (Sigma) in a final volume of 150 μl. The phosphorylation reaction was incubated for 90 min at 37 °C, after which 15 μl of low endotoxin bovine serum albumin (10 mg/ml) was added as a carrier protein. The reaction mixture was dialyzed against 500 ml of phosphate-buffered saline for several hours and then against 500 ml of fresh phosphate-buffered saline overnight to remove free ATP. The final protein typically incorporated over 40% of the total ATP, resulting in a specific activity of at least 800 Ci/mmol using (γ-33P]ATP and 2400 Ci/mmol using (γ-32P]ATP. The final product was checked by SDS-PAGE using a 10% polyacrylamide gel followed by autoradiography.

Cell Culture—SW620, U373, Eahy926, HeLa, COS-7, Jurkat, CHO, and THP-1 cells were grown in RPMI medium (Irvine Scientific, Santa Ana, CA) containing 10% fetal calf serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin/glutamine. HUVECs were obtained from Clonetics Corporation (San Diego, CA) and grown in endothelial cell growth medium, supplemented with 5% fetal calf serum, following the suppliers’ guidelines.

Cell Activation Experiments—SW620 epithelial cells were grown to confluence in 96-well plates and washed four times in RPMI 1640 medium containing 1 mg/ml human serum albumin. To each well was added 200 μl of RPMI 1640 medium containing Re595LPS and sCD14AH as indicated in the figures. After incubating the cells for 6 h at 37 °C, the medium from each well was removed and assayed for interleukin-8 by a standard sandwich enzyme-linked immunosorbent assay method using rabbit anti-human interleukin-8 antibodies.

Binding Assays—The labeled sCD14AH was preincubated at 37 °C for 1 h with an equimolar amount of LBP and a 2-fold molar excess of Salmonella minnesota Re595 LPS. The average molecular mass of Re595 LPS was taken as 2300 Da. Thus, a typical preincubation reaction contained 160 ng of sCD14AH, 240 ng of LBP, and 20 ng of Re595 LPS in phosphate-buffered saline plus 2 mM EDTA in a final volume of 20 μl. The preincubation reaction was then diluted to 400 μl with ice-cold binding buffer (21) (20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM EDTA, and 0.5% bovine serum albumin), and 100 μl was aliquoted into Eppendorf tubes kept on ice (binding reactions).

Actinomycin D detached epithelial cells were resuspended using cell dissociation buffer (Life Technologies, Inc.), and the cell pellet was washed twice with several volumes of binding buffer. The final pellet was resuspended in ice-cold binding buffer at 4 × 107 cells/ml, and 100 μl was added to each of the above binding reactions. Whole cell binding was allowed to proceed for 20 min on ice, after which the cells were pelleted for 60 s at 6,000 × g. The supernatant was removed, and the cells were quickly resuspended in 150 μl of ice-cold binding buffer. The cell suspension was then layered on 200 μl of mineral oil composed of 80% 550 fluid and 20% 200 fluid (Dow Corning, Midland, MI) and centrifuged for 1 min at 10,000 × g. The tubes were frozen on dry ice, and the cell pellet was clipped into a scintillation vial, resuspended in 200 μl of NaOH and assayed for bound counts in Ecoscint scintillation fluid (National Diagnostics, Atlanta, GA).

RESULTS

Expression, Labeling, and Biological Activity of sCD14AH—sCD14 has been shown to facilitate the activation of a variety of mononuclear cells in response to LPS (8–10). To detect cell surface interactions of sCD14 with these cells, we required a labeled sCD14 molecule as a ligand. Initially, this was attempted through iodination using chloramine T or iodogen; however, these procedures rendered sCD14 biologically inactive. In addition, treatment of sCD14 with N-hydroxysuccinimide derivatives of biotin or fluorescein isothiocyanate has also been reported to destroy the biological activity of this protein (22). To overcome the inactivation of sCD14, we have utilized a technique that involves radiolabeling the protein at an artificially introduced protein kinase A site, a method that has been successfully carried out to label a variety of intracellular proteins and soluble ligands (Ref. 23 and references therein). Thus, a sCD14 molecule was engineered that replaced the carboxyl end and the putative GIP anchoring site with a protein kinase A site and a six-histidine tag (see “Experimental Procedures”). This protein, hereafter named sCD14AH, was expressed in a baculovirus system and purified by affinity chromatography (Fig. 1A). sCD14AH was at least 95% pure and consisted of three discernible bands on a Coomassie-stained SDS-PAGE gel. The purified protein was radiolabeled to high specific activity at the protein kinase A site (see “Experimental Procedures”), and the final product displayed the same SDS-PAGE profile as the purified protein when visualized by autoradiography (Fig. 1B). Western blot analysis has confirmed that the three bands are CD14 (data not shown) and full-length CD14 expressed in baculovirus displays a similar SDS-PAGE profile, which has been attributed to heterogeneity in protein glycosylation (24).

Structure function analysis of sCD14 has demonstrated that the ability of the protein to stimulate mononuclear cells in the presence of LPS resides in the amino-terminal half of the molecule (25). In this regard, all of the modifications made in the construction of sCD14AH have been made at the carboxyl terminus of the protein. Nevertheless, to ensure that sCD14AH retains biological activity, we tested the ability of the final radiolabeled protein to stimulate SW620 epithelial cells with low doses of LPS. Labeled sCD14AH activated interleukin-8 expression of SW620 epithelial cells in an LPS-dependent fash-
experimental Procedures.

were subsequently assayed for interleukin-8 as described under “Ex-

sCD14AH in the presence of 10 ng/ml Re595 LPS. Cell supernatants

amounts of CHO cell-expressed sCD14, unlabeled sCD14AH, or labeled

stimulation of SW620 epithelial cell when incubated with varying

either the presence or absence of labeled sCD14AH. Cell supernatants

in the presence of 10 ng/ml Re595 LPS. Cell supernatants

were subsequently assayed for interleukin-8 as described under “Ex-

perimental Procedures.”

ion at concentrations as low as 100 pg/ml and showed maximal

activity at 10 ng/ml LPS (Fig. 24). In addition, the LPS sensi-

itivity and level of stimulation were identical to previously

published results from our lab using sCD14 purified from CHO

cell supernatants (9). To confirm that labeled sCD14AH retains

full biological activity, we compared the sCD14 dose depend-

ence on cellular responses at a single LPS concentration. CHO-

expressed sCD14, unlabeled sCD14AH, and labeled sCD14AH each increased cellular interleukin-8 expression in dose-de-

pendent fashions, and all three sCD14 preparations exhibited

indistinguishable cell-stimulatory activities (Fig. 2B). In addi-

tion, we have confirmed that labeled sCD14AH binds LPS,

since an increase in fluorescence was observed upon adding it
to a mixture of LBP and fluorescein isothiocyanate-labeled LPS

(12) (data not shown). These results demonstrate that affinity-
purified and radiolabeled sCD14AH protein retains the LPS

binding and cell stimulatory activity of native sCD14.

LBP and LPS Enable Binding of sCD14 to Epithelial

Cells—We have utilized labeled sCD14AH as a ligand in whole

cell binding assays in an effort to study cellular interactions of

sCD14. Since the activation of epithelial and endothelial cells by

LPS requires sCD14, and sCD14 is an LPS-binding protein,

we reasoned that cell surface binding of sCD14AH may be

LPS-dependent. Since LBP has been shown to transfer LPS to

sCD14 (11, 12), it was used to facilitate the formation of sCD14-LPS complexes. Therefore, we preincubated sCD14AH and

LBP, at concentrations comparable with those found in

serum (26, 27), in the presence of a small molar excess of LPS. After the preincubation, the resulting complexes were diluted

with ice-cold binding buffer, and whole cell binding assays were

performed with SW620 epithelial cells on ice to prevent inter-

nalization (see “Experimental Procedures”). When labeled

sCD14AH was preincubated by itself, with LBP alone, or with

LPS alone, no specific binding to SW620 epithelial cells could

be measured (Fig. 3). In fact, we have been unable to detect any

specific binding of sCD14AH to SW620 epithelial cells in the

absence of LBP even after preincubating the sCD14AH with

excess molar amounts of LPS for several hours, a condition that

allows sCD14-LPS complex formation (11) (data not shown).

However, when preincubated at 37 °C with both LBP and LPS,
sCD14AH displayed significant binding to these cells (Fig. 3).

This binding was specific, since it was abrogated by the addi-
tion of a 100-fold molar excess of unlabeled sCD14AH in the

preincubation but not by an equivalent amount of human se-

rum albumin. Furthermore, no specific binding of sCD14 can be
detected when it is added to LBP and LPS on ice without a

37 °C preincubation (Fig. 3). Taken together, these results
demonstrate that sCD14 must interact with both LBP and LPS

at 37 °C to bind specifically to SW620 epithelial cells. We have

found that the binding is dependent on the length of the pre-

incubation time but is maximal within 5 min at 37 °C (data not

shown). The simplest interpretation of these results is that

sCD14, LBP, and LPS form a ternary complex at 37 °C that can

subsequently bind to epithelial cells.

To examine more closely the dependence of LBP and LPS on

the cell surface binding of sCD14, we tested the effects of

varying the amounts of these components in the preincubation

with respect to sCD14AH. Fig. 4A shows the LPS dependence

of the binding at three different LBP concentrations. The bind-

ing of sCD14AH initially increased with the amount of LPS

present but was inhibited at high molar ratios of LPS to

sCD14AH. sCD14AH binding also increased with the amount

of LBP present; however, the binding was maximal not at any

particular ratio of LPS to sCD14AH but when the LPS-LBP

ratio was approximately 3:1. These results were confirmed and

![Graph](Image)

**Fig. 2.** Labeled sCD14AH allows epithelial cells to respond to low concentrations of LPS. Panel A shows the activation of SW620 epithelial cells when incubated with varying amounts of Re595 LPS in either the presence or absence of labeled sCD14AH. Panel B shows the stimulation of SW620 epithelial cell when incubated with varying amounts of CHO cell-expressed sCD14, unlabeled sCD14AH, or labeled sCD14AH in the presence of 10 ng/ml Re595 LPS. Cell supernatants were subsequently assayed for interleukin-8 as described under “Experimental Procedures.”

**Fig. 3.** Binding of sCD14 to cells is dependent on prior preincubation with LPS and LBP. Labeled sCD14AH (160 ng) was preincubated at 37 °C in either the presence or absence of an equimolar amount of LBP (240 ng), a 2-fold molar excess of Re595 LPS (20 ng), a 100-fold molar excess of unlabeled sCD14AH (16 μg), or an excess of human serum albumin (16 μg) as indicated at the bottom of the figure. Binding assays were performed in triplicate using SW620 epithelial cells as described under “Experimental Procedures.” In the final binding condition (bar on the far right), the components were added together on ice with no preincubation at 37 °C.
extended when the LBP dependence of the binding was examined at four different LPS concentrations (Fig. 4B). In all cases, the binding increased with the amount of LBP added. When the molar ratio of LPS with respect to LBP is low, the binding rises steeply with increasing amounts of LBP and eventually saturates. Conversely, when the molar amount of LPS is high with respect to LBP, the binding is initially inhibited until the LBP to LPS molar ratio is sufficient to overcome the inhibitory effect of excess LPS. The binding of sCD14AH still increases at high stoichiometric levels of LBP, further indicating that LBP is not acting catalytically in this system to deliver LPS to sCD14. We observe no inhibition of binding even at exceptionally high molar ratios of LBP to sCD14AH or LBP to LPS (Fig. 4B and data not shown). We next examined the effect of cell number on sCD14AH binding (Fig. 4C). The level of sCD14AH bound increased with increasing cell number; however, sCD14AH binding at different cell concentrations exhibited the same LPS dependence and was maximal when the LPS:LBP ratio was approximately 3:1. These results show that while the amount of sCD14AH bound is dependent on cell number, the maximal binding stoichiometry with respect to LPS and LBP is independent of cell number.

The saturability of sCD14 binding to epithelial cells was investigated by determining the binding of a series of dilutions made from a single preincubation of sCD14AH, LBP, and LPS. Surprisingly, the binding was directly proportional to the total amount of the preincubated mixture added to the cells and was linear over at least 4 logs of sCD14AH concentration, from $10^{2}$ to $10^{7}$ M (Fig. 5). These results confirm the idea that once formed complexes of sCD14<sub>z</sub>LBP<sub>z</sub>LPS are relatively stable and are refractory to dilution. Moreover, the inability to saturate the binding of sCD14 under these conditions is reminiscent of the inability to saturate the binding of LBP-LPS complexes to cells bearing mCD14. This phenomenon was attributed to the ability of these complexes to self-aggregate on the cell surface (16).

**LBP- and LPS-directed Binding of sCD14 Does Not Lead to Cellular Activation**—In contrast to the requirement of both LPS and LBP for binding of sCD14AH to cells (Figs. 3 and 4), the sCD14-mediated stimulation of nonmyeloid cells by LPS does not require LBP (Fig. 2 and Ref. 9). Thus, the LBP- and LPS-dependent binding of sCD14 does not appear to be directly involved in events leading to cellular activation. To confirm this, we tested the effects of a panel of monoclonal anti-CD14 and anti-LBP antibodies. These antibodies were added to the preincubation reaction containing sCD14AH, LBP, and LPS, and the resulting complexes were tested for the ability to bind and stimulate interleukin-8 production using SW620 epithelial cells (Fig. 6). The anti-CD14 antibody 63D3 had no effect on sCD14AH binding or cellular stimulation. In contrast, the anti-CD14 antibody 28C5 blocked both binding and activation of SW620 cells. However, while activation was inhibited by the anti-CD14 antibody 18E12, there was no effect on the binding of sCD14AH. Conversely, although binding was abrogated by two anti-LBP antibodies, 18G4 and 2B5, these antibodies had no effect on cellular activation. These results clearly demon-
strate that the LPS- and LBP-dependent binding of sCD14 is not directly involved in interactions leading to cellular activation.

The anti-CD14 antibody 28C5 blocks the activation of myeloid cells by preventing the binding of LPS to mCD14 (16, 21, 28). 28C5 also blocks the binding of LPS to sCD14, and we have observed that the ability of sCD14AH to bind fluorescein isothiocyanate-labeled LPS, as measured by an increase in fluorescence (12), is completely abrogated by this anti-CD14 antibody (data not shown). The fact that the binding of sCD14AH to cells requires a preincubation of this protein with LBP and LPS (Fig. 3), coupled with the ability of 28C5 to completely abrogate the cellular binding of sCD14AH, strongly suggests that the observed binding requires a prior interaction between sCD14AH and LPS.

**Binding of sCD14-LBP-LPS Complexes Does Not Involve mCD14—**Membrane-anchored CD14 has been shown to be a receptor for LBP-LPS complexes in a binding phenomenon that does not saturate (16). Therefore, to address this and ascertain the generality of our results, we tested a variety of cells for binding of sCD14AH that was dependent on the presence of both LBP and LPS and could be blocked by the antibodies 28C5 and 18G4 in the preincubation (Table I). Following these criteria, sCD14AH displayed binding to a variety of cells that respond to LPS in a sCD14-dependent fashion. These cells included the astrocytoma cell line U373, the endothelial cell line Eahy926, and primary HUVECs. In addition, the LBP- and LPS-dependent binding of sCD14AH was detected using HeLa cells, COS-7 cells, CHO cells, and the myeloid cell line THP-1. Binding of sCD14AH to Jurkat cells, a T-cell line, was barely detectable in our assay. We also examined the effects of the anti-CD14 and anti-LBP antibodies when added to cells after the preincubation (Table I). In this situation, the 28C5 antibody no longer inhibited binding, confirming that the effects of this antibody are on the interaction between sCD14AH and LPS. Conversely, 18G4 blocked sCD14AH binding even when added after the preincubation, demonstrating that LBP is a physical part of the final binding complex. These results affirm the idea that sCD14, LBP, and LPS form stable ternary complexes that subsequently bind cells. The fact that a variety of cells believed not to possess mCD14 exhibit this binding and the fact that we still observe binding even in the presence of large quantities of 28C5, added after the preincubation, strongly suggest that membrane CD14 is not responsible for the observed binding of sCD14-LBP-LPS complexes.

Two other experiments indicate that mCD14 is not involved in the LPS- and LBP-dependent binding of sCD14. When the binding of preincubated sCD14AH is compared between CHO cells and CHO cells expressing mCD14, no differences are observed (Fig. 7A). In addition, the treatment of SW620 epithelial cells with PIPLC, which removes GPI-anchored proteins, has no effect on the observed level of binding of sCD14AH to these cells (Fig. 7B).

**Bound sCD14 Is Internalized—**Since LBP has been shown to be involved in the clearance of LPS by macrophages (16) and the observed binding of sCD14 is LBP-dependent but does not lead to activation, cellular internalization of sCD14AH was investigated. These experiments were performed by allowing the binding to proceed to completion at 4 °C and then shifting the cells to 37 °C for varying times. A time-dependent increase in cell-associated counts was observed in healthy cells but not in cells that had been metabolically poisoned (Fig. 8A). The time-dependent increase in total cell-associated labeled sCD14AH was evaluated for its resistance to stripping by protease treatment. The cell-associated sCD14AH was protease-sensitive for cells kept at 4 °C and became protease-resistant as a function of time for cells shifted to 37 °C (Fig. 8B). Taken together, these results reveal that, once bound, sCD14 is actively internalized by the cell.

**DISCUSSION**

Through use of a labeled biologically active sCD14 ligand, we have shown that LPS in conjunction with LBP can facilitate the binding of sCD14 to a variety of cells. Previous work has clearly shown that LBP can act to mediate the transfer of LPS to CD14

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**Table I: LPS- and LBP-dependent binding of sCD14 to various cell types**

Preincubation reactions and binding assays were performed as described under “Experimental Procedures” except for the inclusion of 4 μg of an anti-CD14 (63D3 or 28C5) or anti-LBP antibody (18G4). Antibodies were added either before or after the preincubation at 37 °C as indicated. The numerical values represent the average of three binding assays where the S.E. was less than 20% in all cases. The level of binding of sCD14 to THP-1 cells in the presence of anti-CD14 antibodies was substantially higher, presumably due to the presence of Fe receptors or to the cross-linking of sCD14 to mCD14 by the antibody and was therefore deemed not applicable (NA).

| Cell type | sCD14 alone | LBP and LPS |
|-----------|-------------|-------------|
|           | Before preincubation | After preincubation |
|           | 63D3 | 28C5 | 18G4 | 63D3 | 28C5 | 18G4 |
| SW620     | 3.5  | 54.2 | 52.4 | 10.2 | 9.9  | 55.0 | 51.1 | 9.4  |
| HUVEC     | 2.3  | 15.0 | 15.7 | 4.6  | 3.9  | 17.3 | 17.0 | 3.0  |
| U373      | 2.2  | 12.0 | 11.8 | 4.3  | 3.4  | 12.5 | 12.2 | 3.3  |
| Eahy926   | 1.2  | 44.9 | 48.0 | 11.2 | 2.9  | 45.1 | 46.6 | 4.8  |
| HeLa      | 1.5  | 14.4 | 13.6 | 6.3  | 4.1  | 16.5 | 14.8 | 2.9  |
| COS-7     | 6.2  | 45.8 | 39.1 | 5.1  | 6.8  | 36.7 | 35.2 | 5.5  |
| Jurkat    | 1.1  | 3.1  | 2.2  | 2.2  | 1.0  | 3.0  | 3.2  | 1.9  |
| CHO       | 1.3  | 14.7 | 16.0 | 6.2  | 1.7  | 13.5 | 13.4 | 2.4  |
| THP-1     | 2.0  | 14.8 | NA   | NA   | 2.7  | NA   | NA   | 2.9  |
Double reciprocal plots of the initial rate of LPS transfer at different LPS and sCD14 concentrations indicate that the LBP-mediated transfer of LPS to CD14 acts through the formation of an LPS LBP CD14 ternary complex (29). In addition, erythrocytes coated with LBP and LPS complexes (30), whole bacterial opsonized with LBP (17), and LBP LPS complexes (16) all bind to mCD14 on the surface of cells. The cell surface binding of sCD14AH is dependent on the preincubation of both LBP and LPS at 37 °C. In addition, whole cell binding is inhibited by the anti-CD14 antibody 28C5, which blocks the binding of sCD14 to LPS, only when this antibody is bound to sCD14AH prior to incubation with LBP and LPS. Cell surface binding of sCD14AH is also completely abrogated by the anti-LBP antibody 18G4 even when this antibody is added after preincubating sCD14AH with LBP and LPS, demonstrating that LBP must be part of the final complex that binds to cells. Taken together, these observations provide direct evidence for the formation and subsequent cell surface binding of ternary sCD14AH-LBP-LPS complexes. The physical make-up of the sCD14AH-LBP-LPS complexes is not known; however, sCD14AH binds to cells maximally when the molar ratio of LPS to LBP is approximately 3:1 regardless of the actual molar amount of sCD14AH present.

Since our experiments measure binding using labeled sCD14AH, we do not know the CD14 dependence of this binding, i.e. it remains formally possible that sCD14 is not required for the binding but is merely being carried to the cell surface by LPS and LBP. In fact, this possibility is difficult to rule out using excess cold components, since the binding itself does not saturate. We are currently investigating the radiolabeling of LBP to a high specific activity to address this question. The binding of sCD14AH-LBP-LPS complexes is highly reminiscent of the binding of complexes of LBP and LPS to CHO cells expressing mCD14. However, the variety of non-mCD14-bearing cells that exhibit binding of sCD14AH, coupled with the inability to block the binding after preincubating SW620 epithelial cells with 28C5 or PIPLC, demonstrates that mCD14 is not involved in facilitating the LPS- and LBP-dependent binding of sCD14. Panel A compares the LPS- and LBP-dependent binding of sCD14 to CHO cells expressing (white bars) and not expressing (black bars) membrane CD14. The preincubation reactions contained 160 ng of sCD14AH, 240 ng of LBP, 20 ng of Re595 LPS, and/or 4 μg of 18G4 as indicated at the bottom. Binding assays were performed with the CHO cells in triplicate as described under “Experimental Procedures.” Panel B compares the LPS- and LBP-dependent binding of sCD14AH to SW620 epithelial cells that have been treated (white bars) or not treated (black bars) with PIPLC. The binding assays were preformed in triplicate in either the presence or absence of LPS and LBP as described under “Experimental Procedures.”
sCD14AH. Similar to the binding of sCD14 in our system, the binding of LBP-LPS to mCD14-bearing CHO cells is inhibited by a large excess of LPS, occurs maximally at a low LPS-LBP ratio, is blocked by the anti-LBP antibody 18G4, and does not saturate but is cell number-dependent (16). The simplest interpretation of the observed LBP- and LPS-dependent binding of sCD14AH is that sCD14 can replace the need for membrane-anchored CD14 to bring LPS to the cell surface. Therefore, in the same way that sCD14 can replace mCD14 to stimulate cells in the presence of endotoxin, it appears that sCD14 can replace mCD14 in the clearance of endotoxin. In this regard, a cell surface component other than mCD14 has been proposed to be involved in the binding of LBP-LPS complexes to CHO cells bearing mCD14 (16).

The nature of the cell surface component involved in the binding of sCD14-LBP-LPS complexes is unknown. Several proteins have been identified on various cells that bind LPS (31–35). Most recently, one group studying LPS binding to Western blots of electrophoretically separated membrane proteins discovered an 80-kDa protein that preferentially binds LPS in the presence of serum (36). Interestingly, these studies demonstrated that the binding of LPS to the 80-kDa membrane protein required a combination of both LBP and sCD14. This protein has been observed using membrane preparations of monocytes and endothelial cells and, due to the requirements of sCD14, LBP, and LPS, is a candidate for the cell surface component involved in the LPS- and LBP-dependent binding of sCD14 characterized here.

The ability of GPI-anchored mCD14 to elicit cellular responses to LPS even after conversion to a transmembrane-anchored protein (37), as well as the fact that CD14 itself does not confer on cells the ability to discriminate between certain LPS agonists and antagonists (38), provides direct evidence for the existence of at least one other cell surface effector molecule that cooperates with mCD14. In support of this, activation and binding studies using THP-1 cells have shown that half-maximal responses of these cells occur at concentrations of LPS in the $10^{-11}$–$10^{-10}$ M range, which is almost 100-fold lower than the apparent affinity constant of $[^{3}H]$LPS for these cells. These observations, as well as the low concentrations of LPS antagonist required to inhibit responses to LPS but not the binding of $[^{3}H]$LPS, suggests that the LPS effector molecule is of low abundance relative to mCD14 (13, 39). Similarly, sCD14 has been shown to stimulate a variety of non-CD14-bearing cells in the presence of LPS with half-maximal responses to LPS exhibited in the high picogram or low nanogram range ($10^{-10}$ to $10^{-11}$ M) (Fig. 2 and Refs. 8–10). Despite the use of a biologically active labeled sCD14 molecule, we have been unable to observe specific binding of sCD14, either alone or when preincubated with LPS, to cells. Given the high specific activity of our ligand, an affinity constant of $10^{-11}$ M for sCD14 is certainly within the range of our assay. These results indicate either that the receptor responsible for allowing cellular responses to sCD14-LPS complexes is of extremely low cell surface abundance or that it has a low affinity for sCD14 itself. In the later case, it is possible that sCD14 delivers LPS to the effector molecule and is not a physical part of the final complex leading to cellular activation.

While this manuscript was in preparation, another group reported an LPS-dependent, cell association phenomenon for sCD14 (40). The relationship between their findings and the results reported here is difficult to discern for several reasons. First, these authors iodinated sCD14 using chloramine T, a labeling method that we have found destroys the biological activity of sCD14. Second, the cell association observed required very high concentrations, at least 5 $\mu$g/ml, of LPS to achieve saturation. Third, the observed cell association in that report required that sCD14, LPS, and cells be incubated together at room temperature for several hours, conditions that would certainly allow for internalization. Finally, the authors did not use LBP in their binding assays and therefore would not have observed the LBP- and LPS-dependent binding of sCD14 reported here.

It is clear from the antibody blocking experiments that the LPS- and LBP-dependent binding of sCD14 is not directly involved in cellular activation. The binding of sCD14-LBP-LPS complexes may represent a novel and general LPS clearance mechanism for cells. In support of this, we have shown that sCD14 is internalized by epithelial cells in a time-dependent fashion. In addition, previous work in our laboratory has shown that uptake of $[^{3}H]$LPS by SW620 epithelial cells is serum-dependent and can be blocked by the anti-CD14 antibody 28C5 (9). The ability of a variety of cells to clear LPS may be important, especially near sites of infection where the local concentration of LPS is high. In accordance with a role for LBP in LPS clearance, the binding of LPS in conjunction with either mCD14 (16) or sCD14 increases with available levels of LBP. Therefore, the dramatic rise in LPS levels observed during an acute phase response (41, 42) may function to ensure efficient clearance of LPS.

High concentrations of sCD14 have been shown to inhibit LPS-induced tumor necrosis factor-$\alpha$ production by cells in whole blood (24), dampen LPS-induced activation of monocytes and macrophages (43), and protect mice from LPS-induced lethality (44). Moreover, sCD14 has been shown to inhibit LPS priming of neutrophils in an LBP-dependent fashion, with priming responses progressively decreasing with increasing concentrations of LBP (45). The ability of high levels of sCD14, in combination with LBP, to dampen LPS-induced immune responses may be due to the formation of ternary sCD14-LBP-LPS complexes that are bound and cleared by cells without resulting in cellular activation.

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