

## Soluble CD14 Participates in the Response of Cells to Lipopolysaccharide

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### Summary

CD14 is a 55-kD protein found both as a glycosylphosphatidyl inositol-linked protein on the surface of mononuclear phagocytes and as a soluble protein in the blood. CD14 on the cell membrane (mCD14) has been shown to serve as a receptor for complexes of lipopolysaccharide (LPS) with LPS binding protein, but a function for soluble CD14 (sCD14) has not been described. Here we show that sCD14 enables responses to LPS by cells that do not express CD14. We have examined induction of endothelial-leukocyte adhesion molecule 1 expression by human umbilical vein endothelial cells, interleukin 6 secretion by U373 astrocytoma cells, and cytotoxicity of bovine endothelial cells. None of these cell types express mCD14, yet all respond to LPS in a serum-dependent fashion, and all responses are completely blocked by anti-CD14 antibodies. Immunodepletion of sCD14 from serum prevents responses to LPS, and the responses are restored by addition of sCD14. These studies suggest that a surface anchor is not needed for the function of CD14 and further imply that sCD14 must bind to additional proteins on the cell surface to associate with the cell and transduce a signal. They also indicate that sCD14 may have an important role in potentiating responses to LPS in cells lacking mCD14.

Recent studies have described proteins that enable monocytes and PMN to respond to nanogram per milliliter concentrations of Gram-negative bacterial LPS (endotoxin) (1, 2). LPS is first recognized by soluble serum proteins, either LPS binding protein (LBP)<sup>1</sup> (2) or the abundant factor in serum, termed septin (3). The resulting complex is then bound by CD14 (1), a glycosylphosphatidyl inositol (GPI)-anchored protein on the surface of phagocytic cells (4, 5). Blockade of cell surface CD14 with mAbs prevents binding of LPS/LBP complexes (1), prevents synthesis of TNF by monocytes (1), and prevents activation of adhesion receptors on PMN (6) that occur in response to low levels of LPS. These findings thus suggest a pivotal role for cell membrane (m)CD14 and serum proteins in responses to LPS.

The generality of the above scheme has appeared limited since several cell types respond to LPS but do not express

CD14. For example, human umbilical vein endothelial cells (HUVEC) respond to LPS with increased expression of tissue factor (7), endothelial-leukocyte adhesion molecule 1 (ELAM-1) (8), and IL-6 (9, 10). The astrocytoma line U373 responds to LPS with strong synthesis of IL-6 (10a), and we have recently observed that bovine brain microcapillary endothelial cells and cultured bovine pulmonary arterial endothelial (CPAE) cells respond to LPS with enhanced permeability and cell death (11). CD14 has not been reported on the surface of U373 cells or endothelial cells, and sensitive binding studies with fluorescent or radiolabeled anti-CD14 antibodies have failed to detect any CD14 on HUVEC (12; S. K. Lo and S. D. Wright, unpublished observations). Here we report that CD14 is essential for the response of each of these cell types to LPS, but that the CD14 is derived from the soluble pool of CD14 present in serum, not from the surface of the responding cell.

### Materials and Methods

**Reagents.** LPS from *Haemophilus influenzae* (Hib) (strain 10211) was isolated by magnesium ethanol precipitation (11, 13). Anti-

<sup>1</sup> Abbreviations used in this paper: CPAE, cultured bovine pulmonary arterial endothelial; ELAM-1, endothelial-leukocyte adhesion molecule 1; GPI, glycosylphosphatidyl inositol; HUVEC, human umbilical vein endothelial cells; LBP, LPS binding protein; mCD14, cell membrane CD14; NHS, normal human serum; sCD14, soluble CD14.

CD14 monoclonals 3C10 (14), 60b (15), and 26ic (15) were purified from ascites fluid by chromatography on protein G. mAb 60b was coupled to CNBr-Sepharose to yield 1 mg IgG/ml of packed gel. 3C10 was biotinylated using *N*-hydroxysuccinimide biotin. My4 (anti-CD14) and anti-CD18 antibodies were purchased from Coulter Immunology (Hialeah, FL) and Dakopatts (Copenhagen, Denmark), respectively. The anti-CD14 mAb AML2-23 was purchased from Medarex (Lebanon, NH), and alkaline phosphatase-conjugated streptavidin was purchased from Vector Labs (Burlingame, CA). LBP was purified from human ascites fluid as described (3).

**Depletion of Soluble (s)CD14 from Serum.** A 1-ml column of anti-CD14-Sepharose was equilibrated in PBS and 2 ml of normal human serum (NHS) was passed over at a flow rate of 0.05 ml/min. Depletion of sCD14 from serum was measured using a capture ELISA. Briefly, ELISA Terasaki plates were coated with AML2-23, and then blocked with FCS. Dilutions of human serum were incubated with the plates for 60 min at 21°C, then with biotinylated 3C10 for 60 min at 21°C. Retention of biotin was measured by adding alkaline phosphatase-conjugated streptavidin in a final step. Retained alkaline phosphatase activity was measured with a fluorescence plate reader (Cytofluor 2300; Millipore, Bedford, MA) using the fluorogenic substrate, attophos (JBL Scientific, San Luis Obispo, CA). This assay showed that serum passed over an anti-CD14 column contained <0.1% of the sCD14 present in untreated serum. Removal of sCD14 caused no change in the ability of the serum to mediate adhesion of LPS-coated erythrocytes to macrophages or to mediate stimulation of adhesivity of PMN by LPS. Septin was thus not depleted by this procedure. To confirm that anti-CD14 did not contaminate the CD14-depleted serum, a sensitive ELISA for murine anti-CD14 antibody was performed. ELISA wells were coated with 1 µg/ml sCD14, blocked with nonfat dry milk (1%), then incubated with dilutions of anti-CD14 or serum for 45 min. Retained mouse IgG was detected with an alkaline phosphatase-conjugated anti-mouse IgG and the fluorogenic substrate, attophos. No anti-CD14 could be detected in the serum passed over the 60b column (data not shown). The sensitivity of the assay assures that the final concentration of anti-CD14 was <0.002 µg/ml in assays that used the CD14-depleted serum.

**sCD14.** sCD14 was obtained from two different sources. Serum sCD14 was obtained by passing 20 ml of human serum over a 60b-Sepharose column and eluting with 0.1 mM triethylamine, pH 11.5. The eluted material was dialyzed against PBS, and appeared as a doublet at 55 kD in SDS gels. In some experiments, this sCD14 was additionally purified by chromatography on Mono Q. Alternatively, CD14 was isolated from the urine from a nephrotic patient by affinity chromatography on an MEM-18-Sepharose column as previously described (16).

**Death of Bovine Endothelial Cells in Response to LPS.** CPAE cells were obtained from American Type Culture Collection (Rockville, MD) (CCL 209) and maintained in MEM (Gibco Laboratories, Gaithersburg, MD), 20% FCS (Gibco Laboratories), nonessential amino acids, penicillin (100 µg/ml) and streptomycin (100 µg/ml). Monolayers were grown 24–48 h in microtiter plates and washed in serum-free media before addition of Hib LPS and serum (6.25%) followed by an overnight incubation. Cytotoxic assays using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (M2128; Sigma Chemical Co., St. Louis, MO) were performed as described elsewhere (11, 17). This assay detects living but not dead cells using a quantitative colorimetric assay dependent on viable mitochondrial dehydrogenase activity of viable cells.

**Expression of ELAM-1 on HUVEC.** HUVEC were isolated and established as monolayer cultures on fibronectin-coated Terasaki wells

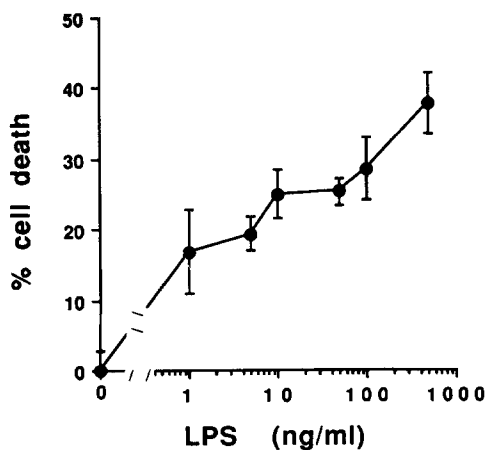
in human serum-containing medium as previously described (18). Some experiments used HUVEC obtained from a commercial source (Clonetics, San Diego, CA) and passaged four times in bovine serum-containing medium according to the manufacturer's instructions. To initiate the experiment, monolayers were washed extensively in MEM containing 10 µg/ml α<sub>2</sub>-macroglobulin (Calbiochem-Behring Corp., San Diego, CA), then incubated for 4 h at 37°C in medium supplemented with serum and Re595 LPS (List Biological, Campbell, CA). The monolayers were then washed, and expression of ELAM-1 on the cell surface was measured as follows. 5 µg/ml of anti-ELAM-1 antibody BB11 (19) was added for 30 min. After extensive washing, alkaline phosphate-conjugated anti-murine IgG was added for an additional 30 min. After a final wash, cell-associated alkaline phosphatase was measured using a fluorogenic substrate as described above for the sCD14 ELISA. Results are reported in arbitrary fluorescence units averaged from triplicate data points. Background fluorescence obtained in the absence of anti-ELAM-1 antibody was subtracted from these values.

**IL-6 Secretion by U373 Cells.** The human astrocytoma cell line U373 (HTB 17; American Type Culture Collection) was obtained from Dr. Guillemont (Santi, France) and maintained in RPMI 1640 supplemented with 10% FCS (Gibco Laboratories). Monolayers of U373 cells in 24-well culture plates (Costar, Cambridge, MA) were washed three times in HBSS (Gibco Laboratories) before addition of different concentrations of LPS from *Escherichia coli* (Sigma Chemical Co.) with 10 ng/ml of urinary sCD14 or 100 ng/ml LBP. In some experiments U373 cells received 10 µg/ml of 3C10, My4, or CD18 antibodies and LPS. The incubations were carried out in AIM serum-free medium (Gibco Laboratories) or in RPMI 1640 (Gibco Laboratories) supplemented with 10% heat-inactivated human A<sup>+</sup> serum (The Blood Bank, University Hospital of Trondheim, Trondheim, Norway) as indicated in the figure legends. After 24 h of incubation the supernatants were harvested and assayed for IL-6 activity by using the IL-6-dependent mouse hybridoma cell line B.13.29 clone 9 as previously described (10, 20).

## Results

We have previously shown that LPS has a marked cytotoxic effect on CPAE endothelial cells (11). This response of endothelial cells to LPS requires serum (11), and occurs at doses of LPS as low as 1 ng/ml (Fig. 1). Concentrations of LPS >500 ng/ml did not decrease the cell viability further. Maximum toxicity varied in experiments from 20 to 70%, but complete cell killing was never observed, regardless of the LPS or serum concentrations used.

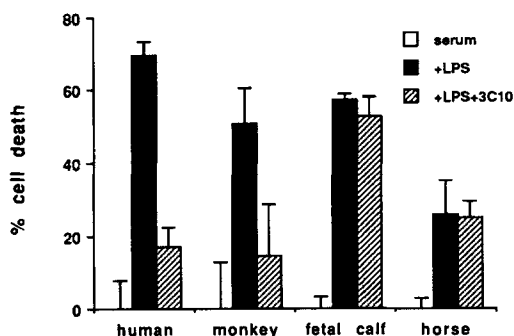
**Anti-CD14 Fails to Block Cytotoxicity if Serum from Nonprimates Is Used.** The requirement for serum and low levels of LPS led us to examine whether CD14 played a role in LPS-induced cell death. Initial studies using bovine endothelial cells cultured in human serum showed a striking blockade of cytotoxicity using the anti-human CD14 mAb 3C10 (11). Similar results were found using a second anti-human CD14 mAb (60b), which blocks the function of human CD14. A third anti-CD14 mAb (26ic), which binds CD14 but does not block interaction of CD14 with LPS/LBP complexes, did not block cytotoxicity in human serum at concentrations as high as 20 µg/ml (data not shown). These results suggest that CD14 serves in recognition of LPS by endothelial cells as it does in mononuclear cells. Further experiments, however, showed that 3C10 did not bind to bovine endothelial



**Figure 1.** LPS-induced cytotoxicity for bovine endothelial cells. LPS was added to CPAE cells in 6.25% human serum and incubated overnight. Viability of the endothelial cells was measured by the MTT colorimetric assay (17). Values are the average of four samples  $\pm$  SD.

cells, and pretreatment of CPAE cells with 3C10 did not affect their sensitivity to LPS added in a subsequent incubation (data not shown). These observations suggested that the CD14 inhibited by anti-CD14 mAb was not expressed at the surface of the endothelial cells.

The specificity of the blocking action of 3C10 was further investigated in studies using bovine endothelial cells cultured in sera from different species. LPS-dependent cytotoxicity was observed in sera from several different species, including human, macaque monkey, fetal bovine, bovine, horse, and rabbit (Fig. 2; and our unpublished results), indicating that all these sera contain the activities necessary for mediating a response to LPS. However, the anti-CD14 antibody 3C10 (2  $\mu$ g/ml) prevented cytotoxicity only with sera from human and macaque sources, not from other species (Fig. 2). Similar results were obtained with a second anti-human CD14 mAb, 60b (data not shown). An ELISA using 3C10 showed a strong signal from both human and macaque serum, but no signal



**Figure 2.** LPS-directed cytotoxicity with sera from various sources and blockage with anti-human CD14 antibody. LPS (100 ng/ml) was added to CPAE cells (where indicated) with sera from different animal sources (6.25%) and incubated overnight followed by measurement of cell viability. 3C10, a CD14 blocking mAb, was added at 2  $\mu$ g/ml where indicated before addition of LPS. Values are the average of four samples  $\pm$  SD.

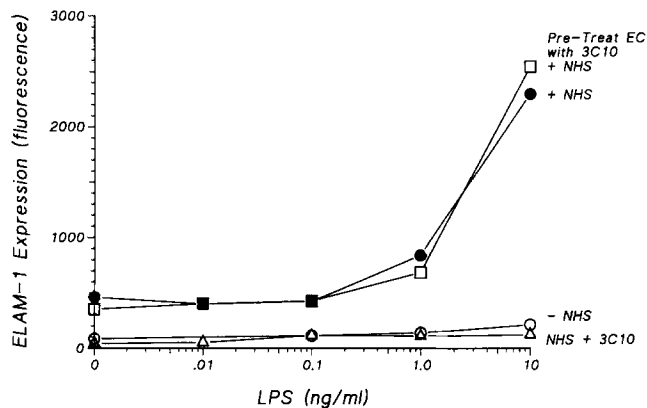
**Table 1.** Depletion of sCD14 from Serum Prevents Response to LPS

Components added	Percent cell death*
Normal serum	0 $\pm$ 2.3
Normal serum, LPS	25.6 $\pm$ 10.2
CD14-depleted serum	0 $\pm$ 1.1
CD14-depleted serum, LPS	0 $\pm$ 5.1
CD14-depleted serum, sCD14, LPS,	23.0 $\pm$ 6.5
CD14-depleted serum, sCD14, 3C10, LPS	6.0 $\pm$ 4.5

\* 6.25% serum (normal or depleted) was added to CPAE endothelial cells, followed by addition of immunopurified sCD14 (4  $\mu$ g/ml from human serum), anti-CD14 3C10 antibody (2  $\mu$ g/ml), and Hib LPS (100 ng/ml) as indicated. Cells were incubated overnight before measuring viability. Values are the average of four samples  $\pm$  SD.

from sera from the other species (data not shown). These results suggest that molecules in the serum rather than on the cell surface represent the targets recognized by anti-CD14 mAbs.

**Depletion of sCD14 from Plasma Blocks Its Ability to Support LPS-mediated Cytotoxicity.** CD14 is found not only on the surface of phagocytes but also in the plasma (16, 21). Bazil and Strominger (22) have shown that CD14 may be released from the surface of monocytes and appears in the plasma of healthy adults at concentrations  $\sim$ 5  $\mu$ g/ml. To determine if the sCD14 in plasma participates in responses of endothelial cells to LPS, human serum was depleted of CD14 by affinity chromatography on anti-CD14-Sepharose. Serum depleted of CD14 in this way failed to support a cytotoxic response of CPAE to LPS (Table 1).



**Figure 3.** Expression of ELAM-1 on HUVEC in response to LPS and serum. Monolayers of HUVEC cultured in bovine serum were washed and incubated for 15 min at 37°C in the presence (open squares) or absence of 50  $\mu$ g/ml 3C10. Monolayers were washed thoroughly, then incubated for 4 h with the indicated concentrations of LPS (Re) in the presence (filled circles) or absence (open circles) of 0.5% NHS. 3C10 (50  $\mu$ g/ml) was added to the NHS in one set (open triangles). ELAM-1 expression was measured as described in Materials and Methods.

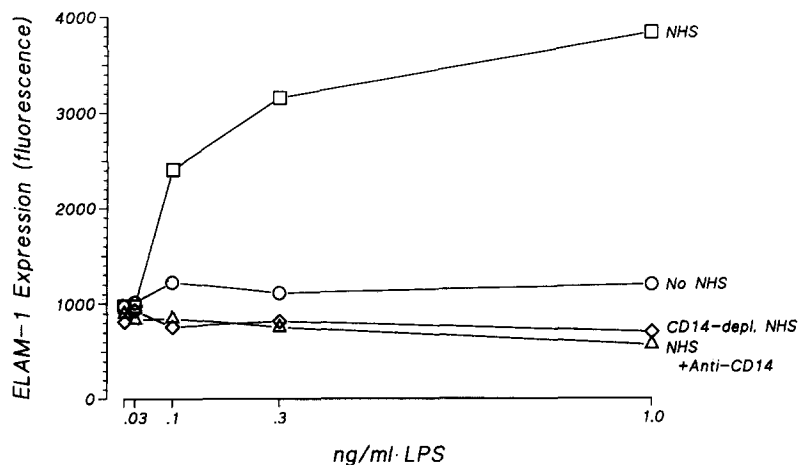
Several observations indicate that the inability of CD14-depleted plasma to support a response to LPS was caused by depletion of CD14, not other factors. LPS-binding proteins in plasma such as LBP or septin were not depleted by the anti-CD14 column since the depleted plasma showed unaltered ability to opsonize LPS-coated erythrocytes for recognition by macrophages (data not shown). Further, the depleted serum was fully capable of supporting responses of CD14-bearing cells to LPS: it showed unaltered ability to enable PMN to respond to LPS with enhanced activity of the leukocyte integrin CR3 and to enable monocytes to respond to LPS with secretion of TNF (data not shown). Anti-CD14 is unlikely to contaminate the CD14-depleted serum since both of these assays are blocked by anti-CD14 (1, 6). Moreover, an ELISA for anti-CD14 in the depleted serum failed to show contamination (see Materials and Methods). Finally, the ability of depleted serum to support a response of endothelial cells to LPS was restored by readdition of sCD14 to the depleted plasma (Table 1). These observations thus indicate that sCD14 is necessary for bovine endothelial cells to respond to LPS.

**sCD14 Is Needed for Induction of ELAM-1 on HUVEC.** LPS has been shown to induce the expression of the adhesion protein ELAM-1 (E-selectin) on HUVEC in serum-containing medium (8). We found that serum is absolutely required for the response of these cells to LPS (Fig. 3), indicating a requirement for soluble proteins. As with bovine endothelial cells, the induction of ELAM-1 by LPS required CD14 since the response was completely blocked with anti-CD14 mAbs 3C10 (Fig. 3) or 60b (Fig. 4), but was unaffected by a control mAb directed against CD18 (IB4; data not shown). mCD14 does not appear to play an essential role in the response of HUVEC to LPS since a pretreatment of HUVEC monolayers with anti-CD14 antibody 3C10 caused no change in the ability of the monolayers to respond to LPS and serum (Fig. 3). On the other hand, depletion of sCD14 from the serum completely abolished its ability to support synthesis of ELAM-1 in response to LPS (Fig. 4), and this response was restored by the addition of sCD14 (Fig. 5). These results confirm that

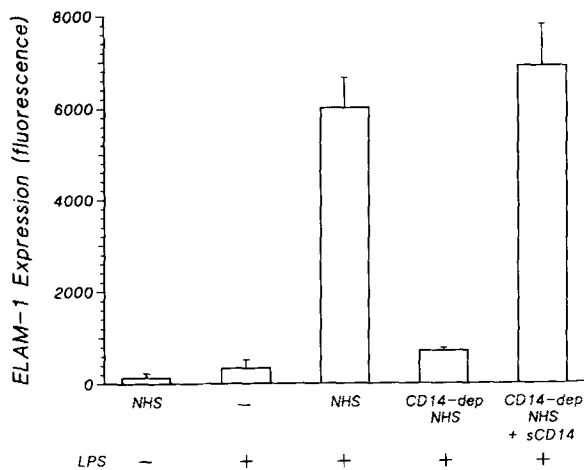
sCD14 in serum plays a crucial role in the response of HUVEC cells to LPS.

**sCD14 Is Required for Secretion of IL-6 by U373 Cells in Response to LPS.** U373 cells are human astrocytoma cells that respond to LPS by secreting IL-6 in a serum-dependent fashion (10a and Fig. 7). Experiments were carried out to study whether CD14 is involved in this induction of IL-6. Different concentrations of LPS in 10% A<sup>+</sup> NHS were added to U373 cells in the absence and presence of 10  $\mu$ g/ml of 3C10, My4, or anti-CD18. Both the anti-CD14 mAbs 3C10 and My4 markedly inhibited LPS-induced IL-6 production whereas CD18 antibodies had no effect (Fig. 6). Since U373 cells express no detectable surface CD14 (10a), these results suggest that CD14 in serum may also be essential for the LPS response in U373 cells.

To confirm a role for sCD14 in the response of U373 cell to LPS, IL-6 secretion was measured in the presence of purified LBP and sCD14. LBP alone was incapable of enabling a response to LPS (10a and Fig. 7). Parallel studies confirmed that the LBP used in these experiments was active in enhancing the responses of monocytes to LPS (data not shown). This result confirms that binding of LPS/LBP complexes to mCD14 does not account for production of IL-6 in U373 cells and indicates that serum contains factors in addition to LBP that are required for cell stimulation. Addition of sCD14 and LBP, on the other hand, enabled strong LPS-dependent stimulation of IL-6 production (Fig. 7). Further studies showed that addition of sCD14 alone, in the apparent absence of LBP, also enabled responses to LPS. Addition of 10 ng/ml sCD14 in AIM serum-free medium resulted in a >10-fold increase in the amount of IL-6 induced by 10 and 100  $\mu$ g/ml of LPS. The ability of LPS to induce IL-6 in the presence of sCD14 was  $\sim$ 10-fold less than in the presence of 10% NHS. Addition of 100 ng/ml LBP to sCD14 did not affect the LPS-mediated response under serum-free conditions. The enhanced response to LPS by sCD14 could be blocked by 3C10 (data not shown). These data thus demonstrate that sCD14 is necessary for U373 cells to respond to LPS.



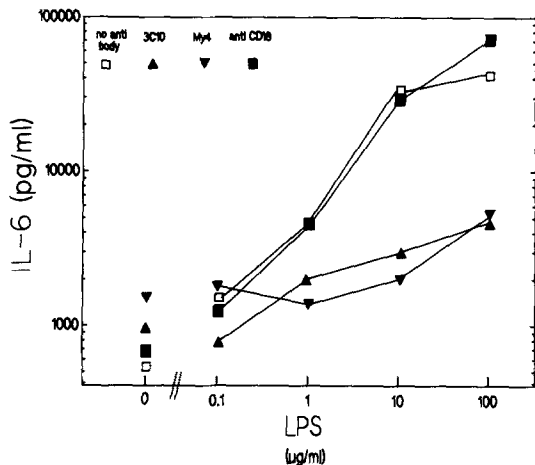
**Figure 4.** Depletion of sCD14 from NHS prevents a response of HUVEC to LPS. Monolayers of HUVEC were grown in human serum containing medium, washed, then incubated for 4 h with the indicated concentrations of Re LPS in the presence of medium alone (circles), 0.5% NHS (squares), 0.5% NHS containing anti-CD14 mAb 60b (triangles), or with CD14-depleted NHS (diamonds). Monolayers were washed, and expression of ELAM-1 was measured as described in Materials and Methods. Results are representative of six separate studies.



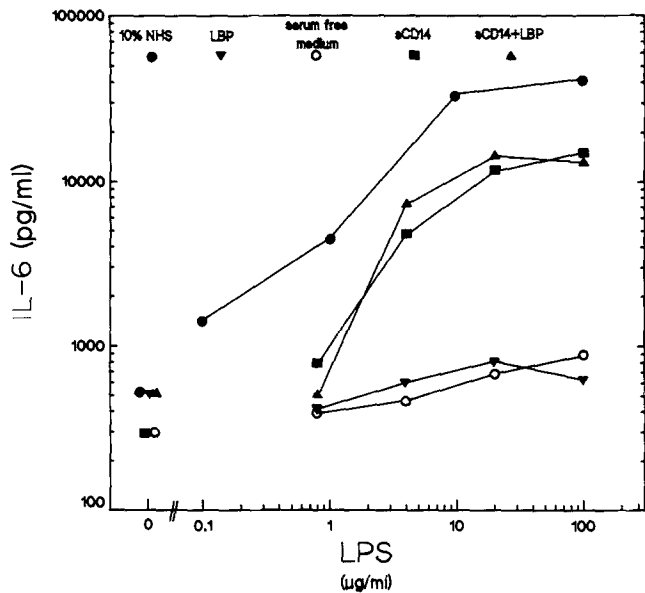
**Figure 5.** Addition of sCD14 restores ability of serum to enable a response to LPS. Monolayers of HUVEC were grown in bovine serum-containing medium, washed, then incubated for 4 h in the presence or absence of LPS (5 ng/ml), NHS (0.5%), CD14-depleted serum (0.5%) reconstituted with 0.5  $\mu$ g/ml of sCD14. The sCD14 used in this study was purified from NHS by sequential chromatography on anti-CD14 and Mono Q. Results are representative of two experiments.

## Discussion

Previous studies have shown that mCD14 on monocytes and PMN is necessary for the responses of these cells to LPS (1, 6). Here we show that CD14 is also necessary for CPAE, HUVEC, and U373 cells to respond to LPS. In contrast with the phagocytic leukocytes studied previously, CPAE, HUVEC, and U373 cells do not express mCD14 at their cell surfaces. The CD14 required by these cells derives, instead, from sCD14 present in serum. These results suggest that sCD14 in the plasma may play a significant role in responses of animals to LPS, and that CD14 may play a role in the responses of



**Figure 6.** Inhibition of LPS-induced IL-6 production in U373 cells by CD14 antibodies. Different concentrations of LPS in 10% NHS ( $\square$ ) with 10  $\mu$ g/ml of 3C10 ( $\blacktriangle$ ), My4 ( $\blacktriangledown$ ), or CD18 antibodies ( $\blacksquare$ ) were added to U373 cells. Supernatants were harvested after 24 h and assayed for IL-6 levels. Addition of antibodies to U373 cells without LPS did not inhibit the spontaneous release of IL-6 from the cells.



**Figure 7.** sCD14 is required for induction of IL-6 in U373 by LPS. Different concentrations of LPS were added to U373 cells in AIM serum-free medium ( $\circ$ ) with 100 ng/ml LBP ( $\blacktriangledown$ ), 10 ng/ml sCD14 ( $\blacksquare$ ), or 100 ng/ml LBP + 10 ng/ml sCD14 ( $\blacktriangle$ ). The LPS response in 10% NHS in RPMI 1640 ( $\bullet$ ) was included for comparison. Supernatants were harvested after 24 h and assayed for IL-6 levels.

many additional cell types, including those that do not express mCD14.

sCD14 is abundant in plasma, accounting for at least 99% of the total CD14 content of blood. Maliszewski (23) has suggested that the sCD14 could act to bind and neutralize LPS/LBP complexes, thus inhibiting responses to endotoxin. Our results suggest the opposite. The sCD14 enables responses of cells that do not express mCD14, and sCD14 certainly does not block the response of monocytes, which respond briskly to LPS in whole blood.

sCD14 is elaborated into the medium by mononuclear cells in vitro (16, 22). These studies have suggested that cell surface CD14 is released into the medium through a proteolytic mechanism, but direct secretion of sCD14, without a membrane-bound intermediate, has not been ruled out. Since sCD14 is thought to lack a GPI anchor (16), these results suggest that the GPI anchor is not necessary for CD14 to perform its role in signal transduction.

How does sCD14, without a GPI anchor, participate in signal transduction? Since sCD14 must interact with cells in some way to stimulate them, we suggest that sCD14 must interact with some additional component on the membrane to bring about association with the cell and signaling of a response. We thus propose that sCD14 binds to an additional receptor subunit on the surface of the endothelial cells and U373 cells. Since sCD14 is not normally associated with the cell surface of these cells, we propose that sCD14 only interacts with this additional subunit after ligation of CD14 by an LPS/LBP on an LPS/septin complex. Precedent for such a mechanism is provided by the receptor for IL-6. Recent studies have shown that the ligand-binding 80-kD subunit of the IL-6 receptor interacts with a signal-transducing 130-kD

subunit only after binding of IL-6 (24). Moreover, both soluble 80-kD as well as membrane-bound 80-kD proteins may bind IL-6, associate with the 130-kD subunit, and transduce a signal (24).

Previous studies have shown that LPS triggers responses in monocytes and PMN by interacting first with the serum proteins LBP or sepsin (1-3, 6). The resulting complex of LPS/LBP or LPS/sepsin then interacts with mCD14 and cellular responses are initiated. By analogy with results obtained with monocytes and PMN, we propose that LPS first interacts with serum-binding proteins such as LBP or sepsin, and the resulting complex then interacts with sCD14 to initiate cel-

lular responses. We wish to point out, however, that a requirement for LBP or sepsin in the function of sCD14 has not been formally proven by our experiments. Indeed sCD14 and LPS alone, without intentional addition of LBP or sepsin, caused strong IL-6 secretion by U373 cells, and a similar response to LPS and sCD14 was observed in both CPAE and HUVEC (data not shown). It is not presently clear whether our preparations of sCD14 are contaminated with LBP or sepsin, or whether a novel mechanism, independent of LBP or sepsin, is involved in the stimulation of cells by LPS and sCD14. The details of the mechanism by which sCD14 stimulates cells are currently under investigation in our laboratories.

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