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CD14 Employs Hydrophilic Regions to “Capture” Lipopolysaccharides

Mark D. Cunningham,* Robert A. Shapiro, ‡ Carrie Seachord, § Kent Ratcliffe, † Linda Cassiano, † and Richard P. Darveau §

CD14 participates in the host innate inflammatory response to bacterial LPS obtained from Escherichia coli and other Gram-negative bacteria. Evidence from several laboratories suggests that different regions of the amino-terminal portion of the molecule may be involved in LPS binding. In this report a series of single-residue serine replacement and charge reversal mutations were generated to further elucidate the mechanism by which this protein may bind a multitude of different LPS ligands. Single-residue CD14 mutation proteins were examined for their ability to bind LPS obtained from E. coli, Porphyromonas gingivalis, and Helicobacter pylori and facilitate the activation of E-selectin from human endothelial cells. In addition, the single-residue CD14 mutation proteins were employed to perform monoclonal epitope-mapping studies with three LPS-blocking Abs that bound tertiary epitopes. Evidence that several different hydrophilic regions of the amino-terminal region of CD14 are involved in LPS binding was obtained. Epitope-mapping studies revealed that these hydrophilic regions are located on one side of the protein surface. These studies suggest that CD14 employs a charged surface in a manner similar to the macrophage scavenger receptor to “capture” LPS ligands and “present” them to other components of the innate host defense system. The Journal of Immunology, 2000, 164: 3255–3263.

The innate host defense system recognizes non-self Ags and elicits an immediate inflammatory response. The response facilitates the movement of serum-soluble and cellular components out of the vasculature and into the specific location in tissue where microbial invasion was detected. The goal of the system is to remove the microbial invader. A key unresolved issue is how the host can immediately recognize the multitude of non-self (microbial) components to which it is exposed and initiate such an instant, profound, and potentially life-threatening response.

Recently the LPS binding protein (LBP) † /CD14 activation pathway (1, 2) has been described as a mechanism of microbial activation of the host inflammatory response. Although inflammatory responses to a wide variety of different microbial components are mediated through this pathway (1, 3–10), little is known concerning how the host recognizes different microbial structures or how it regulates the intensity of the inflammatory response. Studies with LPS suggest that recognition of specific lipid A structural details most likely occurs after CD14 binding (11, 12). Kitchens and Munford (11) by careful titration of LBP and LPS have shown that deacylated LPS inhibits wild-type LPS activation at an uncharacterized site after CD14 binding in the LPS recognition pathway. Further, Delude et al. (12) conclusively demonstrated that inhibition of LPS responses by Rhodobacter sphaeroides lipid A and lipid IVₐ was not due to LPS recognition by CD14. These data are consistent with a proposal by Wright (13) that CD14 serves as a mechanism to concentrate microbial ligands at the host cell surface.

Although CD14 binds and shuttles a wide variety of different microbial ligands, the mechanism of how this is accomplished is not clear. Initial studies with truncation mutants identified the amino-terminal region and three leucine repeat domains as sufficient for Escherichia coli LPS binding and host cell activation. Separate studies demonstrated that four CD14 deletion mutation proteins representing removal of the most hydrophilic regions (labeled 1–4 in Fig. 1) were independently necessary for LPS ligand binding. Subsequently, protease protection experiments, an additional deletion CD14 mutation protein, and mAb epitope mapping identified region 4 as required for E. coli LPS binding (14, 15). Another study confirmed that region 3 is involved in E. coli LPS binding with the use of multiple alanine replacement mutations and monoclonal epitope mapping (16). Additional mutations and monoclonal epitope mapping indicated that region 1 may be involved in LPS signaling, but not binding (17). Work from our laboratory, initially employing a peptide approach and then incorporating single amino acid replacements, identified a residue not located in any of these regions to be involved in LPS binding (see Fig. 1) (18). These data indicate that there are several different regions located throughout the amino-terminal region of CD14 that could be involved in LPS binding. However, little is known about the potential tertiary structure of this region, since there are no known sequence similarities with other protein domains, nor has an x-ray crystallographic structure been reported. Therefore, the molecular mechanism employed by CD14 to bind a wide variety of different microbial ligands remains elusive.

In this report the molecular mechanism underlying the interaction of LPS and CD14 was further investigated. Single-point mutations were generated throughout the amino-terminal portion of CD14, and their effects on binding to three different LPS species were examined. Conformational effects of the CD14 mutations were monitored by selective LPS ligand binding, the ability of the...
CD14 mutation protein to function as a host cell activator with E. coli LPS, and mAb epitope analysis. Based upon these analyses the evidence supports the contention that residues located throughout the amino-terminal region of CD14 are involved in LPS ligand binding. Additionally, mAb epitope mapping studies indicate that residues involved in LPS binding are located on the same surface of the protein. We therefore suggest that CD14 is able to bind multiple microbial ligands by presenting an array of charged residues on one surface of the protein. This mechanism is similar to that proposed for the macrophage scavenger receptor protein, which "captures" microbial ligands with charged lysine residues (19).

Materials and Methods

Reagents, cells, and buffers
Reagent grade chemicals were obtained from Sigma (St. Louis, MO). HUVEC growth medium consisted of medium 199 (Life Technologies, Gaithersburg, MD) containing 4 mM l-glutamine, 90 μg/ml heparin, 1 mM sodium pyruvate, 30 μg/ml endothelial cell growth stimulant (Biomedical Products, Bedford, MA), and 20% FBS (HyClone, Logan, UT). Medium 199 stimulation medium consisted of medium 199 containing 4 mM l-glutamine, 90 μg/ml heparin, 1 mM sodium pyruvate, 1 mg/ml human serum albumin, and 5% pooled normal human serum (Gemini Bioproducts, Calabasas, CA). RPMI 1640 medium was obtained from Life Technologies (Grand Island, NY). IL-8 levels in supernatants were measured using CytoSetn Immunoassay Kit (BioSource, Camarillo, CA). Pooled human sera were obtained from Gemini Bioproducts and were depleted of sCD14 as previously described (20). Protein A-Sepharose was obtained from Repligen (Cambridge, MA). HUVEC were obtained from Clonetics (San Diego, CA) and maintained in HUVEC growth medium. Cells were used at the fourth passage for all experiments described in this report.

Bacterial strains and purification of LPS

_Helicobacter pylori_ ATCC 43504 was obtained from the American Type Culture Collection (Manassas, VA). Porphyromonas _gingivalis_ ATCC 33277 was provided by Aaron Weinberg, Department of Periodontics, University of Washington (Seattle, WA). _E. coli_ BMS A016 (serogroup O6) is a human blood culture isolate provided by Marie Coyle, Harborview Medical Center (Seattle, WA). Strains were examined for purity, properly identified, and stored at −70°C. Cultures were made from frozen bacterial stocks to avoid repetitive subculture. Bacterial cells were grown for LPS purification as previously described (20). Protein A-Sepharose was obtained from Repligen (Cambridge, MA). HUVEC were obtained from Clonetics (San Diego, CA) and maintained in HUVEC growth medium.

Construction and expression of sCD14:Rg

A cDNA fragment encoding the extracellular domain of wild-type CD14 was subcloned upstream of an cDNA fragment encoding the H, CH2, and CH3 domains of human IgG1 in the mammalian expression vector CDMTB as previously described (20). Similarly produced CD40 recombinant globulin (CD40:Rg) was used as a control. Oligonucleotide-directed mutagenesis

Mutations were introduced into CD14:Rg by a two-step PCR procedure (18). Overlapping PCR fragments were first generated in separate reactions and then reannealed and extended in a second PCR. In the first step, oligonucleotides (complement and reverse complement) containing the desired codon changes flanked by 18 nucleotides on each side were synthesized for each mutation. Two fragments were then synthesized and PCR using T7 (in pcDNA3) and the reverse complement oligonucleotide to generate the 5’ fragment and CD14R5 (5’-CAAGTCCTGTGGCTTCCA GAG-3’) and the complementary oligonucleotide to generate the 3’ fragment. After gel purification, −10 ng of each fragment was mixed and used as template in a second PCR reaction using T7 and CD14R5 oligonucleotides. The resulting 480-nucleotide PCR fragment was gel purified, digested with HindIII and XhoI, and used to replace the 5’ HindIII/XhoI fragment from the wild-type CD14:Rg/pCDNA3 plasmid. All nucleotide changes were confirmed by DNA sequencing. The fusion proteins were produced by transient expression in COS cells and were purified from the spent COS cell supernatants using protein A-Sepharose chromatography. Fusion protein stock solutions were found to be free of endotoxin by the Limulus lysate assay obtained from BioWhittaker (Walkersville, MD).

Generation of mouse mAbs

mAbs to human sCD14 were cloned by standard methods after fusion of a murine myeloma line with spleen cells from mice immunized with purified sCD14:Rg as previously described (20). Throughout the cloning procedure, culture supernatants were assayed for Abs using the ELISA method, with purified sCD14:Rg as the solid phase Ag. Select mAbs of the appropriate specificity were produced in high titer purified from the culture supernatants by protein A chromatography, extensively dialyzed into PBS, filter-sterilized, and stored at 4°C. The purified mouse anti-human sCD14 mAbs used in these experiments were 2G7 and 6C8. A commercial mouse anti-human CD14 mAb (MY4) was obtained from Coulter Immunology (Hialeah, FL).

The mouse anti-LPS mAbs used for detection of LPS binding to sCD14:Rg were 3B4 (IgM; anti-E. coli A016), 5B9 (IgM; anti-P. gingivalis ATCC 33277), and 7A9 (IgG1 anti- H. pylori ATCC 43504). They were cloned as described above using spleen cells from mice immunized with purified LPS.

ELISA for detection of LPS binding to immobilized sCD14:Rg

LPS binding to wild-type and mutations of sCD14 were measured in a capture ELISA format (20). Briefly, Immulon II (Dynatech, Chantilly, VA) 96-well plates were coated overnight at 4°C with 0.05 μg/well sCD14 wild-type or point mutation protein. The next day, the wells were emptied, and nonspecific binding sites were blocked with 100 μl/well of 0.5% BSA in PBS for 30 min at 37°C. The plates were then washed three times with PBS. Fifty microliters of LPS diluted in 1% CD14-depleted serum (source of LBP) was added per well, and the plates were incubated at 37°C for 1 h. Unbound LPS was removed with four PBS washes, and bound LPS was detected with specific mouse anti-LPS mAb at 0.5 μg/ml in 0.5% BSA in PBS as previously described (20).

ELISA for detection of anti-CD14 mAb binding to immobilized sCD14:Rg

Immunol II plates were coated with 0.05 μg/well of sCD14 wild-type or point mutation fusion proteins as previously described (18), and the amount of fusion protein that was captured was measured. A commercial mouse anti-human IgG, which reacts with the CD14:Rg fusion protein. mAbs MY4, 2G7, and 6C8 (0.02 μg/ml) were added, and ELISA detection was performed as previously described (20) with goat anti-mouse IgG (Sigma).

IL-8 functional assay

Primary HUVEC cultivation and the IL-8 ELISA were performed as described previously (18, 26), except that 1% sCD14-depleted serum, as prepared as previously described (20), instead of 5% complete serum was used in the medium 199 stimulation medium.

LPS blocking experiments

The ability of the CD14 mAbs to block LPS binding to CD14 was examined. CD14 was captured on a 96-well ELISA plate as previously described (27), and 30 min before the addition of _E. coli_ LPS (100 ng/ml), anti-CD14 mAbs or a negative control Ab (anti-CD40) were added. _E. coli_ LPS binding was detected as previously described (20), except that the anti- _E. coli_

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LPS mAb was biotinylated (used at 3 μg/ml), because the usual second-step goat anti-mouse would react with the mouse mAbs.

Results

Single-amino acid replacement mutations in the amino-terminal region of CD14 were generated, and their effect on LPS binding was examined. The mutational analysis was directed to the amino-terminal portion of the molecule, since previous work had established that this portion of the molecule was sufficient for E. coli LPS ligand binding and subsequent activation of host defense cells (28, 29). Both serine replacement and charge reversal mutations were created for 15 different amino acids (Fig. 1). Charged residues were chosen, since the previous analysis had indicated that charge reversal mutations had the greatest effect on LPS binding (18). The CD14 mutation proteins were then examined for their ability to bind LPS (20) obtained from three different species of bacteria (E. coli, P. gingivalis, and H. pylori) such that a broader range of potential LPS binding sites could be identified as previously described (20).

Initial analysis revealed three different groups of CD14 mutation proteins based upon their LPS binding characteristics. Initially a survey was performed with nearly all the new CD14 mutation proteins. Statistical analysis was not performed, nor were additional assays conducted with all the mutation proteins. Rather, this analysis was used to initially identify those new CD14 mutation proteins that may have an effect on LPS binding. Analysis of the data revealed that three different patterns of LPS binding to the CD14 mutation proteins were observed (Table I). In the majority of mutation proteins (group 1) no effect on LPS binding was observed (26 of 36 examined). In this group, consistent with the initial, more limited analysis, none of the serine replacement mutations reduced LPS binding (a total of 19 were examined). The second group (group 2, indicated in bold in Table I) consisted of

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− E. coli (30 ng/ml), P. gingivalis (300 ng/ml), and H. pylori (300 ng/ml) LPS were mixed with 1% CD14-depleted serum, and the amount of LPS binding was determined as described in the text. Equal amounts of fusion protein were captured as monitored by ELISA with goat anti-human IgG which reacts with all the CD14-Rg fusion proteins. Two experiments were performed for each LPS binding to the CD14 mutation fusion proteins. The results for the mutation proteins are expressed as a percentage of binding compared to wild-type CD14. A +, The LPS bound the mutation protein was not reduced by greater than ~25%; +/−, the LPS reduction was between 25 and 50%; and −, the LPS bound the mutation protein by <50% of the wild-type value. NT, LPS was not tested. Three groups of LPS binding patterns were observed: group 1, no effect on LPS binding; group 2 (bold), significantly reduced in binding one or two species of LPS; group 3 (underline), significantly reduced binding to all LPS species examined (E28K and E37K were characterized previously (24) and are included here for purposes of comparison).
those mutation proteins that demonstrated a reduced binding with either *P. gingivalis* or *H. pylori*, but not *E. coli* LPS (E4K, D9K, E11K, D12K, R53E, D59K). All these were charge reversal mutations. Interestingly, no mutation proteins examined demonstrated reduced *E. coli*, yet had normal *P. gingivalis* or *H. pylori* LPS binding. It is suspected that this may relate to the higher affinity for CD14 displayed by *E. coli* LPS (20), although this possibility has not been formally examined. The third group (group 3, indicated by underlining in Table I) consisted of two CD14 mutation proteins that displayed reduced binding to all three LPS ligands examined (D10K and R14E).

Additional binding assays confirmed that charge reversal mutations in select CD14 residues reduced LPS binding

Additional, more comprehensive binding assays were performed with representative CD14 mutation proteins from each group. These assays confirmed the three different patterns of LPS binding to the CD14 mutation proteins mentioned above (see Table I and Fig. 2). The serine replacement mutation proteins in group 1 did not significantly reduce the binding of any LPS species (D10S, E11S, D12S, R14S, R53S, D57S, and D59S; *p* > 0.1, not significant by two-sample t test). The charge reversal mutations in group 3 reduced the binding to the all LPS species examined to near background levels (D10K and R14E; *p* < 0.05 or 0.001, significant or highly significant compared with wild-type CD14 depending upon the species examined by two-sample t test). However, similar to our previous results (18), not all charge reversal mutations reduced LPS binding (for example, D57K).

The CD14 mutation protein representatives from group 2 were of particular interest, because they displayed preferential reductions in LPS binding, suggesting that specific residues may be more important for some LPS species. For example, the binding of *E. coli* LPS was not significantly reduced (*p* > 0.7, by two-sample t test), whereas the binding of both *P. gingivalis* (*p* < 0.05, by two-sample t test) and *H. pylori* LPS (*p* < 0.05, by two-sample t test) was significantly reduced when binding to wild-type CD14 and that to mutation protein E11K were compared. A preferential reduction in LPS binding was also observed for mutation protein D59K (*E. coli* LPS, *p* > 0.3, not significant; *P. gingivalis* LPS, *p* < 0.001, highly significant; *H. pylori* LPS, *p* > 0.1, not significant; by two-sample t test; when the binding of LPS to CD14 mutation protein D59K was compared with wild-type CD14). The binding of all three LPS species to CD14 mutation protein D12K was significantly reduced compared with that to wild-type CD14 (*p* < 0.05, significant by two-sample t test). However, the reduction of *P. gingivalis* LPS was significantly greater than that observed with *E. coli* (*p* < 0.05, significant by two-sample t test, when *P. gingivalis* and *E. coli* LPS binding to D12K were compared with wild-type CD14). Although all LPS species demonstrated reduced binding to CD14 mutation protein R53E, the reduction was only significant (*p* < 0.05, by two-sample t test) for *P. gingivalis* compared with wild-type CD14.

The differential abilities of *E. coli*, *P. gingivalis*, and *H. pylori* LPS to bind mutant CD14 proteins D12K and D59K were further investigated (Fig. 3). Concentration-dependent increases in the binding of all three LPS species examined were observed with both mutant CD14 proteins. Consistent with earlier analysis (Fig. 2), *E. coli* LPS bound to D59K to nearly wild-type levels, and that to D12K was slightly reduced (a significant reduction in *E. coli* LPS binding to CD14 mutation protein D12K was only observed at 30 ng/ml LPS). In contrast, *P. gingivalis* and *H. pylori* LPS binding to CD14 mutation protein D12K was significantly reduced at all LPS concentrations examined (*p* > 0.05, by two-sample t test). Examination of LPS binding to CD14 mutation protein D59K revealed that *P. gingivalis* (*p* < 0.05, by two-sample t test), but not *E. coli* or *H. pylori*, LPS was reduced compared with wild-type CD14. These analyses are consistent with the observation that charge reversal mutations in CD14 residues D12 and D59 have preferential effects on LPS ligand binding.

The ability of mutant CD14 protein to activate IL-8 secretion is consistent with *E. coli* LPS binding

The ability of the CD14 single-point mutation proteins to activate IL-8 secretion in HUVECs with *E. coli* LPS was examined. An assay that employed CD14-depleted serum that was supplemented with the different CD14 mutation proteins was used to examine the ability to elicit IL-8 secretion from human endothelial cells (Fig. 4). Several mutation proteins were examined that did not demonstrate significantly lower *E. coli* LPS binding (E11S, D57K, D59S) and also did not display a significant difference in IL-8 secretion.

![FIGURE 2](http://www.jimmunol.org/). The abilities of LPS from three different species of bacteria to bind CD14 mutation proteins. The binding assays were performed as described in Materials and Methods and Table I. Each assay was performed on at least four separate occasions in duplicate. The mean for each LPS compared with wild-type CD14 is presented.
Three separate experiments were performed, and these CD14 single-point mutation proteins did not alter the amount of IL-8 secreted by human endothelial cells in response to 30 ng/ml E. coli LPS by >20% that of wild-type protein (data not shown). Fig. 4 presents the data obtained with those mutant CD14 proteins that displayed significant reductions in either E. coli LPS binding or the binding of P. gingivalis or H. pylori (group 1, D10K and R14E; group 2, E11K, D12K, R53E, and D59K from Fig. 2). The effect of increasing LPS concentrations was examined. Each CD14 mutant was able to elicit IL-8 secretion; however, the amount of LPS necessary to facilitate secretion varied significantly among the CD14 mutants. Mutants D10K, D12K, and R14E that displayed the poorest ability to bind E. coli LPS were also the poorest activators of IL-8 secretion. Little or no IL-8 secretion occurred with these CD14 mutations proteins at concentrations of LPS <3 ng/ml. In contrast, E11K and D59K, two CD14 mutation proteins that did not display a significant reduction in E. coli LPS binding (p > 0.7

FIGURE 3. The ability of increasing concentrations of E. coli (A), P. gingivalis (B), and H. pylori (C) LPS to bind wild-type CD14 and CD14 mutation proteins D12K and D59K. In creasing concentrations of LPS were mixed with 1% CD14-depleted serum and added to plates previously coated with 0.5 μg/well wild-type sCD14:Rg (●), D12K (▲), D59K (X), or CD40:Rg (■). After 1 h of incubation at 37 C, LPS bound to sCD14:Rg was detected with mouse anti-LPS mAbs (see Materials and Methods). The experiments were performed on three separate occasions, and the averages from duplicate wells from a representative experiment are shown.

FIGURE 4. Human endothelial cell IL-8 secretion in response to CD14 mutation proteins and E. coli LPS. Increasing concentrations of E. coli LPS (as indicated on the x-axis) in 1% CD14-depleted sera were mixed with 0.5 μg/ml of thrombin-cleaved wild-type CD14 and the CD14 mutation protein D10K, E11K, D12K, R14E, R53E, or D59K and added to endothelial cells as described (see Materials and Methods). The experiments were performed on three separate occasions, and the averages from duplicate wells from a representative experiment are shown.

FIGURE 5. Anti-CD14 mAbs block E. coli LPS binding to CD14. In this experiment the ability of the anti-CD14 mAbs to block E. coli LPS binding CD14 was examined. CD14 was captured on a 96-well ELISA plate as described in Materials and Methods, and 30 min before the addition of E. coli LPS (100 ng/ml), anti-CD14 mAbs or a negative control Ab (anti-CD40) were added at the concentrations indicated on the x-axis. Two separate experiments were performed with similar results; a representative experiment is shown.
and $p > 0.3$, respectively), facilitated IL-8 secretion similar to wild-type CD14 (Fig. 4). The relative ability of mutant CD14 proteins to bind E. coli LPS was similar to their respective ability to facilitate E. coli LPS-mediated IL-8 secretion. One exception to this pattern is that mutant D12K bound E. coli LPS slightly better than R14E (Fig. 2); however, R14E was slightly better at facilitating IL-8 secretion. Interestingly, this mutation occurs in the hydrophilic region previously identified as being involved in LPS signaling (17). However, the differences in relative binding and activation were small between these CD14 mutation proteins and do not represent a significant alteration in the general pattern that demonstrates that reductions in mAb binding to CD14 mutation proteins occur independently of their LPS binding properties. Conversely, the three CD14 mutation proteins from group 3 all bound at least one CD14 mAb (one of these, R14E, bound all the mAbs; another, E37K, bound two of three of the Abs). These CD14 mutation proteins displayed a significant reduction with all the LPS species examined, making it more difficult to rule out potential conformation effects of these charge reversal mutations. However, based upon the mAb data, the mutations did not induce conformational changes that altered Ab binding. Collectively, these data clearly demonstrate that reductions in mAb binding to CD14 mutation proteins occur independently of their LPS binding properties.

A panel of anti-CD14 mAbs that block LPS binding and bind native tertiary structure was employed to perform epitope-mapping studies

Binding studies with the different CD14 mutation proteins employing three different anti-CD14 mAbs were performed. The generation of these Abs has been previously described (27). These Abs blocked LPS binding (Fig. 5). The blocking of E. coli LPS to CD14 is shown in Fig. 5; however, these Abs also blocked P. gingivalis and H. pylori LPS binding (data not shown). In fact an analysis of 35 master wells with LPS-blocking activity failed to identify any Ab that displayed selective blocking of these three different types of LPS (data not shown). In addition, these Abs bound tertiary epitopes on CD14, as determined by their inability to bind heat-denatured CD14 by ELISA (data not shown). The inability of these Abs to bind denatured CD14 was further analyzed by immunoblot analysis that demonstrated that these Abs did not bind to SDS and heat-denatured CD14 after SDS-PAGE (data not shown). Further studies (23 different anti-CD14 master wells that blocked LPS binding were examined) failed to reveal an Ab that bound a heat-denatured (or presumably linear) epitope by ELISA (data not shown).

Epitope mapping of mAbs to CD14 revealed that hydrophilic regions 1, 3, and 4 involved in LPS binding are located on the same surface of CD14

Each mAb displayed a different pattern of binding to the CD14 mutation proteins, demonstrating that each Ab bound a different epitope (Table II). In addition, as expected, the mAbs provided a measure of protein conformation independent of LPS binding. For example, CD14 mutation proteins from group 1 (D10S, E11S, D12S, D57K, and D59S), which demonstrated no observable phenotype with respect to LPS binding or host cell activation, demonstrated reduced or no binding to at least one of the mAbs. These data demonstrate that although the CD14 mutation did not influence LPS binding, mAb binding was affected. Conversely, the three CD14 mutation proteins from group 3 all bound at least one CD14 mAb (one of these, R14E, bound all the mAbs; another, E37K, bound two of three of the Abs). These CD14 mutation proteins displayed a significant reduction with all the LPS species examined, making it more difficult to rule out potential conformation effects of these charge reversal mutations. However, based upon the mAb data, the mutations did not induce conformational changes that altered Ab binding. Collectively, these data clearly demonstrate that reductions in mAb binding to CD14 mutation proteins occur independently of their LPS binding properties.

Examination of the CD14 mutation proteins that affected each mAb revealed two different patterns. The first pattern was observed with mAb MY4. Mutation proteins D9K, D10K, D12S, and D12K, but no other mutation proteins, reduced or eliminated MY4 binding. These residues are located at or near hydrophilic region 1 and are located nearly adjacent to each other in the linear sequence of the protein.

m Abs 2G7 and 6C8 represent the second pattern. mAb 2G7 failed to react with CD14 mutation proteins located in hydrophilic region 1, similar to MY4 (D9K, D10K, E11S, E11K, and D12K), although a slightly different pattern was observed. In contrast to MY4, this mAb also failed to bind CD14 mutation protein E37K, a CD14 residue located in hydrophilic region 3 on the linear sequence (see Fig. 1). mAb 6C8 binding was also reduced by mutations located in two different hydrophilic regions found in the

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Table II. Comparison of the ability of CD14 mAbs MY4, 2G7, and 6C8 to bind immobilized sCD14 wild-type and point mutation fusion proteins

* ELISA Immunlon II plates (Dynatech) were coated with 0.05 µg/well of CD14 wild-type or point mutation fusion proteins, and the amount of fusion protein which was captured was monitored with goat anti-human IgG which reacts with the CD14-Rg fusion protein (see legend to Table I). mAbs MY4, 2G7, and 6C8 were added, and ELISA detection was performed. A minimum of two experiments was performed for each mAb binding to the CD14 mutation fusion proteins. The results for the mutation proteins are expressed as a percentage of binding compared to wild type CD14. +, The LPS bound the mutation protein was not reduced by greater than ~25%; +/−, the LPS reduction was between 25 and 50%; and −, the LPS bound the mutation protein by <50% of the wild-type value. NT, the mAb was not tested.
DISCUSSION

The data presented in this manuscript demonstrate that single-residue mutations located throughout the amino-terminal region of CD14 affect LPS binding. This is consistent with other reports employing deletion or multiple alanine replacement mutational analysis to identify regions of CD14 required for E. coli LPS binding (16, 29). In addition, similar to other previous investigations (15, 16, 29), binding to a panel of LPS-blocking anti-CD14 mAbs was employed as an independent measure of potential conformational changes induced by the mutations. All the CD14 mutation proteins examined in this study bound at least one Ab, consistent with the idea that no major conformational changes were induced by the single amino acid substitutions.

Charge reversal mutations had the greatest effect on LPS binding and resulted in the identification of CD14 mutation proteins that were significantly reduced in their ability to bind to at least one LPS species (group 2: E11, D12, and R53, D59; group 3: D10 R14 from this study and E28 and E37 from Ref. 18). These data now indicate that residues located in highly hydrophilic regions 1, 3, 4, and E28 (near region 2) contribute to the LPS binding site (Fig. 1). There are two possibilities for the inability of LPS to bind to these different CD14 mutation proteins. The residue altered in the mutation protein is located at or near an LPS binding site of the protein, and the reversal in side group charge alters the binding site sufficiently to make it incompatible for LPS binding. This would suggest that the LPS binding site is tertiary in nature, incorporating residues located throughout the amino-terminal portion of the molecule, as opposed to a linear sequence located in one region. Alternatively, the different charge reversal mutations may have induced a minor conformational change in a distally located LPS binding region. Definitive resolution of this issue most likely will require determining the protein structure after crystallization.

However, independent evidence consistent with the idea that the CD14 LPS binding site is tertiary in nature and is comprised of residues located throughout the amino-terminal region was obtained. mAb epitope analyses revealed that hydrophilic regions 1, 3, and 4 are located in close enough physical proximity so that residues in each region influence mAb binding.

A major component of the epitope for mAb 2G7 is found in highly hydrophilic region 1 of CD14 (residues D9 to D12, Table II). Both group 2 and 3 LPS binding CD14 mutation proteins are found in this cluster of charged residues. There is good evidence that residue E11, located in this cluster, is a contact residue for Ab 2G7. The replacement of the side chain carboxyl group of E11 with a serine OH nearly eliminated the binding of mAb 2G7, yet had no effect on LPS binding (Table II; see text) or E. coli LPS-mediated host cell activation (see text). A single Ala replacement of residue D59 also eliminates MEM 18 binding, suggesting that 6C8 and MEM 18 share at least one residue in their epitope (15). However, CD14 mutation proteins D10S, D10K, and R14E, which are located at or near hydrophilic region 1, also partially or completely reduced the binding of mAb 6C8. Because D10S had no detrimental effect on LPS binding or host cell activation, and its only phenotype was a partial reduction in Ab 2G7 binding, it is likely that this residue is located at or near the epitope for both Abs. Similarly, although conformational effects cannot be ruled out, the reduction in 6C8 binding observed for CD14 mutation proteins D10K and R14E are probably due to it being located close to or in the epitope for Ab 6C8.

What are the structural implications for hydrophilic regions 1, 3, and 4 being located in close enough physical proximity so that residues in each region influence Ab binding? Ab epitopes on protein surfaces usually contain six to eight contact residues that bind the complementarity-determining region of the Ab. The side groups of other residues on the protein can affect binding to the Ab by steric hindrance or charge repulsion if they are located in close tertiary proximity to the contact residues. Because all the anti-CD14 mAbs examined in this study recognized native, but not denatured protein, due to the likelihood of discontinuous epitopes, any residue could theoretically influence Ab binding if it were located in the area necessary for Ab binding. The surface area on proteins typically recognized by Abs is ~700–900 A^2 (30, 31). Therefore, it is safe to conclude that in the native protein hydrophilic regions 1, 3, and 4 all exist on the same side of the protein surface.

We therefore propose that CD14 presents to its various microbial ligands an array of different potential binding interactions by orientating several of its groups of charged residues to one side of the protein, in close physical proximity to each other. We suggest...
that numerous charged residues located throughout the amino-terminal region of CD14 may participate in ligand binding, each contributing to the total energy of binding and accounting for nanomolar concentration binding constants for ligands such as E. coli LPS. This proposal is consistent with the mutational analysis studies presented here and previously (13) that revealed numerous serine or alanine replacement mutations that did not display a difference in binding to E. coli LPS. If, indeed, the binding site consists of numerous residues, then a mutation in any one may not reduce the affinity sufficiently to be measured experimentally. In contrast, charge reversal mutations may interrupt binding by charge repulsion, effectively blocking the LPS ligand from docking.

The selective binding data from CD14 mutation proteins in group 2 indicate that different LPS ligands interact differently with the CD14 LPS binding site. It is tempting to speculate that different LPS binding characteristics of P. gingivalis, H. pylori, and E. coli to CD14 may be related to the different structures and cell-stimulating properties of these LPS species. In addition to differences in core sugar composition, P. gingivalis lipid A differs from E. coli lipid A by the absence of an ester-linked phosphate at the 4’ position and fatty acids at the 3 and 3’ positions and the presence of fatty acids possessing 16–17 carbon atoms (24). The H. pylori lipid A structure has not been specifically discerned, but compositional analysis has shown that it contains long 3-hydroxy fatty acids (16–18 carbon atoms) and has an unusual phosphorylation pattern compared with that of E. coli lipid A (23). In addition, both P. gingivalis and H. pylori LPS are unusual, in that, unlike E. coli LPS, they are poor stimulators of cytokines from monocytes (32–35) and do not stimulate E-selectin expression from human endothelial cells (32). However, less potent monocyte activation may be due to poor LBP binding (20), and currently there is no evidence that CD14 LPS binding patterns influence downstream signaling events. Although the possibility that different LPS binding patterns influence downstream signaling events cannot be ruled out, it is more likely that the differences in LPS binding observed here were due to lower binding affinities of these LPS for CD14 compared with E. coli (20). It is possible that the structure of these LPS species does not permit as many interactions with charged residues on CD14, making it easier to detect decreased binding with select CD14 mutation proteins in our assays.

This hypothesis is consistent with the data generated from several different laboratories that have investigated the LPS ligand properties of this molecule. It proposes that LPS ligands bind this charge “catch” region in a nonspecific fashion, i.e., LPS ligands are capable of interacting with a variety of different charge residues. This tactic is similar to that previously reported for the macrophage scavenger protein (19), where the collagenous domain has been proposed to “provide a selectively sticky surface that functions as a kind of molecular flypaper for the high affinity binding of specific polyanions.” The hypothesis also provides an explanation for the apparent lack of ligand-specific recognition (12). The exact mechanism by which CD14 binds multiple non-self ligands must await the precise structural determination afforded by protein crystalization. Nevertheless, this hypothesis is consistent with the idea (13) that no one pattern recognition receptor is capable of non-self recognition. Rather, the innate host defense system employs multiple proteins with different binding affinities, transfer properties, and locations to arrive at a consensus about non-self and mount an appropriate response.

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