Helicobacter pylori Lipopolysaccharide Can Activate 70Z/3 Cells via CD14

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Helicobacter pylori persistently colonizes the human gastrointestinal tract and is associated with chronic gastritis and, in some cases, peptic ulcer disease or gastric neoplasms. One factor in the persistence of this organism may be its inability to elicit a strong inflammatory response. Lipopolysaccharide (LPS) is a proinflammatory substance found in the cell walls of all gram-negative bacteria. *H. pylori* LPS has been found by several different measures to be less active than LPS from *Enterobacteriaceae*. This study addresses the role of CD14 and LPS-binding protein in the cellular response to *H. pylori* LPS. We report that *H. pylori* LPS activates mammalian cells expressing CD14 at much lower LPS concentrations than those for control cells not expressing CD14. The maximal activation of CD14-70Z/3 cells by *H. pylori* LPS also requires LPS-binding protein. *H. pylori* LPS at concentrations as high as 30 µg/ml does not elicit an interleukin-8 (IL-8) response from the epithelial cell line SW620 in the presence of CD14; 10 ng of *Escherichia coli* LPS per ml elicits a maximal IL-8 response. Furthermore, in contrast to some other types of LPS with little activity, *H. pylori* LPS does not inhibit the CD14-70Z/3 cell response to *E. coli* LPS. From these studies, we conclude that *H. pylori* LPS, though much less active than *E. coli* LPS, stimulates cells via CD14.

Helicobacter pylori persistently colonizes the human stomach, which causes chronic gastritis and may lead to peptic ulcer disease or to gastric neoplasia (2). Although these organisms do not appear to invade the gastric epithelium, infection induces chronic inflammation in the lamina propria. Because this inflammatory response may be detrimental to *H. pylori*, it has been hypothesized that there has been selection for strains that do not strongly activate the host response (1, 20).

For gram-negative bacteria like *H. pylori*, an important proinflammatory molecule is the lipopolysaccharide (LPS) component of the outer membrane (23). The lipid A portion of the LPS molecule is largely responsible for such activity, but compared with that from the *Enterobacteriaceae*, the *H. pylori* lipid A has an unusual fatty acid composition (6) and phosphorylation pattern (18). As with another persistent colonizer of the human gastrointestinal tract, *Bacteroides fragilis* (30), the LPS of *H. pylori* has low biological activity in many assays, including rabbit pyrogenicity, B-cell mitogenicity, the ability to gel *Limulus* lysates (19), and activation of endothelial cells (4). The ability of *H. pylori* LPS to activate phagocytic cells is 1,000- to 10,000-fold less than that of *Escherichia coli* LPS (20).

Activation of both monocytic and polymorphonuclear phagocytes by LPS from most gram-negative bacteria usually involves the cell surface receptor CD14 (16, 32) and is facilitated by LPS-binding protein (LBP), which is present in human serum (26). One of the central questions in the biology of *H. pylori* infections is how the organism is able to persist in a host that recognizes its presence, as indicated by an immune response. Since previous work has shown that the LPS of *H. pylori* has low biological activity (4, 19, 20), we asked whether the mechanism involves CD14. One hypothesis is that

the decreased activity results from the inability of CD14 to recognize *H. pylori* LPS; alternatively, it may be that there is recognition but the decreased activity is due to other steps in the activation process. A difference in the degree to which LBP facilitates LPS activation via CD14 also could help explain the differences in activation by *Enterobacteriaceae* LPS and *H. pylori* LPS. In the studies reported here, we sought to address these questions. Furthermore, recognition of LPS by LBP and CD14 is critical under such clinical conditions as sepsis due to gram-negative organisms. We reasoned that if *H. pylori* could be fully recognized by CD14 but not evoke a very strong signal, it might be useful as a competitor to occupy receptor sites and inhibit responses to LPS from *Enterobacteriaceae*. We compared the relative activities of *H. pylori* LPS and *E. coli* LPS in two assay systems: 70Z/3 cells expressing CD14 (16) and CD14-dependent activation of an epithelial cell line (21).

MATERIALS AND METHODS

Preparation of LPS. The LPS from *H. pylori* 84-183 was prepared by the hot-phenol-water method of Westphal and Jann (31), exactly as described previously (20). After chemical analyses, which demonstrated protein contamination of less than 5% (20), the LPS preparation was stored in sterile distilled water at 4°C before use in these assays. *E. coli* O111:B4 LPS and D31m4 LPS (Re chemotype) were prepared as previously described (16).

Anti-CD14 MAbs. The monoclonal antibodies (MAbs) 28C5, 18E12, and 63D3 were prepared and used as previously described (28, 29). 28C5 and 18E12 have been previously shown to block LPS activation of cells via CD14 (28, 29). 63D3 binds to a different CD14 epitope than 18E12 and 28C5 do, and 63D3 does not inhibit LPS activation of cells via CD14 (28, 29). All three MAbs are immunoglobulin G1 (IgG1).

Activation of CD14-70Z/3 cells. 70Z/3 cells were transfected with human CD14 as previously described (16). Activation assays with CD14-70Z/3 cells were done for 24 h as previously described (16). Each concentration of LPS was tested in duplicate; the mean and range are presented in this report. To examine the effects of LBP, the CD14-70Z/3 cells were cultured in OptiMem 1 (Gibco) in the presence of various concentrations of recombinant human LBP. The LBP was a gift from Peter Tobias.

Activation of SW620 cells to produce IL-8. The methods described by Pugin et al. were used (21). Briefly, SW620 cells (American Type Culture Collection)

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FIG. 1. Response of 70Z/3 cells or CD14-transfected 70Z/3 cells to 24-h stimulation with *E. coli* O111:B4 LPS or *H. pylori* LPS. Surface IgM (sIgM) expression was measured by flow microfluorometry, and the average channel number (above the average channel number of unstimulated cells) is plotted on the ordinate. The values shown are the averages and ranges of two determinations (the ranges are smaller than the symbols) from one of five similar experiments.

were seeded at 5×10^4 cells/well in a 96-well plate. After 72 h of culture, the cells were washed and stimulated with LPS in the presence of 2% normal human serum for 6 h. The supernatants were harvested, and the amount of interleukin-8 (IL-8) was determined by capture enzyme-linked immunosorbent assay. The capture antibody was polyclonal goat anti-human IL-8 (R and D Systems), and the detection antibody was polyclonal rabbit anti-human IL-8 (Endogen). The horseradish peroxidase-conjugated antibody was goat anti-rabbit IgG (Tago Immunologicals). A standard curve of recombinant human IL-8 (Genzyme) was used to convert optical density values into concentrations of IL-8. Twofold dilutions of the experimental samples were made, and the amount of IL-8 was

determined by comparison to the standard curve. The means and standard deviations of three determinations are shown here.

RESULTS

CD14. We first asked whether CD14 facilitates the activation of 70Z/3 cells by *H. pylori* LPS. The response of control 70Z/3 cells and CD14-transfected 70Z/3 cells to *E. coli* and *H. pylori* LPS is shown in Fig. 1. The 70Z/3 cells expressing CD14 responded to 100-fold-lower concentrations of *E. coli* O111:B4 LPS than did the control 70Z/3 cells, as has been reported previously (16). *E. coli* LPS was about 10,000-fold more active than *H. pylori* LPS, in both CD14-70Z/3 cells and control 70Z/3 cells, consistent with prior observations about differences between *E. coli* and *H. pylori* LPS (19, 20). However, *H. pylori* LPS stimulated a much better response in CD14-transfected 70Z/3 cells than in control-transfected 70Z/3 cells, suggesting that *H. pylori* LPS is stimulating the 70Z/3 cell via CD14.

To confirm that the increased responsiveness of CD14-70Z/3 cells to *H. pylori* LPS was due to the expression of CD14, the effect of anti-CD14 MAb on the LPS-induced response was determined (Fig. 2). Using either 1 ng of *E. coli* D31m4 LPS or 3 μ g of *H. pylori* LPS per ml, we found that the inhibitory anti-CD14 MAbs 18E12 and 28C5 inhibited the response of CD14-70Z/3 cells. Another anti-CD14 MAb, 63D3, which has been shown to have little effect on *E. coli* LPS activation of cells via CD14 (29), had no effect on *E. coli* activation of cells or on *H. pylori* activation of cells. These data confirm that *H. pylori* LPS stimulates 70Z/3 cells via CD14.

LBP. The response of CD14-70Z/3 cells to LPS derived from *Enterobacteriaceae* is maximal in the presence of the LBP (27). To determine whether the response to *H. pylori* LPS was enhanced by LBP, we stimulated CD14-70Z/3 cells with *H. pylori* LPS in serum-free media; *E. coli* D31m4 LPS was used as a positive control. The concentration of recombinant human LBP was varied from 0.1 pg/ml to 100 ng/ml. As expected, LBP dramatically enhanced the response to *E. coli* D31m4 LPS (Fig. 3). Similarly, LBP substantially enhanced the response of CD14-70Z/3 cells to 3 μ g of *H. pylori* LPS per ml. As observed before (Fig. 1), 10,000 times more *H. pylori* LPS than *E. coli* LPS was required to elicit the same response. An approxi-

FIG. 2. Inhibition of response of CD14-70Z/3 cells to LPS by anti-CD14 MAbs. The response of CD14-70Z/3 cells to 1 ng of *E. coli* D31m4 LPS or 3 μ g of *H. pylori* LPS per ml was measured as for Fig. 1. Cells were preincubated for 2 h with 1 mg of MAb 18E12, 28C5, or 63D3 per ml. The values shown are the averages and ranges of two determinations from one of three similar experiments. sIgM, surface IgM.

FIG. 3. Augmentation of the response of CD14-70Z/3 cells to LPS by LBP. The response of CD14-70Z/3 cells to 300 pg of *E. coli* D31m4 LPS or 3 µg of *H. pylori* LPS per ml was measured as a function of LBP concentration. The values shown are the averages and ranges of two determinations (the ranges are smaller than the symbols) from one of three similar experiments. sIgM, surface IgM.

FIG. 4. *H. pylori* LPS stimulation of SW620 epithelial cells to produce IL-8. SW620 cells were stimulated with the indicated LPS in 2% normal human serum for 6 h, and the supernatants were collected. IL-8 was measured by enzyme-linked immunosorbent assay. The results shown are the means and standard deviations from three determinations.

mately 10-fold excess of LBP was needed for maximal response to *H. pylori* LPS compared to the amount needed for maximal response to *E. coli* D31m4 LPS.

Soluble CD14. Another CD14-dependent activity of LPS is the ability to stimulate certain epithelial cell lines to secrete cytokines (21). We compared the ability of *E. coli* O111:B4 LPS and *H. pylori* LPS to stimulate SW620 cells to secrete IL-8. Normal human serum was used as a source of CD14 and LBP. In this assay, the SW620 cells secreted 632 pg of IL-8 per ml in the absence of LPS; as expected, the addition of as little as 1 ng of *E. coli* LPS per ml substantially increased IL-8 secretion (Fig. 4). The *H. pylori* LPS was essentially inactive in this assay at concentrations as high as 30 µg/ml. Thus, *H. pylori* LPS does not activate SW620 cells via the soluble-CD14 pathway.

Effect of *H. pylori* LPS on *E. coli* LPS activation of CD14 70Z/3 cells. Some types of relatively inactive LPS molecules can inhibit the activity of biologically active LPS. Two examples of this phenomenon that have been studied recently involve *Rhodobacter sphaeroides* lipid A (10, 14, 25) and deacylated *E. coli* LPS (12). To determine whether *H. pylori* LPS could inhibit the activation of CD14-70Z/3 cells by *E. coli* D31m4 LPS, we pretreated CD14-70Z/3 cells with a range of concentrations of *H. pylori* LPS for 2 h and then added 100 pg of *E. coli* D31m4 LPS per ml. As shown in Fig. 5, there was no inhibition of the response to *E. coli* LPS by even a 10,000-fold excess of *H. pylori* LPS. This experiment implies that *H. pylori* LPS is not able to inhibit cellular activation induced by *E. coli* LPS, despite the fact that both types of LPS activate cells via CD14.

DISCUSSION

H. pylori LPS is known to have limited biological activity, compared to that of LPS derived from the *Enterobacteriaceae*, and in this study we tried to establish some of the mechanisms involved. It is important to know how *H. pylori* LPS stimulates cellular responses, for several reasons. *H. pylori* LPS is one of

a group of LPS, lipid A, and partial structures that have little activity (10, 13, 25). Most of these have been shown to stimulate cells via membrane CD14; for *H. pylori* LPS, this question had not yet been addressed. Furthermore, most of the inactive lipid A species are competitive inhibitors with active LPS; the competitive activity of *H. pylori* LPS is unknown.

Several different structural features appear to be important for the biological activity of LPS. One is the presence of phosphate groups at both the 1 and 4' positions; monophosphoryl

FIG. 5. *H. pylori* LPS does not inhibit the CD14-70Z/3 cell response to *E. coli* D31m4 LPS. CD14-70Z/3 cells were incubated with the indicated concentration of *H. pylori* LPS for 2 h. *E. coli* D31m4 LPS (100 pg/ml) was added to half the test wells. The response of CD14-70Z/3 cells to 100 pg of *E. coli* D31m4 LPS per ml alone was 79 (not shown). The values shown are the averages and ranges of two determinations from one of two similar experiments. sIgM, surface IgM.

lipid A is much less active than the diphosphoryl form (23). The fatty acids also are critical. *R. sphaeroides* lipid A, which has an unusual lipid composition, is only minimally active (10, 22). Deacylated *E. coli* LPS, lacking the secondary fatty acids (13), and the lipid A precursor, lipid IV_A, containing only the four primary hydroxymyristate groups, have very little ability to stimulate human cells to respond (5, 24). Interestingly, lipid IV_A and deacylated *E. coli* LPS have normal ability to stimulate LPS responses in murine cells (23). The C₁₈ and C₁₆ long-chain fatty acids found in *H. pylori* LPS (compared to the C₁₄ fatty acids of *E. coli* LPS) and the fact that *H. pylori* LPS contains a single phosphate at the 1 position may explain its relative lack of activity.

The murine pre-B cell line 70Z/3 has been a very useful tool for studying the role of CD14 in cellular activation by LPS and other agonists, since the 70Z/3 cell normally expresses no CD14 (15, 16, 29). Ligands such as *E. coli* LPS, which can stimulate 70Z/3 cells directly, stimulate CD14-70Z/3 cells at much lower concentrations, especially in the presence of LBP (16). Peptidoglycan, another ligand which stimulates cells via CD14, activates CD14-70Z/3 cells but not control-transfected 70Z/3 cells (8); peptidoglycan activation does not require LBP.

In this series of experiments, we found that CD14-70Z/3 cells respond to 1,000-fold-lower concentrations of *H. pylori* LPS than control-transfected 70Z/3 cells do. Activation was inhibited by the same anti-CD14 MAb previously found to inhibit LPS-induced activation of CD14-70Z/3 cells (29). Maximal activation required LBP. In addition, *H. pylori* LPS did not stimulate an epithelial cell line to produce IL-8 in the presence of human serum. This is in agreement with the recent demonstration that *H. pylori* LPS does not stimulate human umbilical cord endothelial cells to synthesize L-selectin, a function which also requires soluble CD14 (3). In terms of CD14 and LBP dependence, *H. pylori* LPS behaves similarly to both active LPS derived from *Enterobacteriaceae* and inactive analogs. In contrast, *H. pylori* LPS does not block *E. coli* LPS activation of CD14-70Z/3 cells. In this respect *H. pylori* LPS has a different profile from that of the other inactive LPS and lipid A analogs.

Our assays of membrane CD14 binding to LPS require that the LPS be labeled to a high specific activity (9). This has been done by metabolic labeling, fluorescence labeling, or photoaffinity derivatization (9). We have not been able to use these methods effectively with *H. pylori* LPS, so it has not been possible for us to measure *H. pylori* LPS binding to CD14-CHO cells. However, binding assays have limitations; when studying deletion mutants of membrane CD14, we could not detect LPS binding by many CD14 mutants that were partially functional LPS receptors (28). We presume that cellular activation is simply a more sensitive measure of LPS-membrane CD14 interaction than a binding assay is. Recently, others have shown that soluble CD14 binds *H. pylori* LPS, although less well than *E. coli* LPS (3). Taking these data together it seems clear that soluble CD14 binds *H. pylori* LPS but that the complex is not able to stimulate epithelial or endothelial cells.

H. pylori LPS does not block activation of cells by active LPS, which sets it apart from other compounds, such as *R. sphaeroides* lipid A (10) or deacylated LPS (13). These compounds appear to inhibit at multiple steps in the activation process, many of which occur after the LPS-CD14 binding step (12, 14). Several workers have hypothesized that there must be an LPS receptor other than CD14 in macrophages (5, 12). We certainly know that that is the case in 70Z/3 cells, since the untransfected cells respond well to LPS despite the absence of detectable CD14 (16). Although several laboratories have published conflicting data suggesting what the "second receptor"

might be (7, 11, 17), the nature of this receptor is currently unknown. However, an LPS recognition system other than CD14 exists in 70Z/3 cells and probably exists in macrophages, too. We presume that *H. pylori* may lack the ability to activate the "second receptor."

The findings from this study confirm and extend previous observations on the low biological activity of *H. pylori* LPS. An important question concerns how *H. pylori* is able to persist for decades in the stomach of an infected person when there is both immune recognition of the organism by the host and inflammation in the tissue. Either or both of these phenomena might normally be expected to limit the tenure of the microbe in its niche. We propose that over the protracted course of *H. pylori* colonization of humans and our prehuman ancestors, there has been natural selection for *H. pylori* strains that do not evoke a very strong inflammatory response.

According to this hypothesis, strains that induced powerful responses would be at a disadvantage due either to the early demise of their host or to the rapid development of atrophic gastritis, leading to the loss of a competition-free ecological niche. The presence of LPS with strong proinflammatory activity would thus be detrimental. This model predicts that there has been selection for LPS molecules in *H. pylori* that results in minimum cellular activation. The inability of *H. pylori* LPS to block the activity of *E. coli* LPS specifically suggests that there has been selection for *H. pylori* LPS molecules that bind poorly to cellular receptors such as CD14 or the proposed "second receptors."

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