Structure-Function Analysis of CD14 as a Soluble Receptor for Lipopolysaccharide*

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CD14 is a glycoposphatidylinositol-linked protein expressed by myeloid cells and also circulates as a plasma protein lacking the glycosylphosphatidylinositol anchor. Both membrane and soluble CD14 function to enhance activation of cells by lipopolysaccharide (LPS), which we refer to as receptor function. We have previously reported the LPS binding and cell activation functions of a group of five deletion mutants of CD14 (Viriyakosol, S., and Kirkland, T.N. (1995) J. Biol. Chem. 270, 361–368). We have now studied the functional impact of these mutations on soluble CD14. We found that some deletions that abrogated LPS binding in membrane CD14 have no effect on LPS binding in soluble CD14. In fact, some of the soluble CD14 deletion mutants bound LPS with an apparent higher affinity than wild-type CD14. Furthermore, we found that all five deletions essentially ablated soluble CD14 LPS receptor function, whereas only two of the deletions completely destroyed membrane CD14 LPS receptor function. Some of the mutants were able to compete with wild-type CD14 in soluble CD14-dependent assays of cellular activation. We concluded that the soluble and membrane forms of CD14 have different structural determinants for LPS receptor function.

Septic shock remains a major problem in clinical medicine. It is estimated that 200,000 cases of septic shock occur each year in the U.S. alone, with a mortality rate approaching 50% (1, 2). Septic shock remains the most common cause of death in intensive care units (1, 2). The syndrome of septic shock is caused by overproduction of a host of inflammatory cytokines by monocytes and macrophages in response to microbial products. These include tumor necrosis factor, IL-1, IL-6, and IL-8 (reviewed in Refs. 3 and 4). Macrophages also secrete a wide variety of other compounds in response to microbial products, including platelet activating factor, prostaglandins, enzymes, and reactive oxygen intermediates (4, 33). Endothelial cells respond directly to lipopolysaccharide (LPS), as well as to cytokines secreted by macrophages (5–7). The expression of tissue factor by endothelial cells can activate the clotting cascade (8), and endothelial cell production of nitric oxide may play a role in vasodilatation (9). Production of all these mediators can culminate in hypotension, intravascular coagulation, poor organ perfusion, multi-organ failure, and ultimately, death (1).

A major breakthrough in our understanding of the molecular mechanisms of septic shock has been the realization that CD14 is a receptor for both LPS and peptidoglycan (10–12), two of the most abundant constituents in the bacterial cell wall. We will use the term “LPS receptor” to indicate the ability of CD14 to facilitate the activation of cells by LPS. CD14 functions both as a cell membrane receptor and a soluble receptor for bacterial LPS (5–7, 10). CD14 is expressed as a glycoposphatidylinositol-linked protein on the surface of macrophages, monocytes, and polymorphonuclear phagocytes (13), and many laboratories have shown that CD14 is a critical part of the LPS recognition system in those cells (10, 11, 14, 15). Soluble CD14 (sCD14) also plays a crucial role in the LPS response of endothelial and epithelial cells (5–7). It is noteworthy that sCD14 did not function as an effective soluble receptor for peptidoglycan despite the fact that sCD14 bound peptidoglycan very well (16).

CD14 has also been reported to be a membrane receptor for a very wide range of ligands. The bacterial products include lipoparabinomannan (17), lipoetichoic acid (18), a uronic acid polymer (19), spirochete lipoproteins (20), and a surface protein of the pathogenic fungus Blastomyces dermatitidis (21). CD14 has also been reported to bind to apoptotic cells (22). Both sCD14 and membrane CD14 have been reported to bind to phospholipids (23, 24). This wide spectrum of reactivity fits the definition of a “pattern recognition receptor,” as proposed by Janeway (25). Thus, CD14 seems to be an important part of the innate immune system. The structural basis for this wide spectrum of activity is not known.

We have previously characterized the LPS receptor activity of a group of deletion mutants of CD14 expressed as membrane proteins on Chinese hamster ovary cells (26) and the mouse pre-B cell, 70Z/3 (27, 28). In this report, we evaluate the activity of this same group of CD14 mutants as soluble LPS receptors and report that they have different activities than when expressed as membrane receptors. This implies that soluble and membrane forms of CD14 are functionally distinct in their roles as LPS receptors.

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‡ The abbreviations used are: IL, interleukin; LPS, lipopolysaccharide; Re LPS, S. minnesota Re595 LPS; FITC-LPS, fluorescent Re LPS; sCD14, soluble CD14; PBS, phosphate-buffered saline; LBP, LPS-binding protein; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.
**Structure-Function Analysis of Soluble CD14**

LPS Binding Assays—Three different methods were used to determine LPS binding activity: an ELISA, a fluorescence assay, and sucrose density gradient sedimentation velocity measurements. The ELISA method was done as described with modification (30). CD14 or mutant CD14 was coated on a 96-well plate overnight in 0.2 μL acetate buffer, pH 5.0. The plate was blocked with 0.5% human serum albumin in the acetate buffer for 30 min at 37 °C. Dilutions of Re LPS diluted in human serum albumin with PBS were added to each well and incubated for 10 min at 37 °C. The plate was washed four times with PBS, and affinity purified rabbit antibody to Re LPS was added (29). The plate was incubated for 1 h at 37 °C and washed with PBS, 50 μL/well of a 1:2000 dilution of anti-rabbit antibody conjugated to alkaline phosphatase was added for 1 h at 37 °C. The plate was washed five times, the alkaline phosphatase substrate was added, and the OD was measured at 405 nm.

For the fluorescence assay, 10 ng/mL FITC-LPS and variable quantities of sCD14 were analyzed in a fluorometer as published (32). The affinity was determined by using a variety of concentrations of CD14 and measuring the increase in fluorescent signal. Seven concentrations of CD14 or mutant CD14 were added to FITC-RelPS (10 ng/mL) and the increase in fluorescence was determined. The data were plotted as a double reciprocal plot (1/fluorescence versus 1/concentration). The x intercept was used to determine 1/Kd. All determinations were done twice with essentially identical results.

The sucrose density gradient sedimentation velocity experiments were done by mixing 40 μM sCD14 with 840 μM sCD14 wild type or mutants for 15 min at 37 °C. The complex was analyzed on a linear 5–20% sucrose gradient resting on a cushion of 40% sucrose as described previously (32). The gradient was centrifuged at 55,000 rpm in a VT865 rotor (Sorvall) for 80 min. 350–μL fractions were collected, and the amount of [3H]LPS was determined by liquid scintillation counting. The experiments were done twice with essentially identical results.

**Biological Assays of sCD14 Activity**—LPS and sCD14 induction of IL-6 in U373 cells was done as described by Pugin et al. (6). Briefly, U373 cells were cultured in a 96-well plate at a density of 5 × 10^4 cells/well and grown overnight at 37 °C. The cells were activated, and the supernatant was harvested after 16 h. In all experiments, the CD14 was tested for its ability to stimulate cells in the absence of LPS, and it was found to be inactive. In some experiments, U373 cells were stimulated with 1 ng/mL human IL-1 γ for 16 h. The concentration of IL-6 in the supernatants was determined by ELISA. For the ELISA assay, IL-6 was captured with goat anti-human IL-6 neutralizing antibody (R&D) and detected by rabbit anti-IL-6 (Endogen). The rabbit antibody was detected with the goat anti-rabbit IgG-horseradish peroxidase (Tago). The color reaction was developed with 3,3',5,5'-tetramethylbenzidine substrate, and the OD was measured at 450 nm. The amount of IL-6 was calculated compared with a standard curve of the recombinant human IL-6 (Genzyme).

**RESULTS**

**Reactivity with Monoclonal Antibodies**—The mutants generally fell into two groups (Table 1). The DDED, PFPQD, DDED/PQPD, and AVEVE deletions all had poor reactivity with 28C5, MY-4, 60bca, UCHM-1, and MO-2 but good reactivity with 18E12 and 63D3. 28C5, MY-4, and 60bca have been shown to inhibit CD14 binding to LPS (10, 26). 63D3 and 18E12 must bind to epitopes C-terminal to glycine 152, because they do not react with the 152-amino acid CD14 truncation (28). The DRRQ crystallization was reactive with all monoclonal antibodies tested except for MEM-18. These patterns of reactivity are thus what we would predict from previous studies with soluble (31) and membrane CD14 (26, 28).

**LPS Binding**—CD14 binding of LPS was measured in three different ways: ELISA, binding of FITC-LPS, and sucrose density gradient analysis of complexes. The results of a typical ELISA assay are shown in Fig. 2. Four of the five deletion mutants bound LPS approximately as well as wild-type CD14. The DDED/PQPD deletion mutant was a maximum of 4.5 times more LPS than the wild-type CD14.

We also examined the soluble CD14 deletion mutants in the FITC-LPS binding assay. A representative tracing of wild-type CD14 binding FITC-LPS is shown (Fig. 3). Using our preparations of sCD14, there appears to be no need for the LPS-binding protein to observe the increase in fluorescent signal that is associated with the CD14 binding of LPS. Fig. 3 shows a trac-
ELISA assays were done with mutant or wild-type CD14 as described under “Experimental Procedures.” An OD of >90% of the OD wild-type CD14 was considered ++ + +, 76–90% was assigned ++ +, 51–75% was assigned ++, and 11–50% was assigned +.

| DELETION | 28C5 | MY4 | 60ba | MEM-18 | UCHM-1 | MO-2 | 18E12 | 63D3 | Polyclonal |
|----------|------|-----|------|--------|--------|------|-------|------|-----------|
| DDED     | 0    | 0   | 0    | +++    | +      | 0    | +++   | +    | +++       |
| PQPD     | +    | 0   | 0    | +++    | +      | 0    | +++   | +    | +++       |
| DDED/PQPD | 0    | 0   | 0    | +++    | 0      | 0    | +++   | +    | +++       |
| AVEVE    | 0    | +++ | +++  | +++    | 0      | 0    | +++   | +    | +++       |
| DPRQY    | +++  | +++ | +++  | +++    | 0      |+++   | +++   | +    | +++       |

**TABLE II**

**Apparent affinity of soluble CD14 for LPS**

Data are derived from the fluorescence assay of LPS binding. Seven concentrations of CD14 or mutant CD14 were added to FITC-ReLPS (10 ng/ml), and the increase in fluorescence was determined. The data were plotted as a double reciprocal plot (1/Δ fluorescence versus 1/concentration). The x intercept was used to determine 1/Kp. All determinations were done twice with essentially identical results.

| CD14                  | Kp (M⁻¹) |
|-----------------------|----------|
| Wild-type             | 7.4 × 10⁻⁶ |
| DDED deletion         | 1.7 × 10⁻⁶ |
| PQPD deletion         | 1.7 × 10⁻⁶ |
| DDED/PQPD deletion    | 2 × 10⁻⁷  |
| AVEVE deletion        | 3.3 × 10⁻⁶ |
| DPRQY deletion        | <10⁻⁶    |

**Fig. 2.** An ELISA assay for CD14 binding to Re LPS. The relative amount of LPS binding (compared with wild-type CD14) is shown as a function of increasing amount of LPS. The mean and standard deviation of three determinations is shown; this is one of four representative experiments. Open circles, DDED; closed circles, PQPD; open triangles, DDED/PQPD; closed triangles, DPRQY; open squares, AVEVE.

**Fig. 3.** The fluorescence intensity of FITC-LPS (10 ng/ml) interacting with the indicated concentrations of sCD14 or sCD14 deletion mutant is shown on the y axis; time is shown on the x axis. Two separate experiments are superimposed. Wild-type sCD14 (1 μg/ml) is depicted by circles; FITC-LPS is added at 0 s, and the protein is added at 70 s. DDED/PQPD (1 μg/ml) is shown as a line without symbols; FITC-LPS is added at 50 s, and the protein is added at 120 s. The abrupt declines in the baseline are due to the opening of the shutter to add reagents.

of the 6His tag on LPS binding in this assay, we compared wild-type CD14 and the DDED/PQPD deletion mutant, with and without the 6His tag. The binding curves were identical regardless of the presence of the 6His tag (data not shown). We obtained estimates of apparent Kp of all the mutants by measuring the increase in fluorescence intensity a function of concentration of CD14 (Table II). The estimated apparent Kp of the CD14/LPS interaction for the deletion mutant varied from 2 × 10⁻⁸ (DDED/PQPD) to <10⁻⁶ (DPRQY). Although the fluorescence assay allows us to measure affinity of sCD14 for LPS, results obtained with LBP and bactericidal/permeability increasing protein show that the assay does not report an LPS aggregation state (34).

The functional consequences of LPS binding by different proteins can be very different. For example, both LBP and bactericidal/permeability increasing protein bind FITC-LPS with an increase in fluorescence similar to those seen in Fig. 3, but LPS complexes with bactericidal/permeability increasing protein forms aggregates, and LPS complexed with LBP is dispersed (34). To address the aggregation state, sucrose density gradient experiments were done to evaluate the sedimentation velocity of LPS complexes with mutant CD14. Fig. 4 shows the results. As expected, LPS alone migrates to the bottom of the tube indicating that it is aggregated (Fig. 4A). Wild-type CD14-LPS complexes are primarily at the top of the gradient indicating that the complexes are relatively small (Fig. 4B). We interpret these data as we have in the past (32, 34); sCD14 has dissociated the large LPS aggregates by forming complexes containing one or a few LPS molecules/sCD14. The complexes of LPS with the PQQD, DDED/PQPD, and AVEVE deletion mutants (data not shown) all resembled LPS-wild-type CD14 complexes (Fig. 4B). LPS complexed with the DDED deletion (Fig. 4C) or the DPRQY deletion (Fig. 4D) was more widely distributed in the gradient but generally had a higher sedimentation velocity than wild-type CD14-LPS complexes. Overall, these studies showed that sCD14 mutants with high affinity for LPS (as measured by the fluorescence assay) also formed low molecular weight LPS-CD14 complexes.

**Biological Activity of the Deletion Mutants—**The biological activity of the various forms of CD14 were determined using a U373 epithelial cell assay for sCD14 activity. Soluble wild-type
or mutant CD14 alone did not activate these cells. The concentration of Re LPS was 10 ng/ml, and the concentration of CD14 or deletion mutant varied from 0 to 1 μg/ml. Over this concentration range, there was very little, if any, activation of U373 cells by the CD14 mutants as assessed by IL-6 release (Fig. 5). Once again, the DDED/PQPD deletion mutant behaved the same with or without the 6His tag (data not shown). Similar results with the CD14 were seen with *E. coli* O111:B4 LPS stimulating U373 cells to produce IL-6 and Re LPS stimulating SW620 cells to produce IL-8 (data not shown). At higher concentrations, some of the mutants did stimulate cellular responses (see below).

**Ability of Mutant CD14 to Inhibit Activation of Cells by Wild-type sCD14**—Because many of the CD14 mutants bound LPS but did not activate cells, we asked whether they would compete with wild-type CD14 and inhibit sCD14-dependent cell activation. A typical experiment is shown in Fig. 6. Panel A shows the response to 10 ng/ml Re595 LPS in the presence of 10 or 50 μg/ml mutant CD14. High concentrations of the DDED and the DPRQY mutants activated U373 in the presence of LPS. In the experiment shown in panel B, the concentration of Re LPS was 10 ng/ml, and the concentration of wild-type sCD14 was 200 ng/ml. The concentration of mutant CD14 was varied from 0.1 to 50 μg/ml. The PQPD, DDED/PQPD, and AVEVE mutants inhibited activation of U373 cells by wild-type CD14, in a concentration-dependent fashion. To ensure that this phenomenon was not related to the 6His tag, the DDED/PQPD mutant with the 6His tag removed was tested and was found to inhibit LPS activation as well as the tagged molecule. In other experiments with Re LPS, *E. coli* D31m4 LPS and *E. coli* O111:B4 LPS, the DDED/PQPD mutant was consistently inhibitory at concentrations of 1 μg/ml or above.

To determine whether or not the inhibition seen with mutant CD14 was specific for responses stimulated by LPS, U373 cells were stimulated with 1 ng/ml IL-1 β in the presence or absence of wild-type or mutant CD14. In all instances, there was no inhibition of IL-1 β stimulation of IL-6 by the DDED, DDED/PQPD, or the AVEVE CD14 deletion mutants (data not shown).

These studies were done with wild-type and mutant CD14, in the absence of other plasma proteins, which might modulate the interaction of LPS with CD14. In an effort to more closely mimic the *in vivo* situation, other experiments were done using diluted normal human serum as a source of wild-type CD14. Fig. 7 shows the results. In this experiment, the PQPD and AVEVE mutants partially inhibited wild-type CD14 LPS receptor function. In contrast, at concentrations of 10–50 μg/ml, the DDED/PQPD deletion mutants almost completely inhibited LPS-induced IL-6 production by U373 cells in the presence of serum.

**DISCUSSION**

The objective of this study was to compare membrane and soluble forms of CD14 mutants directly in several different assay systems. They included monoclonal antibody reactivity, LPS binding, and LPS activation of cells. The reactivity with monoclonal antibodies of the sCD14 mutants was almost identical to the pattern of reactivity seen with the membrane form of the protein. There appear to be two classes of epitopes within the N-terminal half of the protein, the 28C5, MY-4, 60bca, MO-2 group (26, 28), and the MEM-18, CHRIS 6 group (31). Although both of these monoclonal antibodies inhibit CD14 binding of LPS and activation of cells, they clearly react with different epitopes by mutational analysis. The epitopes recognized by 63D3 and 18E12 appear to be the C-terminal half of the protein, because they do not react with the N-terminal
brane CD14 binding of [3H]LPS (36). The DDED, PQPD, and open bars represent the apparent $K_D$ of LPS with almost 40-fold higher apparent affinity than wild-type CD14. Unexpectedly, the DDED/PQPD double deletion bound AVEVE deletions all bound slightly better than wild-type and was very similar to the apparent $K_D$ estimated for sCD14 (32). In fluorescent signal, we could estimate apparent $K_D$ varying the protein concentration and measuring the increase was the most quantitative LPS binding assay (32). By measuring the increase was essentially identical to previous estimates for sCD14 (32) and was very similar to the apparent $K_D$ estimated for membrane CD14 binding of [3H]LPS (36). The DDED, PQPD, and AVEVE deletions all bound slightly better than wild-type CD14. Unexpectedly, the DDED/PQPD double deletion bound LPS with almost 40-fold higher apparent affinity than wild-type CD14. As expected from a previous study (31), the DPRQY bound LPS very poorly in the fluorescence assay. The sucrose density gradient assays gave additional information about the type CD14. As expected from a previous study (31), the DPRQY bound LPS very poorly in the fluorescence assay. The sucrose density gradient assays gave additional information about the nature of the complexes formed. All mutants formed complexes with [3H]LPS that had a lower sedimentation velocity than LPS alone, which suggests that the LPS is disaggregated when bound to the CD14 mutants. Even the DPRQY mutant, which bound LPS poorly in the fluorescence assay, formed some complexes.

The preparations of CD14 we used bound LPS rapidly in the absence of LBP. We cultivated our baculovirus-infected High 5 insect cells cultured in this medium containing 5% fetal calf serum be reported elsewhere, we found that CD14 derived from insect cells cultured in this medium containing 5% fetal calf serum required LBP for efficient binding of LPS in the fluorescent LPS binding assay, in contrast to CD14 prepared from insect cells cultured in serum-free medium. We are currently comparing sCD14 prepared in serum and in the absence of serum to try to determine the basis of the need for LBP.

Physiologic concentrations of all but one of the sCD14 mutants we have reported here are unable to activate U373 and SW620 cells in the presence of LPS, despite their ability to bind LPS. We expected that the DDED deletion would lack activity because of previous reports that this region was critical for sCD14 LPS receptor function (37). The DPRQY region has previously been identified as critical for LPS binding activity of sCD14 (31). Darveau and co-workers (30) reported that a charge reversal point mutation of E47R (which is located between the PQPD and the AVEVE regions) selectively affected...
the binding of Porphyromonas gingivalis LPS (30). The E47R mutant was also a less biologically active receptor for P. gingivalis LPS in an endothelial cell activation assay (30).

The observation that all of our sCD14 mutants were biologically inactive LPS receptors was unexpected. When these same deletions were evaluated as cell membrane LPS receptors on Chinese hamster ovary cells and 70Z/3 cells, we found that receptor function was modestly impaired in the DDED and PQQPD double deletions (26, 28) (Table III). The DDED/PQQPD double deletion and the AVEVE deletion were almost inactive as membrane LPS receptors (26). The DPRQY deletion was minimally capable of binding detectable amounts of LPS as membrane receptors in several different types of assay (26). Clearly, the critical regions in CD14 for LPS receptor function are in the membrane and soluble forms of the receptor. It is widely believed that membrane CD14 is only part of a receptor complex and that sCD14-LPS complexes must also bind to a signal-transducing receptor. The discrepancy between critical regions of sCD14 and mCD14 suggests that these two forms of CD14 may be interacting with different signal-transducing receptors.

The ability of some of these mutants to competitively inhibit sCD14 and membrane CD14 receptor function makes sense because of the high affinity binding of LPS without LPS receptor function was modestly impaired in the DDED and PQQPD deletions (26, 28) (Table III). The DDED/PQQPD double deletion and the AVEVE deletion were almost inactive as membrane LPS receptors (26). The DPRQY deletion was minimally capable of binding detectable amounts of LPS as membrane receptors in several different types of assay (26). Clearly, the critical regions in CD14 for LPS receptor function are in the membrane and soluble forms of the receptor. It is widely believed that membrane CD14 is only part of a receptor complex and that sCD14-LPS complexes must also bind to a signal-transducing receptor. The discrepancy between critical regions of sCD14 and mCD14 suggests that these two forms of CD14 may be interacting with different signal-transducing receptors.

The ability of some of these mutants to competitively inhibit sCD14 and membrane CD14 receptor function makes sense because of the high affinity binding of LPS without LPS receptor activity. The DDED/PQQPD double deletion is particularly striking in this regard. This mutant is capable of complete inhibition of sCD14 receptor function in a concentration-dependent manner. The inhibition is specific for LPS, because responses to IL-1β are not affected. The DDED/PQQPD double deletion can also inhibit sCD14 receptor function in human serum, indicating that it is capable of LPS binding in the presence of other LPS-binding proteins. The ability of these sCD14 mutants to inhibit LPS toxicity in vivo suggests that they may be able to inhibit LPS toxicity in vivo.

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