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Identification of CD14 Residues Involved in Specific Lipopolysaccharide Recognition

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CD14 is a key molecule responsible for the innate host inflammatory response to microbial infection. It is able to bind a wide variety of microbial ligands and facilitate the activation of both myeloid and nonmyeloid cells. However, its specific contribution to the innate recognition of bacteria is not known. Presently there is no information on the contribution of individual CD14 residues to *Escherichia coli* lipopolysaccharide (LPS) binding or on the molecular basis of the interaction between CD14 and LPS from other bacteria. LPS obtained from *Porphyromonas gingivalis*, a bacterium associated with chronic inflammatory disease, binds CD14 and activates myeloid cells but does not facilitate the activation of nonmyeloid cells. The transfer and binding of these two LPS species to soluble CD14 recombinant globulin proteins with single point mutations was examined. Functional activity of the mutant proteins was monitored by E-selectin expression on human umbilical cord endothelial cells. The analysis identified a charge reversal mutation in a single residue, E47, that demonstrated selective binding to *E. coli* LPS but not to *P. gingivalis* LPS. E-selectin activation assays indicated that proteins with mutations at position E47 maintained their structural integrity. Other mutations, including a charge reversal mutation of residue E58, did not significantly reduce the binding of either LPS ligand or the ability of the molecule to facilitate E-selectin activation. These data demonstrate that CD14 can selectively recognize different LPS ligands.

CD14 is now clearly established as a key molecule involved in the recognition of bacteria by the innate host defense system (23, 25). It is able to facilitate the expression of inflammatory molecules produced by both myeloid and nonmyeloid cells in response to gram-positive and gram-negative bacteria (5, 14, 18, 19). The well-known inflammatory activation by *Escherichia coli* lipopolysaccharide (LPS) is mediated through CD14 (7). Several studies have revealed that LPS initially binds LPS-binding protein (LBP), an acute-phase serum protein which facilitates the transfer of LPS to CD14 (22, 26). Myeloid cells contain CD14 as a glycosylphosphatidylinositol-anchored membrane protein (mCD14) which accepts LBP-delivered LPS. LPS complexes with a serum-soluble form of CD14 (sCD14) to facilitate activation of nonmyeloid cells (19). A previous study had determined that the amino-terminal portion of CD14 was sufficient for *E. coli* LPS binding and activation (13). Mutational analysis in this region has identified discrete regions required for *E. coli* LPS binding (12, 15, 24).

LPS molecules obtained from different bacteria differ with respect to both structure and ability to stimulate the innate host defense system. For example, LPSs obtained from *Helicobacter pylori* and *Porphyromonas gingivalis*, two bacteria associated with chronic inflammatory disease, bind sCD14 but fail to activate nonmyeloid cells (1, 2). In addition, although LPSs obtained from these bacteria employ mCD14 to activate myeloid cells (21), they are less effective than *E. coli* LPS (2, 17). Analysis of LBP binding and CD14 transfer has revealed that these LPS molecules bind LBP poorly and therefore have significantly lower rates of transfer to CD14 (1). However, poor transfer and lower levels of binding of *P. gingivalis* LPS to sCD14 could not account for the lack of nonmyeloid cell activation (1). One possible explanation for the lack of nonmyeloid cell activation is that *P. gingivalis* LPS binds CD14 differently than *E. coli* LPS.

Little is known about how CD14 is able to recognize different microbial ligands and facilitate the activation of either nonmyeloid or myeloid cells (14, 18). Pugin et al. (18) have proposed that CD14 fits the criteria for a pattern recognition receptor, a hypothesis for microbial recognition by the innate host defense system originally proposed by Janeway (11). As such, it is able to recognize and respond to a wide variety of nonself or microbial antigens. Alternatively, Wright (25) has proposed that CD14 is part of a winnowing process in which CD14, LBP, and other proteins each partially recognize microbial components and concentrate a subset of microbial antigens for presentation to the different components of the innate recognition activation cascade. In either case, a clear description of the binding specificity of CD14 has not been performed.

Although previous work has described regions of CD14 (12, 13, 15, 24), no single point mutations involved in LPS binding have been reported. In addition, no studies have described the residues necessary for binding molecules other than *E. coli* LPS. To begin to determine the potential contribution of CD14 to innate recognition, a comparison of the binding of two LPS ligands that differ both structurally (16, 20) and functionally (2) in their ability to mediate innate host responses to CD14 was performed. A site-specific mutation in CD14 was obtained that demonstrated selective binding and transfer of *E. coli* LPS but not *P. gingivalis* LPS. These data are suggestive of specificity of binding and transfer of different LPS molecules via the CD14 pathway and implicate CD14 as a target for the treatment of bacterially induced chronic inflammatory disease.

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MATERIALS AND METHODS

Media, buffers, and reagents. Reagent-grade chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Pooled human sera were obtained from

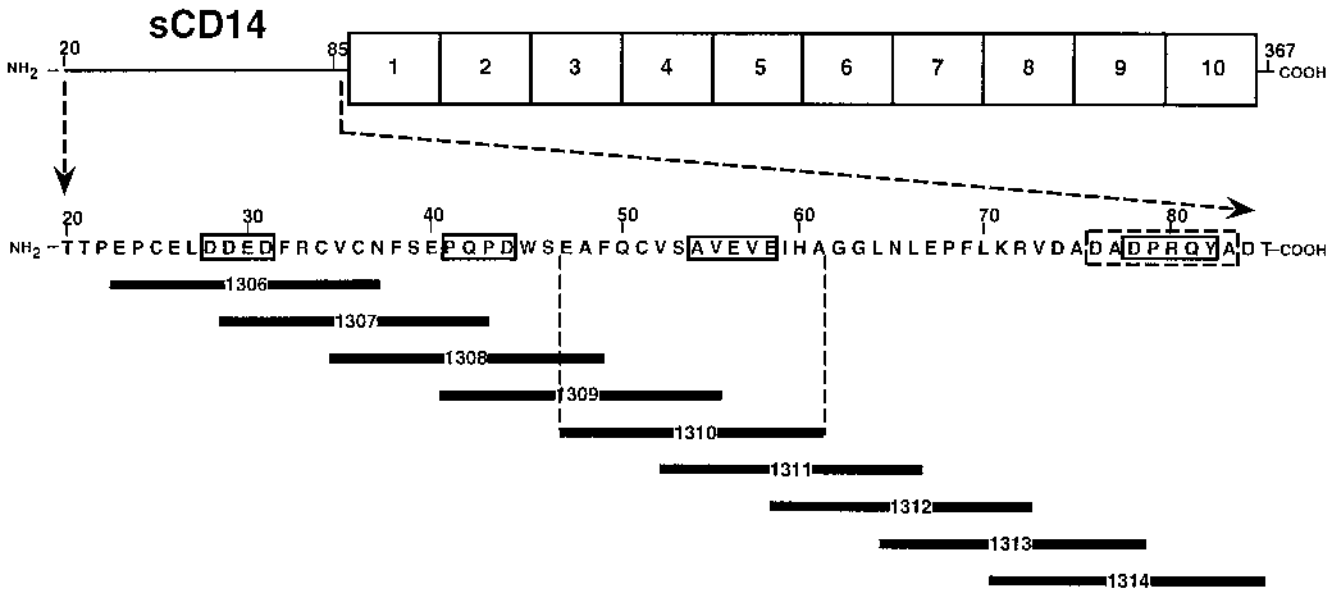

FIG. 1. Schematic representation of human CD14. The residues are numbered beginning with the signal sequence peptide. The boxed numbers 1 through 10 refer to the number of leucine repeats found in the complete molecule. An enlargement of amino acid residues 20 to 85 of human CD14 is shown. Boxed residues (solid [24] and dashed [12]) represent sites previously identified as participants in binding of *E. coli* LPS. The residues included in peptides 1306 to 1314 are shown.

Gemini Bioproducts, Calabasas, Calif., and were depleted of sCD14 as previously described (1). Protein A-Sepharose was from Repligen, Cambridge, Mass. LPSs from *E. coli* BMS A016 and *P. gingivalis* ATCC 33277 were purified as previously described (1). The molecular weights of *E. coli* and *P. gingivalis* LPSs were estimated to be 9,170 and 7,042, respectively.

Generation of CD14 peptides. Nine peptides, 15 amino acids in length and corresponding to the amino terminus of human CD14, were synthesized on a Gilson Multiple Peptide Synthesizer AMS422 with 9-fluorenylmethoxycarbonyl amino acids (6). The assembled peptides were cleaved from the resin and deprotected by a 2-h reaction with trifluoroacetic acid-water-thioanisole-ethanedithiol (100:5:5:2.5). Following precipitation with cold ether, the peptides were dissolved in formic acid, diluted with water, and lyophilized.

Construction and expression of sCD14:Rg. A cDNA fragment encoding the extracellular domain of wild-type CD14 was subcloned upstream of a cDNA fragment encoding the hinge, CH2, and CH3 domains of human immunoglobulin G1 in the mammalian expression vector CDM7B− as previously described (1). Similarly produced CD40 recombinant globulin (CD40:Rg) (9) was used as a control.

Oligonucleotide-directed mutagenesis. Mutations were introduced into CD14:Rg by a two-step PCR procedure. 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 by PCR with T7 (in pcDNA3) and the reverse-complement oligonucleotide to generate the 5′ fragment and with cd14R5 (5′-CAAGTCCTGTGGCTTCCAG AG-3′) and the complementary oligonucleotide to generate the 3′ fragment. After gel purification, ~10 ng of each fragment was mixed and used as the template in a second PCR reaction with T7 and cd14R5 oligonucleotides. The resulting 480-nucleotide PCR fragment was gel purified, digested with *Hin*dIII and *Xho*I, and used to replace the 5′ *Hin*dIII/*Xho*I 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 purified from the spent COS cell supernatants by protein A-Sepharose chromatography as previously described (8). Fusion protein stock solutions were stored at −20°C in 50% glycerol–phosphate-buffered saline (PBS) and found to be free of endotoxin by the limulus lysate assay (BioWhittaker, Walkersville, Md.).

HUVEC E-selectin assay. Primary human umbilical vein endothelial cells HUVEC cultivation and the E-selectin enzyme-linked immunoassay (ELISA) were performed as described previously (2) except that 2% sCD14-depleted serum was used in the M-199 stimulation medium instead of 5% complete serum.

sCD14 used in E-selectin activation experiments was obtained by cleaving sCD14:Rg overnight at room temperature at the thrombin cleavage site engineered between sCD14 and the immunoglobulin G tail sequences (1 μg of human thrombin [Sigma] per 5 μg of sCD14:Rg in 100 mM Tris–200 mM NaCl–5 mM $CaCl_2$–0.2% 2-mercaptoethanol).

ELISA for detection of LPS binding to immobilized sCD14:Rg. LPS binding to wild-type and mutant sCD14 was measured in a capture ELISA format. Briefly, 96-well Immulon II plates were coated overnight at 4°C with 50 μl of goat anti-human immunoglobulin G antiserum (Antibodies, Inc., Davis, Calif.) diluted 1/1,000 in PBS per well. The next day, the wells were emptied, and nonspecific binding sites were blocked with 100 μl of 0.5% bovine serum albumin (BSA) in PBS per well for 30 min at 37°C. The wells were emptied, and 50 μl of sCD14:Rg or CD40:Rg at 1 μg/ml diluted in 0.5% BSA–PBS was added per well. The plates were incubated for 2 h at 37°C. The plates were then washed three times with PBS. Fifty microliters of LPS diluted in 0.5% BSA–PBS was added per well, and the plates were incubated at 37°C for various intervals. Unbound LPS was removed with four PBS washes, and bound LPS was detected with specific mouse anti-LPS monoclonal antibody at 0.5 μg/ml in 0.5% BSA–PBS as previously described (1).

RESULTS

Peptide inhibition of LPS binding to sCD14. A peptide approach was taken to determine if a difference in the abilities of these two LPS species to bind or transfer to CD14 could be detected. Peptides corresponding to the amino terminus of CD14 were examined for the ability to inhibit binding of either *E. coli* or *P. gingivalis* LPS to CD14. It has been previously shown that this region of the molecule binds *E. coli* LPS and functions to activate interleukin 6 (13). Nine peptides, 15 amino acids in length and sequentially overlapped by 5 amino acids, were constructed corresponding to the amino-terminal amino acids E24 to T85 of CD14 (Fig. 1). Peptides were preincubated in sCD14-depleted serum with either *E. coli* or *P. gingivalis* LPS and then added to immobilized sCD14:Rg. All peptides shown in Fig. 1 were examined for inhibition of LPS binding to sCD14 on at least three separate occasions. The abilities of peptides 1309, 1310, and 1311 to inhibit LPS binding are shown in Fig. 2. It was found that one peptide, designated 1310, corresponding to amino acids E47 to A61, significantly inhibited binding of *P. gingivalis* LPS ($P < 0.001$ [two-sample t test] at both 400 and 200 μg of peptide per ml) compared to 1309 or 1311. None of the other peptides examined displayed any inhibition of LPS binding to CD14. Inhibition of binding of *P. gingivalis* LPS to CD14 by 1310 was dependent on peptide concentration. A slight inhibition of binding of *E. coli* LPS to CD14 by peptide 1310 was noted at 400 μg of peptide per ml ($P < 0.05$ [two-sample t test], 1310 versus 1309) but was not statistically significant compared to

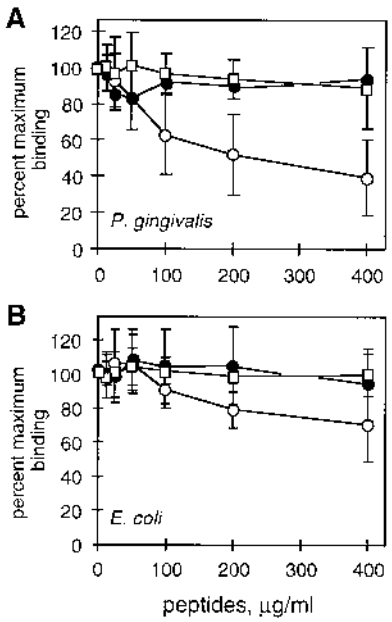

FIG. 2. Abilities of CD14 peptides 1309, 1310, and 1311 to block binding of LPS to immobilized sCD14:Rg. Increasing concentration of peptides 1309 (□), 1310 (○), and 1311 (●) were mixed with 1% CD14-depleted serum and 200 ng of *P. gingivalis* LPS per ml (A) or 10 ng of *E. coli* (B) LPS per ml. The mixtures were preincubated for 1 h at 37°C and then transferred to plates that had been previously coated with 1-μg/ml sCD14:Rg (50 μl/well; 0.5 μg/well). After 1 h of incubation at 37°C, LPS bound to sCD14:Rg was detected by using mouse anti-LPS monoclonal antibodies (see Materials and Methods). The results are presented as the averages of three separate experiments (duplicate wells for each experiment) ± the interassay standard deviations (error bars).

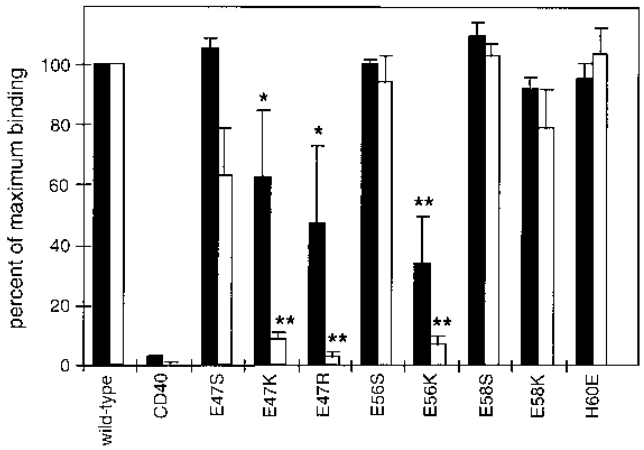

FIG. 3. ELISA detection of *E. coli* and *P. gingivalis* LPSs bound to immobilized sCD14 wild-type and point mutation fusion proteins. sCD14 point mutations of residues between E47 and A61 (within peptide 1310) were examined for the ability to bind *E. coli* and *P. gingivalis* LPSs. *P. gingivalis* LPS (300 ng/ml) and *E. coli* LPS (30 ng/ml) were mixed with 1% CD14-depleted serum and added to plates previously coated with 1 μg (50 μl/well; 0.5 μg/well) of either wild-type sCD14:Rg or CD40:Rg per ml. The results are presented as the averages of three separate experiments (duplicate wells for each experiment) ± the interassay standard deviations. Dark bars, *E. coli* LPS; light bars, *P. gingivalis* LPS; *, $P < 0.05$ versus wild type; *, $P < 0.001$ versus wild type (two-sample *t* test).

1311 ($P > 0.1$ [two-sample *t* test], 1310 versus 1311). No significant inhibition of binding of *E. coli* LPS to CD14 was observed at peptide concentrations of 200 μg/ml ($P > 0.2$ [two-sample *t* test]) when peptide 1310 was compared to 1309 or 1311. The ability of peptide 1310 to significantly inhibit binding of *P. gingivalis* LPS to CD14 suggested that this region of the molecule may be more important for binding of this LPS than in *E. coli.*

Point mutations in sCD14 residues significantly reduce LPS binding. Based upon the peptide inhibition results, selected residues in CD14 between positions E47 and A61 were subjected to site-specific mutagenesis. Three negatively charged residues (E47, E56, and E58) were mutated. In addition, the histidine at position 60 (H60) was replaced by glutamic acid.

The binding of LPS to the mutated CD14 proteins was compared with binding to wild-type CD14 by ELISA (Fig. 3). Amino acid substitutions at E58 and H60 had little or no effect on binding of either *E. coli* LPS or *P. gingivalis* LPS ($P > 0.2$ [two-sample *t* test]). Substituting S for E at position 47 (E47S) reduced *P. gingivalis* LPS slightly, but the reduction was not statistically significant. A similar substitution at position 56 (E56S) did not significantly affect the ability of either LPS species to bind to CD14 ($P > 0.2$ [two-sample *t* test]). In contrast, the binding of both *E. coli* and *P. gingivalis* LPSs to CD14 mutant E56K was reduced to less than 50% of that of the wild type ($P < 0.001$ [two-sample *t* test]). Mutations E47K and E47R displayed a differential effect on LPS binding. Binding of *E. coli* LPS to E47K and E47R was reduced to 65 and 49%, respectively, of the wild-type level ($P < 0.05$ for E47K and $P < 0.01$ for E47R [two-sample *t* test]). Binding of *P. gingivalis* LPS to E47K and E47R was more severely reduced, to 9 and 3%, respectively ($P < 0.001$ [two-sample *t* test]).

The differential abilities of *E. coli* and *P. gingivalis* LPSs to bind mutant CD14 proteins E47K and E47R were further investigated. Binding assays were performed with various concentrations of LPS (Fig. 4). Concentration-dependent increases in binding of *E. coli* LPS were observed with both mutant CD14 proteins E47K and E47R. At the highest con-

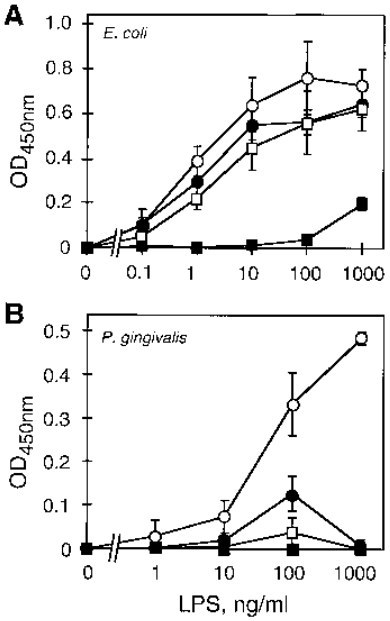

FIG. 4. The ability of increasing concentrations of *E. coli* and *P. gingivalis* LPSs to bind E47K and E47R relative to wild-type sCD14. Increasing concentrations of *E. coli* LPS (A) or *P. gingivalis* LPS (B) were mixed with 1% CD14-depleted serum and added to plates previously coated with 1 μg (50 μl/well; 0.5 μg/well) of wild-type sCD14:Rg (○), E47K (●), E47R (□), or CD40:Rg (■) per ml. After 1 h of incubation at 37°C, LPS bound to sCD14:Rg was detected by using mouse anti-LPS monoclonal antibodies (see Materials and Methods). The results are presented as the averages of three separate experiments (duplicate wells for each experiment) ± the interassay standard deviations (error bars).

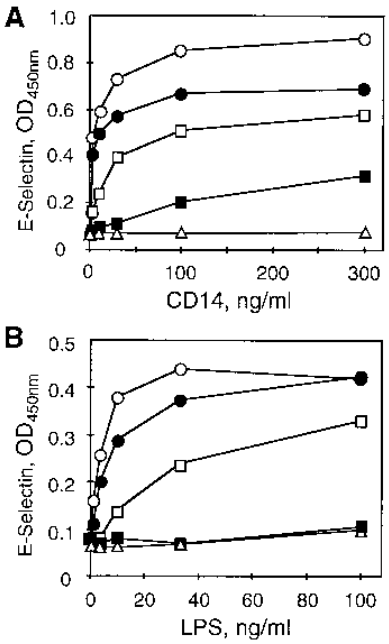

FIG. 5. Abilities of E47K, E47R, and E56K to mediate *E. coli* LPS stimulation of E-selectin expression on HUVEC compared to that of wild-type sCD14. (A) Increasing concentrations of wild-type sCD14 (○), E47K (●), E47R (□) or E56K (■) or buffer only (△) was mixed with 2% sCD14-depleted serum and 30 ng of *E. coli* LPS per ml. (B) Increasing concentrations of LPS were mixed with 70 ng of wild-type sCD14 (○), E47K (●), E47R (□), or E56K (■) per ml or with buffer only (△). The mixtures from A and B were immediately applied to fourth-passage HUVEC in 96-well plates. E-selectin expression was detected after 4 h of incubation by using a mouse anti-E-selectin monoclonal antibody. The experiments were performed three times, and the results shown are the averages for duplicate wells from a representative experiment.

centration of *E. coli* LPS examined (1 μg/ml), there was no significant difference in the binding of *E. coli* LPS to the wild-type and mutant proteins. Binding of *P. gingivalis* LPS to E47K and E47R was significantly reduced at all LPS concentrations ($P < 0.05$ at 10 ng/ml and $P < 0.001$ at 100 and 1,000 ng/ml for both E47K and E47R [two-sample t test]). At the highest concentration of *P. gingivalis* LPS examined (1 μg/ml), no binding to E47K or E47R was observed.

The ability of mutant CD14 proteins to activate E-selectin expression is consistent with binding of *E. coli* LPS. The functional activity of the CD14 single point mutation proteins to activate E-selectin expression in HUVEC was monitored with *E. coli* LPS. Examination of *P. gingivalis* LPS was not possible since it does not activate E-selectin (2), intercellular adhesion molecule, or interleukin 8 expression on endothelial cells. Those proteins which did not demonstrate a significantly lower level of LPS binding (E47S, E56S, E58S, E58K, and H60E) (Fig. 3) also did not display a significant difference in E-selectin activation (data not shown) ($P > 0.05$ for each protein compared to the wild type [two-sample t test] based upon four separate experiments). Figure 5 contains the data obtained with those mutant CD14 proteins that displayed significant reductions in binding of *E. coli* LPS (E47K, E47R, and E56K). The effects of both increasing concentrations of CD14 mutant protein (Fig. 5A) and increasing LPS concentrations (Fig. 5B) were examined. Each CD14 mutant was able to facilitate activation of E-selectin; however, both the amount of protein necessary to facilitate expression and the level of E-selectin expression varied significantly among the CD14 mutants. Mutant E56K, which displayed the poorest ability to bind *E. coli* LPS, was also the poorest activator of E-selectin expression. Minimal activation occurred at 300 ng of E56K CD14 protein per ml, and no activation was observed when increasing LPS concentrations were added to 70 ng of E56K CD14 protein per ml. In contrast, E47K CD14 protein was able to activate E-selectin expression almost as well as the wild type at 3 ng of protein per ml (Fig. 5A). At 70 ng of protein per ml, E47K CD14 protein was similar to the wild type in its ability to activate E-selectin expression with increasing concentrations of *E. coli* LPS (Fig. 5B), consistent with its ability to bind higher concentrations of *E. coli* LPS. The relative abilities of mutant CD14 proteins E47K, E47R, and E56K to bind *E. coli* LPS are similar to their respective abilities to activate E-selectin.

DISCUSSION

The abilities of *P. gingivalis* and *E. coli* LPSs to bind sCD14 were examined to determine if this molecule recognizes these LPS ligands differently. These LPS species differ both structurally and functionally. Detailed lipid A analysis of *P. gingivalis* LPS has revealed the absence of a 4′-ester-linked phosphate and 3- and 3′-ester-linked fatty acids, as well as the presence of fatty acids containing 16 or 17 carbon atoms, compared to the *E. coli* LPS (16). Functionally, *P. gingivalis* LPS does not activate nonmyeloid cells (2), although it binds sCD14 (1). These observations provided a system with which to investigate whether CD14 recognizes different LPS ligands or LPS-LBP complexes in a specific manner.

The analysis revealed that CD14 was able to selectively recognize two different LPS species. Mutations in amino acid E47 to either lysine (E47K) or arginine (E47R) were unable to accept transfer of *P. gingivalis* LPS from LBP. In contrast, E47K and, to a lesser extent, E47R could accept LBP-delivered *E. coli* LPS and were able to facilitate activation of E-selectin expression. The mutation which identified the selective transfer of these LPS species was very specific. Other, similar mutations reversing the charge of other residues located in the same region of the molecule had no effect on either LPS binding or activation. This analysis did not permit us to distinguish if E47K or E47R was either unable to accept *P. gingivalis* LPS bound to LBP or, after successful transfer to CD14, bound too weakly to be detected in our assay system. Regardless of the mechanism, the ability of a single residue to differentially affect LPS binding demonstrates a previously unobserved ligand specificity for CD14.

An earlier study had shown that the amino-terminal portion of CD14 was sufficient for both *E. coli* LPS ligand binding and signal transduction (13). This region in CD14 does not display significant homology to sequences of other proteins of known structure (4, 24). Additional studies had identified a series of mutants with deletions in this region of the protein which failed to bind *E. coli* LPS (24). Independent evidence that residues located within one of these deletion mutants are directly involved in binding of *E. coli* LPS was obtained by protease protection and monoclonal epitope mapping experiments (12, 15). E47 is located in a region of the molecule not previously identified by any of these studies (Fig. 1). Examination of the *P. gingivalis* LPS ligand identified this residue as being important for LPS transfer; however, binding of *E. coli* LPS was partially affected in the mutants E47K and E47R, suggesting an additional role for this residue in the binding of this LPS. In addition, this analysis identified E56 as a key residue within one previously identified deletion mutation (A54 to E58) which had been shown to affect binding of *E. coli* LPS (24). The mutant protein E56K demonstrated reductions in binding of both *E. coli* and *P. gingivalis* LPSs to nearly

background levels, and the mutation dramatically reduced the ability of the molecule to facilitate E-selectin activation. A similar mutant protein, E58K, had no effect on LPS binding or E-selectin activation. In the absence of structural data for CD14, the exact contribution of residues E47, E56, and other previously identified regions (12, 15) to LPS binding could not be resolved. However, it is clear that recognition of the LPS ligand depends on specific contributions of select CD14 residues. This has implications for pattern recognition (14, 18) in innate host defense. Regardless of whether there is partial (25) or full (18) recognition by CD14, the mutations described here provide a molecular basis for microbial recognition.

The role of CD14 in determining the ability of a cell to distinguish between two different LPS molecules is not known. This has a particular relevance in the case of binding of *P. gingivalis* LPS, since previous work has shown that *P. gingivalis* LPS was able to bind CD14 and activate myeloid cells but not nonmyeloid cells (references 1 and 2 and unpublished data). Little is known about the events that occur after binding of LPS to CD14 which result in cellular activation. However, recently it has been shown that proteins in the LPS activation cascade distal to CD14 can recognize LPS ligands (3). It is possible that similar proteins in nonmyeloid cells are not activated by the *P. gingivalis* LPS-CD14 complex due to the altered binding characteristics of this LPS compared with *E. coli* LPS. This could explain the differential activation of mCD14 but not sCD14 displayed by this LPS (2). Additional experimentation will be required to test this hypothesis.

The specificity of binding or transfer of LPS to CD14 identifies this molecule as a potential new target for immunomodulation of chronic inflammatory diseases. This may be particularly relevant in the treatment of human periodontal disease, in which there is a clear association with colonization by *P. gingivalis* (10). This disease consists of repeated cycles of bacterial colonization after effective mechanical debridement and short-term antibiotic treatment. Long-term administration of antibiotics to protect against recolonization is not desirable due to both the development of resistance and the disruption of the normal bacterial flora. Administration of an immunomodulatory drug with the properties of peptide 1310 could limit the destructive inflammatory effect of the infection without exerting selective pressure or altering the normal flora. The ability to specifically attenuate the inflammatory response may have applications in other chronic disorders, such as *H. pylori* infection, which is suspected of being associated with several long-term sequelae, including cancer (4a).

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R.A.S. and M.D.C. contributed equally to this work.

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