Helicobacter pylori and Porphyromonas gingivalis
Lipopolysaccharides Are Poorly Transferred to Recombinant Soluble CD14

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Helicobacter pylori and Porphyromonas gingivalis are gram-negative bacteria associated with chronic inflammatory diseases. These bacteria possess lipopolysaccharides (LPSs) that are able to activate human monocytes to produce tumor necrosis factor alpha but fail to activate human endothelial cells to express E-selectin. With Escherichia coli LPS, tumor necrosis factor alpha activation requires membrane-bound CD14 and E-selectin expression requires soluble CD14 (sCD14). Therefore, the ability of H. pylori and P. gingivalis LPSs to transfer to and bind sCD14 was examined by using immobilized recombinant sCD14 and human serum or recombinant LPS-binding protein (LBP). H. pylori and P. gingivalis LPSs were transferred to sCD14 when serum or LBP was present. However, the transfer of these LPSs to CD14 in serum was significantly slower than the transfer of E. coli LPS. Quantitation of the transfer rates by Michaelis-Menten kinetics yielded $K_m$ values of 6 and 0.1 nM for H. pylori and E. coli LPSs, respectively. The amount of P. gingivalis LPS required to obtain half-maximum binding to CD14 was approximately 10-fold greater than the amount of E. coli LPS required. The slower transfer rates displayed by these LPSs can be explained by the poor binding to LBP observed in direct binding assays. These results are consistent with the proportionately lower ability of these LPSs to activate monocytes compared with E. coli LPS. However, the ability of H. pylori and P. gingivalis LPSs to bind LBP and transfer to sCD14 demonstrates that the lack of endothelial cell CD14-dependent cell activation by these LPSs occurs distal to sCD14 binding.

Lipopolysaccharide (LPS) is a potent stimulator of both myeloid and nonmyeloid cells involved in host defense (6, 51). The host’s ability to rapidly recognize and signal responses to bacterial challenge is believed to represent an important component of our innate resistance to infection. Recently, both LPS-binding protein (LBP) and CD14 have been implicated as key mediators in this process (33, 50). LBP (molecular mass, $\sim$60 kDa) is present in normal serum and increases in concentration during the acute-phase response (44). CD14 is a 55-kDa glycoprotein found both on monocytes and in serum, where it binds LPS and initiates the cellular response to this molecule (19, 34, 51). CD14 has recently been suggested to act as a pattern recognition receptor and accordingly is becoming appreciated as a key molecule for bacterial recognition and the initiation of the inflammatory response by the host (33, 50).

On the basis of studies with LPS from Escherichia coli and Salmonella species, a mechanism describing the early molecular events leading to LPS activation of proinflammatory responses has been proposed (46). LPS is transferred to and binds CD14 via LPS-LBP complexes (45). An LPS binding site has been identified on LBP (10, 42). Studies have shown that LPS binds CD14 in the absence of LBP, but LBP enhances binding when it is present (9, 51). In addition, data suggest that LBP can transfer LPS to CD14 at substoichiometric concentrations of LBP, suggesting a catalytic function (9).

CD14 is found either on the surface of myeloid cells, including macrophages and neutrophils, as a glycosylphosphatidylinositol-anchored membrane protein (mCD14), or as a soluble form in serum (sCD14) (5, 6, 34, 43). mCD14 has been shown to be required for the production of a variety of inflammatory cytokines in response to LPS (46, 51). sCD14 is required for LPS stimulation of both cytokines and cell adhesion molecules from nonmyeloid cells, including epithelial cells, astrocytes, smooth muscle cells, and endothelial cells (6, 23, 34). Recent studies have begun to unveil the molecular interactions between LPS and CD14 that result in activation (7, 20, 45). The N-terminal region of CD14 is required for both LPS binding and signal transduction (17, 47). Specific regions of the amino terminus of CD14 have been implicated as necessary for LPS binding (26). Strong evidence for the existence of an effector/receptor molecule distal to mCD14 on monocytes/macrophages has been presented (4, 18, 20, 24). LPS activation through sCD14 is believed to require an uncharacterized receptor on the surface of nonmyeloid cells (34).

The gram-negative bacteria Helicobacter pylori and Porphyromonas gingivalis are important chronic inflammatory disease etiologic agents of the stomach and periodontium, respectively (8, 16, 53). Tissue damage resulting from colonization with H. pylori and P. gingivalis is due to both the production of bacterial virulence factors and an aberrant inflammatory host response (25, 31). However, it is not clear which bacterial antigens or host defense systems are responsible. H. pylori and P. gingivalis LPSs can stimulate tumor necrosis factor alpha, interleukin-1$\beta$, and the production of other inflammatory mediators in human monocytes (2, 30, 32). P. gingivalis LPS stimulation of monocytes/macrophages can be blocked with MY4, a monoclonal antibody that blocks CD14-dependent activation (35, 38), demonstrating that activation requires mCD14. In contrast, these LPSs do not activate human endothelial cells to produce E-selectin or stimulate neutrophil adhesion, an sCD14-mediated process with E. coli (2, 52). It is not clear how LPSs from these bacteria can activate mCD14-dependent pro-
cesses on myeloid cells but not sCD14 processes on nonmyeloid cells. Studies with *E. coli* LPS suggested that the structural differences in the LPSs of these bacteria could account for the lack of E-selectin activation (28, 30, 40). However, it is not known how these differences relate to the ability of *H. pylori* and *P. gingivalis* LPSs to interact with the CD14 pathway. To begin addressing these issues, an enzyme-linked immunosorbent assay (ELISA) using immobilized sCD14 recombinant globulin (sCD14-Rg) was developed and used to analyze the transfer of these LPSs to sCD14 with and without serum. It was found that purified LPSs from *H. pylori* and *P. gingivalis* bound sCD14-Rg in the presence of LBP recombinant globulin (LBP-Rg) or serum. Quantitative binding analysis revealed, however, that *H. pylori* and *P. gingivalis* LPS molecules bound LBP poorly, and the serum-dependent transfer of these LPSs to sCD14 was significantly slower than that of *E. coli* LPS.

**MATERIALS AND METHODS**

**Media, buffers, and reagents.** Reagent-grade chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Pooled human serum (PBS) was obtained from Biologics, Calabasas, Calif. Protein A-Sepharose was purchased from Repligen, Cambridge, Mass. Trypticase soy broth used for growing *E. coli* was supplied by BBL Microbiology Systems, Cockeysville, Md. Enriched Trypticase soy broth for cultivation of *P. gingivalis* consisted of (per liter) 30 g of Trypticase soy broth, 1 g of yeast extract (Difco, Detroit, Mich.), 1 g of glucose, 0.4 g of anhydrous NaCl, 0.4 g of cysteine, 10 mg of hemin, and 10 mg of vitamin K. The agar medium used for growing *H. pylori* has been previously described (37) and consisted of Trypticase soy broth with 1.5% agar (Bacto Agar; Difco) supplemented with 10% fetal bovine serum and 0.1% yeast extract.

**Bacterial strains and purification of LPSs.** *H. pylori* ATCC 43504 was obtained from the American Type Culture Collection. Strains ATCC43504 and ATCC33277 were provided by Marie Coyle, Harborview Medical Center, Seattle, Wash. Strains ATCC43504 and ATCC33277 were grown for 3 to 4 days on enriched Trypticase soy broth with 1.5% agar (Bacto Agar; Difco) and consisted of Trypticase soy broth with 1.5% agar (Bacto Agar; Difco) supplemented with 10% fetal bovine serum and 0.1% yeast extract. Growth of *H. pylori* ATCC 43504 was grown overnight in Trypticase soy broth at 37°C with shaking in room air. Purified LPSs from *P. gingivalis* ATCC 33277 and *H. pylori* ATCC 43504 were prepared by the cold MgCl₂-ethanol procedure (3). Purified LPSs from *E. coli* ATCC33277 and *P. gingivalis* ATCC33277 were cloned by the phenol-water method (33). All LPS preparations were suspended in distilled water and were found to be free from contaminating nucleic acid and protein by measuring optical density at 280 nm (OD₉₀₀) and OD₂₆₀. The molecular weight analysis of the LPSs and fatty acid composition revealed that the composition of these purified LPSs agreed with published data (27, 29, 40) and allowed calculation of average molecular weights. Molecular weights for *E. coli*, *P. gingivalis*, and *H. pylori* LPSs were estimated to be 19,700, 7,000, and 8,050, respectively.

**Generation of mouse monoclonal antibodies.** Monoclonal antibodies to LPSs of *E. coli* A1016, *H. pylori* ATCC 43504, and *P. gingivalis* ATCC 33277 were cloned by the method of antibody purification after fusion of a murine myeloma line with spleen cells from mice immunized with purified LPS preparations (21, 41). Throughout the cloning procedure, culture supernatants were assayed for antibodies by using the ELISA method with purified LPSs as the solid-phase antigen. Select monoclonal antibodies of the appropriate specificity were produced in high-titer culture supernatants. The antibodies were purified from the culture supernatants by protein A or protein G chromatography, extensively dialyzed into phosphate-buffered saline (PBS), filtered, and sterilized at 4°C. The purified mouse anti-LPS monoclonal antibodies used for detection of LPS binding to CD14-Rg were 3B4 (immunoglobulin M [IgM]; anti-*E. coli* A1016), 6E12 or 5B9 (IgM; anti-*P. gingivalis* ATCC 33277), and 7A9 (IgG1; anti-*H. pylori* ATCC 43504).

The anti-human CD14 mouse monoclonal antibody 2G7 was cloned as described above, using spleen cells from a mouse that had been immunized with sCD14-Rg. A complementary mouse anti-human CD14 (MY4) monoclonal antibody was obtained from Coulter Immunology, Hialeah, Fla.

**Construction and expression of sCD14-Rg and LBP-Rg.** A CDNA fragment encoding the extracellular domain of CD14 (39) was obtained by PCR using a CD14 template that was provided by Bristol-Myers Squibb, New York, N.Y. The two fragments were inserted into the mammalian expression vector pCDM7B (14, 54). Similarly, two PCR fragments encoding LBP were amplified from a human liver cDNA library obtained from Clontech, Palo Alto, Calif., using the following oligonucleotide primers: 5′ GTC CCA AGC TTC CAT CAT GGG CCT GGC AAG A 3′, 5′ GAG TCA GCC GGT ATC ATG 3′, 5′ GTC TTT ACA GCG GCC AGC 3′, and 5′ CGG ACA TCT ATG TAT TGG ACA 3′. This fragment was subcloned upstream of a CDNA fragment encoding the human IgG1 heavy and CH2 and CH3 domains of human IgG1 in the mammalian expression vector pCDM7B (14, 54). Similarly, two PCR fragments encoding the constant region were used. The construction and expression of sCD14-Rg and LBP-Rg were cloned as described above, using spleen cells from a mouse that had been immunized with sCD14-Rg. The anti-human CD14 mouse monoclonal antibody 2G7 was cloned as described above, using spleen cells from a mouse that had been immunized with sCD14-Rg. A complementary mouse anti-human CD14 (MY4) monoclonal antibody was obtained from Coulter Immunology, Hialeah, Fla.

**RESULTS**

**Generation and functional characterization of sCD14-Rg and LBP-Rg fusion proteins.** The construction and expression of recombinant molecules consisting of the protein of interest fused to the constant region of an immunoglobulin have been used to characterize a number of different proteins (13). Advantages of this method include the ability to manipulate expressed proteins by using techniques such as immuno- precipitating antibodies such as purification by protein A affinity chromatography and ELISA capture. In addition, we found that COS cells expressed and secreted significantly more sCD14-Rg than when constructs without the immunoglobulin constant region were used. Therefore, sCD14-Rg and LBP-Rg were used as therapeutic agents.

**ELISA for detection of LPS binding to immobilized sCD14-Rg.** Each well of Immunol II 96-well plates (supplied by Dynatech, Chantilly, Va.) was coated overnight at 4°C with 50 μl of sCD14-Rg (or CD40-Rg) (10 μg/ml; 0.5 μg per well). The next day, the plates were washed with PBS, and nonspecific binding sites were blocked with 0.5% human serum albumin (HSA) in acetate buffer (100 μl per well) for 30 min at 37°C. The plates were then washed three times with PBS. Fifty microliters of LPS diluted in 0.5% HSA in PBS with the addition of either serum, LBP-Rg, or buffer only were 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, per well, 50 μl of specific mouse anti-LPS monoclonal antibody at 0.5 μg/ml in 0.5% HSA in PBS. The plates were incubated for 1 h at 37°C. The plates were then washed three times with PBS, and bound mouse anti-LPS antibody was detected with, per well, 50 μl of a cocktail of Fab fragments of goat anti-IgG- and IgM-horseradish peroxidase conjugates obtained from Jackson Immunoresearch Laboratories, West Grove, Pa., diluted 1:200,000 in 0.5% HSA in PBS. The plates were incubated for 1 h at 37°C. Finally, the plates were washed five times with PBS, and 100 μl of tetramethylbenzidine chromagen reagent was added to each well. The reaction was stopped with 100 μl of 1 N H₂SO₄ per well, and the plates were read in an ELISA plate reader. Preliminary experiments performed with irrelevant primary antibodies or with the goat anti-IgG- and IgM-horseradish peroxidase second-step antibodies alone did not show any binding above background.

Experiments examining the kinetics of LPS binding to sCD14-Rg used 64 μg of fusion protein per ml (3.2 μg per well). Preliminary experiments examining the binding of an anti-CD14 monoclonal antibody (MY4) to increasing amounts of sCD14-Rg absorbed to ELISA wells showed that protein binding capacity of the wells had not been reached at 1 μg/ml, thus allowing addition of higher concentrations of sCD14-Rg. Each well of Immunol II 96-well plates was coated overnight at 4°C with 50 μl of goat anti-human IgG (antibody; Antibodies, Inc., Davis, Calif.) diluted 1:20,000. The next day, the wells were emptied, and nonspecific binding sites were blocked with 0.5% bovine serum albumin (BSA) in PBS (100 μl per well) for 30 min at 37°C. The wells were emptied, and 50 μl of LBP-Rg or CD40-Rg at 1 μg/ml diluted in 0.5% BSA in PBS was added to each 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 in 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 as described for the sCD14 binding ELISA.

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were constructed, expressed, and purified as described in Materials and Methods. A schematic representation and a gel showing the purified forms of both proteins under reduced and nonreduced conditions are shown in Fig. 1. sCD14:Rg and LBP:Rg exist as homodimers under nonreduced conditions because of two interchain disulfide bonds in the hinge domain of the IgG tail (Fig. 1B). sCD14:Rg was evaluated for its ability to mediate E. coli LPS stimulation of E-selectin expression on HUVEC (Fig. 2). For this experiment, PHS was specifically depleted of sCD14 by immunoabsorption with an anti-CD14 monoclonal antibody (MY4, an anti-CD14 monoclonal antibody known to inhibit CD14-mediated activation of cells by LPS (6), was overcome by increasing concentrations of sCD14:Rg (Fig. 2A). E-selectin expression was also used to evaluate the functional activity of LBP:Rg when combined with sCD14:Rg in the absence of serum. E-selectin expression was detected when LBP:Rg and sCD14:Rg were combined with E. coli LPS (Fig. 2B). No E-selectin expression was observed in the absence of either fusion protein or LPS (not shown). In addition, LBP:Rg was unable to stimulate E-selectin expression when CD40:Rg was substituted for sCD14:Rg, demonstrating specificity in the reaction sequence.

Analysis of E. coli, H. pylori, and P. gingivalis LPS binding to sCD14:Rg. LPS binding to sCD14 was evaluated by ELISA with sCD14:Rg as the solid-phase antigen and monoclonal antibodies to E. coli, H. pylori, and P. gingivalis LPSs as the primary detecting antibodies (see Materials and Methods). As shown in Fig. 3, LPSs from all three bacteria bound sCD14:Rg, and binding was LPS concentration dependent. Both serum and LBP:Rg augmented binding. The ability of LBP:Rg to replace serum was consistent with reports identifying the critical role of this serum protein in the transfer of E. coli LPS to CD14 (34, 36). No binding was detected when CD40:Rg was substituted for sCD14:Rg, demonstrating complete blocking of nonspecific binding. Low-level binding to sCD14:Rg was detected in the absence of serum only at high concentrations of E. coli and H. pylori LPSs. In contrast to E. coli, the addition of LBP:Rg resulted in more H. pylori and P. gingivalis LPS transfer to sCD14:Rg compared with serum.

Kinetic analysis of E. coli LPS binding to sCD14:Rg. The influence of serum on the ability of E. coli LPS to bind sCD14:Rg was further investigated. The concentration of sCD14:Rg added to the ELISA plate was increased to 64 μg/ml (see Materials and Methods) to increase the possibility that serum would yield a linear increase with time at several different LPS concentrations (conditions necessary for analysis of transfer by Michaelis-Menten kinetics). LPS binding to sCD14:Rg was measured as a function of time, using various concentrations of serum and LPS (Fig. 4). Serum significantly increased the rate of LPS transfer to sCD14:Rg at all concentrations of LPS examined. For example, the transfer of LPS to sCD14:Rg was essentially complete after 5 min in 0.5% serum, whereas LPS

![FIG. 1. (A) Schematic diagram of sCD14:Rg and LBP:Rg. Expression plasmids containing sCD14 and LBP CDNA were transiently transfected into COS cells, and sCD14:Rg and LBP:Rg were purified from the culture supernatants (see Materials and Methods). (B) Sodium dodecyl sulfate–8 to 16% polyacrylamide gel electrophoresis of purified sCD14:Rg and LBP:Rg in the absence or presence of 5% 2-mercaptoethanol. Approximately 4 μg of protein was loaded per lane. Lanes: 1, molecular weight standards; 2 and 3, sCD14:Rg reduced (lane 2) and nonreduced (lane 3); 4 and 5, LBP:Rg reduced (lane 4) and nonreduced (lane 5).](http://iai.asm.org/)

![FIG. 2. Stimulation of E-selectin expression on HUVEC with sCD14:Rg and LBP:Rg. (A) Increasing concentrations of purified sCD14:Rg were mixed with sCD14-depleted sera (○) or with buffer only (□). As a control, duplicate sCD14:Rg concentrations were preincubated for 30 min at room temperature with a mouse anti-CD14 (MY4) blocking monoclonal antibody before being combined with sCD14-depleted sera (○). E. coli LPS (10 ng/ml, final concentration) was added to each preparation, and then the mixtures were added to fourth-passage HUVEC in 96-well plates. (B) Increasing concentrations of sCD14:Rg were mixed with LBP:Rg (○; 4 μg/ml, final concentration) or no LBP:Rg (□). CD40:Rg, an irrelevant fusion protein, was also mixed with LBP:Rg in place of sCD14:Rg (□). E. coli LPS (10 ng/ml, final concentration) was added to each mixture, and the mixtures were immediately applied to fourth-passage HUVEC in 96-well plates. E-selectin expression was detected after 4 h of incubation, using a mouse anti-E-selectin monoclonal antibody. The experiment was performed three times, and the results shown are the average of duplicate wells from a typical experiment.](http://iai.asm.org/)
binding was still increasing with 0.031% serum at 15 min (Fig. 4A). Significant serum-independent binding of LPS to sCD14:Rg was observed at LPS concentrations of 0.1 mg/ml, and serum was required for binding when 0.01 mg of LPS per ml was used.

The data presented above are in agreement with studies showing that LBP delivers LPS to sCD14 like a catalytic lipid transfer protein (9, 36). In addition, LBP has structural homology with cholesterol ester transfer protein, which follows Michaelis-Menten kinetics in the transfer of cholesterol esters between lipoprotein particles (22, 36). Therefore, the possibility of determining a Michaelis constant, or \( K_m \), for serum-independent binding of LPS to sCD14:Rg was investigated. From the data presented in Fig. 4C, we identified a serum concentration at which the measured initial velocity (as determined by the formation of the LPS-sCD14:Rg complex on the ELISA plate) was linear with time. In addition, LPS binding to sCD14:Rg after 10 min of incubation was linearly proportional to the serum concentration (data not shown). Therefore, a serum concentration of 0.031% was used to determine the \( K_m \) of \( E. coli \) LPS transfer to sCD14:Rg within 10 min. As described in the legend to Fig. 5, these conditions required that concentrations of \( E. coli \) LPS of \(<10\) nM be examined because of interference from serum-independent binding at higher concentrations. Analysis of the data in a Hanes-Woolf plot (12, 49) revealed a straight line consistent with Michaelis-Menten kinetics (Fig. 5B). Estimation of the \( K_m \) from the intersection of the line with the x axis gave a value of 0.1 nM \( E. coli \) LPS. This result was confirmed by plotting the data in the format of the direct linear plot of Eisenthal and Cornish-Bowden (not shown). The reaction sequence for LPS to CD14 can be written as follows:

\[
\text{LBP} + \text{LPS} \rightarrow \text{LBP:LPS} + \text{CD14} \rightarrow \text{LPS:CD14} + \text{LBP}
\]

\[
(E + S_1) \rightarrow (E:S_1 + S_2) \rightarrow (S_1:S_2 + E)
\]

The symbols \( E \) (enzyme) and \( S \) (substrate) are used to compare the interaction of LPS, LBP, and CD14 with that of an enzymatic reaction. The ability to express the transfer of LPS to CD14 in kinetic terms permitted a more quantitative comparison of the transfer of each LPS to sCD14:Rg.

FIG. 3. ELISA detection of \( E. coli \), \( H. pylori \), and \( P. gingivalis \) LPSs bound to immobilized sCD14:Rg in the presence of LBP:Rg or serum. Increasing concentrations of LPS were mixed with either 1 \( \mu \)g of LBP:Rg per ml (■), 1% PHS (○), or buffer only (□) and added to wells previously coated with 10 \( \mu \)g of sCD14:Rg per ml (50 \( \mu \)l per well; 0.5 \( \mu \)g per well). CD40:Rg was used as an irrelevant fusion protein control and was immobilized onto wells at the same concentration (●). After 3 h of incubation at 37°C, LPS bound to sCD14:Rg was detected with mouse anti-LPS monoclonal antibodies (see Materials and Methods). The experiments were performed three times, and the results are presented as the average of duplicate wells from one experiment.

FIG. 4. Influence of serum on the rate of \( E. coli \) LPS transfer to sCD14:Rg. \( E. coli \) LPS at 1, 0.1, and 0.01 \( \mu \)g/ml was mixed with 0.5 (■), 0.125 (○), 0.031 (□), and 0 (●) % serum. The mixtures were added to wells previously coated with 64 \( \mu \)g of sCD14:Rg per ml (50 \( \mu \)l per well; 3.2 \( \mu \)g per well). The plates were incubated for various intervals in a shallow water bath for up to 15 min at 37°C. LPS bound to sCD14:Rg was detected with a mouse anti-\( E. coli \) LPS monoclonal antibody (see Materials and Methods). The results are shown with the no-LPS background subtracted and are expressed as the mean of two separate experiments ± interassay standard deviation. When error bars are not seen, they fall within the symbol.
Kinetic analysis of H. pylori and P. gingivalis LPS binding to sCD14:Rg. Experiments were performed to determine if H. pylori LPS binding to sCD14:Rg also followed Michaelis-Menten kinetics. A serum concentration (0.25%) and a time interval (15 min) at which the rate of LPS bound to sCD14:Rg was linear with time and proportional to serum concentration, respectively, were identified (data not shown). H. pylori LPS transfer to sCD14:Rg as a function of LPS concentration was examined under those conditions (Fig. 6A). H. pylori LPS concentrations of <50 nM were used to analyze the data in a Hanes-Woolf plot format. As with E. coli, a straight line was obtained, demonstrating a Michaelis-Menten relationship. The $K_m$ was estimated to be ~6 nM H. pylori LPS. This result was also confirmed by plotting the data in a direct linear plot (Fig. 6B) or buffer (●) and added to wells in a 96-well ELISA plate previously coated with 64 μg of sCD14:Rg per ml. After 15 min of incubation, LPS bound to sCD14:Rg was detected with a mouse anti-LPS monoclonal antibody (see Materials and Methods). Nonspecific binding to wells with coating buffer only was never higher than 0.1 to 0.2 OD$_{450}$ unit and was subtracted from the total absorbance of each well. The results are presented as the average of three separate experiments (duplicate wells per experiment) ± interassay standard deviation. When error bars are not seen, they fall within the symbol. (B) The results obtained at LPS concentrations of less than 1.5 nM with serum are plotted in a Hanes-Woolf plot format. $s$, substrate concentration in nanomolar LPS; $v$, velocity expressed as OD$_{450}$ in 10 min.

Comparison of half-maximum binding of E. coli, H. pylori, and P. gingivalis LPSs to sCD14. In the absence of obtaining a $K_m$ for P. gingivalis LPS transfer, the concentration of LPS that yielded half-maximum saturation binding was determined. Concentrations of P. gingivalis and H. pylori LPSs required to obtain half-maximum binding to sCD14:Rg after 3 h of incubation were compared with that of E. coli LPS under conditions of excess sCD14:Rg. As shown in Fig. 7, the serum-dependent transfer of each LPS to CD14 was found to be saturable. The concentrations required to achieve half-maximum binding to sCD14:Rg under those conditions were estimated to be 2, 15, and 22 nM for E. coli, H. pylori, and P. gingivalis LPSs, respectively.

Analysis of E. coli, H. pylori, and P. gingivalis LPS binding to LBP. The studies presented above suggested that serum LBP was a significant factor in determining the rate of LPS transfer to sCD14. For example, serum significantly increased the rate of E. coli LPS transfer to sCD14 at all concentrations of LPS examined. Serum was also required for the transfer of H. pylori and P. gingivalis LPSs. In addition, LBP:Rg significantly increased the amount of P. gingivalis and H. pylori LPSs, but not E. coli LPS, transferred to sCD14 (compare Fig. 3B and C with Fig. 3A), suggesting that LBP in serum was rate limiting for LPS transfer with these two species. Therefore, the role of serum LBP in the transfer of LPS to CD14 was examined by direct binding assays. The relative amount of LPS bound to LBP:Rg was determined in a capture assay format. Dose-response detection of LPS binding to LBP was evident with all three LPS preparations examined (Fig. 5A). However, the amount of LPS required to detect binding was significantly lower with E. coli LPS than with H. pylori or P. gingivalis. Binding to LBP was observed at <10 ng of E. coli LPS per ml. In contrast, more than 500 ng of H. pylori LPS per ml and 1,000 ng of P. gingivalis LPS per ml were required to detect significant LPS binding to LBP. The failure to detect lower concentrations of LPSs resulted from the low affinity of LPS binding to LBP. The studies presented above suggested that serum LBP was a significant factor in determining the rate of LPS transfer to sCD14. For example, serum significantly increased the rate of E. coli LPS transfer to sCD14 at all concentrations of LPS examined. Serum was also required for the transfer of H. pylori and P. gingivalis LPSs. In addition, LBP:Rg significantly increased the amount of P. gingivalis and H. pylori LPSs, but not E. coli LPS, transferred to sCD14 (compare Fig. 3B and C with Fig. 3A), suggesting that LBP in serum was rate limiting for LPS transfer with these two species. Therefore, the role of serum LBP in the transfer of LPS to CD14 was examined by direct binding assays. The relative amount of LPS bound to LBP:Rg was determined in a capture assay format. Dose-response detection of LPS binding to LBP was evident with all three LPS preparations examined (Fig. 5A). However, the amount of LPS required to detect binding was significantly lower with E. coli LPS than with H. pylori or P. gingivalis. Binding to LBP was observed at <10 ng of E. coli LPS per ml. In contrast, more than 500 ng of H. pylori LPS per ml and 1,000 ng of P. gingivalis LPS per ml were required to detect significant LPS binding to LBP. The failure to detect lower concentrations of E. coli LPS under conditions of excess sCD14:Rg. As shown in Fig. 7, the serum-dependent transfer of each LPS to CD14 was found to be saturable. The concentrations required to achieve half-maximum binding to sCD14:Rg under those conditions were estimated to be 2, 15, and 22 nM for E. coli, H. pylori, and P. gingivalis LPSs, respectively.

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The ability to respond to bacterial infection is a hallmark of the innate host defense system. This response naturally includes an inflammation component that allows the host to recognize and eliminate potential pathogens. It has become increasingly clear that both LBP and CD14 are important molecular components of the host that recognize bacterial LPS. E. coli LPS is known to be a potent inflammatory mediator; however, the inflammatory potential of LPS obtained from either H. pylori or P. gingivalis is less clear. Recently, LPSs obtained from these two bacteria have been shown to stimulate CD14-dependent pathways in human myeloid but not nonmyeloid cells. Since nonmyeloid activation by LPS has been shown to require sCD14, we examined the ability of these bacteria to interact with this component of innate host defense. In this report, we show that LPSs obtained from P. gingivalis and H. pylori bind sCD14 and that serum or LBP is required. Prior to this study, the ability of these low biologically reactive LPSs to directly bind sCD14 was not known.

The ability to bind sCD14 was detected with an ELISA that measured the transfer of LPS to immobilized sCD14:Rg. The assay detected as little as 0.5 ng of E. coli LPS per ml (50 pM) bound to sCD14:Rg, demonstrating a sensitivity not reported elsewhere. These LPS concentrations are roughly equivalent to those required for E-selectin activation in vitro and the amount of LPS found circulating in the blood of patients with sepsis. This increased sensitivity may result from the fact that the sCD14:Rg molecule is bivalent, which may facilitate more effective removal of LPS from solution. However, consistent with other studies with monovalent CD14, serum or LBP was required for E. coli LPS to bind sCD14 at low, but not high, concentrations of LPS. In addition, the relatively poor binding of H. pylori and P. gingivalis LPSs to LBP:Rg and sCD14:Rg compared with that of E. coli LPS correlates with the poorer ability of these LPSs to activate adherent human monocytes. Quantitation of LPS binding demonstrated that serum-dependent transfer was saturated at <1 nM LPS (~10 ng/ml). Serum-independent binding occurred at approximately 10 nM LPS and approached saturation at 100 nM LPS (Fig. 5). No significant serum-independent binding to sCD14 was observed with any of the concentrations of H. pylori and P. gingivalis LPSs examined.

We wanted to compare the abilities of the various LPS preparations to bind CD14. However, we were restrained by the fact that LPS binding to sCD14 is a measure of both LPS transfer to and affinity for an sCD14 binding site. Therefore, the kinetics of E. coli, H. pylori, and P. gingivalis LPS transfer to sCD14 were compared by determining a K_m, in half-maximum binding assays. A Michaelis-Menten relationship was established for E. coli and H. pylori LPS binding to sCD14:Rg, permitting an estimation of a K_m for both (nanomolar LPS at 1/2 V_max). The K_m, but not the V_max, was used as the basis of comparison because it negated potential anti-LPS antibody affinity differences. A Michaelis-Menten relationship could not be established for P. gingivalis LPS. Although not as quantitative as K_m measurements, the half-maximum binding results allowed a comparison of the abilities of the various LPSs to be transferred to sCD14:Rg. Compared with E. coli LPS, a potent activator of E-selectin expression, H. pylori and P. gingivalis LPSs transferred poorly to sCD14:Rg.
Evidence that the low rates of transfer to sCD14 could be explained by poor LBP binding was obtained. Direct binding assays revealed that it took significantly more H. pylori and P. gingivalis LPS than E. coli LPS to bind serum LBP. In these assays, LPS binding was detected with anti-LPS monoclonal antibodies. Potential differences in antibody affinities and the type of epitope bound by the different monoclonal antibodies precluded us from quantifying the amounts of the different LPS species bound. However, the slower rate of P. gingivalis and H. pylori LPS binding to LBP removed any differential effects that the antibodies might have had and provided convincing evidence that poor binding to LBP resulted in low transfer rates to sCD14.

The data presented in this study provide the first evidence that H. pylori and P. gingivalis LPS binding to LBP may be rate limiting for myeloid cell activation. The poor binding of H. pylori and P. gingivalis LPS to LBP suggests that these LPS molecules cannot enter into the activation pathway as well as E. coli LPS. This view is consistent with the lesser ability of these LPS molecules to activate human monocytes compared with E. coli LPS (2, 30, 32, 38). Although differences in core structure may influence LPS binding to LBP and transfer to CD14, lipid A is recognized as the LPS moiety primarily responsible for interacting with the LBP-CD14 pathway (42). Differences in lipid A structure may account for differences in LBP binding and/or transfer to CD14. P. gingivalis lipid A differs from E. coli lipid A by the presence 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 to 17 carbon atoms (29). 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 to 18 carbon atoms) and has an unusual phosphorylation pattern compared with that of E. coli lipid A (27).

It has been shown that H. pylori and P. gingivalis LPSs fail to stimulate E-selectin expression on HUVEC even when concentrations 10,000-fold higher than necessary for E. coli LPS activation are used (10 µg/ml versus 1 ng/ml) (2). However, the binding data presented in this report demonstrated that there was not a 10,000-fold reduction in the ability of these LPSs to bind sCD14. The less than 50-fold-lower transfer rates for H. pylori and P. gingivalis LPSs compared with E. coli LPSs were determined to be due to lower LBP binding and not a failure to bind sCD14. The data presented in this report suggest that these LPSs should have bound sCD14 in the HUVEC functional assays sufficiently to activate E-selectin expression. Furthermore, the inability of H. pylori and P. gingivalis LPSs to activate E-selectin expression is not due to the slower transfer of LPS to sCD14, since ample time to form LPS-LBP complexes was provided in those experiments (2). The binding or recognition of LPS by LBP and sCD14 is only one step in activation of nonmyeloid cells, and that is evidently not sufficient for signal transduction. Evidence has recently been obtained that in monocytes, LPS interacts with components past CD14 (18, 20, 24). Although the reasons for the poor ability of H. pylori and P. gingivalis LPSs to activate E-selectin are not known, they may include improper binding to sCD14 or a failure to be recognized by proteins distal to CD14 (4). Whatever the molecular explanation, there is clearly a difference in the CD14-dependent response to LPS between monocytes and endothelial cells.

The ability of P. gingivalis and H. pylori LPSs to stimulate monocytes/macrophages (mCD14) and not endothelial cells (sCD14) may partially explain the dysfunctional inflammatory responses associated with these bacteria. The relative importance of myeloid versus nonmyeloid activation is not known, but it is likely that both cell types need to respond to bacterial infection. Further work will be required to determine if this unique attribute of these LPS molecules is related to the disease process.

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