Porphyromonas gingivalis Lipopolysaccharide Is Poorly Recognized by Molecular Components of Innate Host Defense in a Mouse Model of Early Inflammation

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Porphyromonas gingivalis is a gram-negative bacterium that is associated with periodontitis. It has been hypothesized that destruction of bone and periodontal connective tissue is associated with colonization of the subgingival crevicular space by P. gingivalis, although how these bacteria overcome innate host defenses is largely unknown. To examine the early cellular and molecular events of P. gingivalis interaction with host tissues, we compared lipopolysaccharide (LPS) isolated from this bacterium with Escherichia coli LPS, a potent inflammatory mediator, in a mouse model of acute inflammation. In these studies, mice were given intramuscular injections of either P. gingivalis LPS or E. coli LPS and then sacrificed after 4 h. Reverse transcriptase-PCR analysis showed that expression of mRNAs for E- and P-selectins was higher in E. coli LPS-injected muscles than in P. gingivalis LPS-injected or control phosphate-buffered-saline-injected muscles. Similarly, monocyte chemotactic protein 1 and fibroblast-induced cytokine mRNAs were expressed in E. coli LPS-injected muscles whereas their expression was reduced or absent in P. gingivalis LPS-injected samples. These results were confirmed by in situ hybridization whereby stronger hybridization for selectin mRNAs was observed in the endothelium of capillaries from E. coli LPS-injected samples than in that from P. gingivalis LPS-injected muscles. In addition, many monocytess expressing monocyte chemotactic protein 1 mRNA and polymorphonuclear leukocytes expressing fibroblast-induced cytokine mRNA were observed in E. coli LPS-injected muscles whereas only a few cells were identified in P. gingivalis LPS-injected muscles. These results demonstrate that compared with E. coli, P. gingivalis has a low biologically reactive LPS as measured by its weak activation of inflammation. This may allow P. gingivalis to evade innate host defense mechanisms, resulting in colonization and chronic disease.

Porphyromonas gingivalis is a gram-negative bacterium that is associated with periodontitis, a chronic inflammatory disease which results in the degradation of alveolar bone and periodontal connective tissue, leading to tooth loss. While the pathology of this disease has been well characterized (for a review, see reference 24), the mechanism by which bacteria induce periodontitis is not well understood. It is believed that the persistent colonization of bacteria on the tooth root surface is responsible for the destructive inflammatory response characteristic of the disease. A variety of virulence factors which contribute to the maintenance of the organism on the tooth root surface have been reported (24). However, little is known about how this organism initially interacts with innate host defense components and colonizes normally sterile periodontal tissue.

One of the first and most important components of the innate host response to bacterial infection is inflammation. Inflammation results in the margination of leukocytes from the vasculature into tissues at the infection site through the orchestrated expression of selectin and soluble inflammatory mediators. The initial tethering of leukocytes to vascular endothelium is mediated by a group of adhesion receptors called selectins (27). Increased levels of selectins on endothelial cells have been demonstrated by immunohistochemistry on tissue sections from patients with periodontitis (9, 14). This is followed by expression of integrins on the leukocyte surface and their binding to their counter receptors, intracellular cell adhesion molecules, found on the endothelial cell surface (27).

It is hypothesized that activation of integrins is provided by leukocytes through the secretion of a new superfamily of chemotactic cytokines (chemokines) which are resistant to degradation and selectively target leukocyte subsets (for a review, see reference 25). One chemokine, monocyte chemoattractant protein 1 (MCP-1), is a potent attractant and activator of monocytes and is synthesized by a variety of cell types including monocytes, fibroblasts, and endothelial cells (7). Another chemokine, fibroblast-induced cytokine (FIC), has recently been cloned from NIH 3T3 cells and found to be similar to MCP-1 (11).

It has been shown that P. gingivalis does not directly stimulate E-selectin expression in human umbilical vein endothelial cells (HUVEC) and is a poor activator of tumor necrosis factor alpha and interleukin-1β in monocytes (6, 23); both cytokines are indirect activators of selectin expression in humans. On the basis of these observations and the previously reported low biological activity of P. gingivalis lipopolysaccharide (LPS) (8, 22), we hypothesized that P. gingivalis may evade initial host defenses as a result of poor or dysfunctional recognition (6).

The hypothesis contends that an inadequate or improper initial response by the host can lead to tooth root colonization. Since it is difficult to monitor colonization of the tooth root surface in humans, we have developed a mouse model which measures expression of initial components of innate host defense. In this

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TABLE 1. Oligonucleotide primers and in situ probes

<table>
<thead>
<tr>
<th>Molecule</th>
<th>5’ oligonucleotide primer</th>
<th>3’ oligonucleotide primer</th>
<th>Primer/insitu probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>CACCAAGGCTGTCCTG</td>
<td>TGGTTGAGTGGCC</td>
<td>9 oligonucleotide primer</td>
</tr>
<tr>
<td>P-selectin</td>
<td>ATGCGGCTGTCCTG</td>
<td>TGGTTGAGTGGCC</td>
<td>9 oligonucleotide primer</td>
</tr>
<tr>
<td>FIC</td>
<td>ATGCGGCTGTCCTG</td>
<td>TGGTTGAGTGGCC</td>
<td>9 oligonucleotide primer</td>
</tr>
<tr>
<td>E-selectin</td>
<td>ATGAAACACTGGTGGAGTC</td>
<td>TGGTTGAGTGGCC</td>
<td>9 oligonucleotide primer</td>
</tr>
</tbody>
</table>

MODEL, local expression of a select group of inflammatory mediators was examined 4 h after an injection of bacterial LPS by both reverse transcriptase-PCR (RT-PCR) and in situ hybridization. It was found that *P. gingivalis* LPS, in contrast to *Escherichia coli* LPS, was a poor inducer of E- and P-selectin and the chemokines MCP-1 and FIC.

MATERIALS AND METHODS

Induction of acute inflammation. BALB/c mice approximately 12 weeks of age were given injections into the gastrocnemius muscle of either 0.002, 0.02, 0.1, or 0.2 mg of *E. coli* LPS (O111:B4; Sigma) or *P. gingivalis* LPS purified as previously described (6). Compositional analysis demonstrated the presence of hexadecanoic acid, 3-hydroxy-15-methylhexadecanoic acid, and the sugars mannose, rhamnose, galactose, glucose, glucosamine, and galactosamine, in agreement with previous reports (12, 21). Control mice received phosphate-buffered saline (PBS). Mice were sacrificed after 4 or 24 h, and the muscles were excised. For frozen sections, muscles were embedded in optimal-cutting-temperature compound (Miles, Elkhart, Ind.) and cryosectioned. For paraffin sections, muscles were embedded in paraffin and sectioned. For paraffin sections, muscles were embedded in paraffin and sectioned.

Probes for in situ hybridization. Probes for in situ hybridization were either RNA generated from cDNAs or oligonucleotide probes designed from known cDNA sequences. The MCP-1 RNA probe was made from a mouse MCP-1 cDNA (confirmed by standard dideoxynucleotide-chain termination sequencing) in a TATA PCRII vector (Imvirogen, San Diego, Calif.). Template was prepared by PCR directly from the plasmid with M13 forward and reverse primers to excise a 250-bp fragment containing both the SfI and T7 promoters. After phenol-chloroform extraction and inositol precipitation, SfI RNA polymerase and uridine 5’-[32P]triphosphate, triethylammonium salt (Amersham, Arlington Heights, Ill.), were used to generate an antisense RNA probe. The RNA probe was purified on a Sephacryl G-50 column to remove unincorporated radiolabel, precipitated, and resuspended in triethylammonium buffer containing 0.1% sodium dodecyl sulfate. Oligonucleotide probes were synthesized with a DNA synthesizer (Applied Biosystems, Foster City, Calif.) and ethanol precipitated. A cocktail of probes was used as follows: two oligonucleotides were used for E-selectin, while three oligonucleotide probes were used for P-selectin, MCP-1, and FIC. Oligonucleotide 3’ primers for in situ hybridization are listed in Table 1.

The cocktail of oligonucleotides was labeled with [32P]dATP tetra(triethylammonium) (Dupont-New England Nuclear, Boston, Mass.) with terminal deoxynucleotidyl transferase (GIBCO-BRL) and purified on an NENSORB 2 nucleic acid purification cartridge (Dupont-New England Nuclear) as specified by the manufacturer.

In situ hybridization. Paraffin sections and frozen sections were deparaffinized in xylene (for paraffin sections only) or fixed in 4% paraformaldehyde (for frozen sections only), treated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0), dehydrated in graded alcohol, delipidated in chloroform, and allowed to dry at room temperature. Sections were probed for in situ hybridization as described previously for oligonucleotide probes (16, 26) and RNA probes (17). Sections probed with oligonucleotide probes were exposed for 21 days, whereas those probed with the MCP-1 RNA probe were exposed for 5 days. Emulsion-coated slides were stored desiccated at 4°C. After development, the sections were stained with hematoxylin and eosin and evaluated. Coded sections were evaluated and confirmed blinded.

RNA preparation and analysis. Total RNA from muscle samples was purified with the RNA-Stat 30 kit (Tel-Test “B” Inc., Friendswood, Tex.) as specified by the manufacturer. cDNA was synthesized from 3 μg of RNA by the SUPERSCRIPT preamplification system (GIBCO-BRL) as specified by the manufacturer. A 5-μl volume of a 1:30 dilution of cDNA in total volume of 50 μl was used for PCR analysis. Anti sense oligonucleotides were used in the in situ hybridizations were individually paired with a 5’ sense oligonucleotide and used in a PCR (35 cycles at an annealing temperature of 54°C) to assess the specificity of each oligonucleotide for its target mRNA. 5’ sense oligonucleotides are listed in Table 1. The amplified PCR products were then analyzed by electrophoresis on 2% agarose gels. The identities of the bands were confirmed by sequence analysis. β2-Microglobulin was detected with specific oligonucleotide primers (Clontech Laboratories, Inc., Palo Alto, Calif.) to control for mRNA and cDNA synthesis.

RESULTS

Development of an in vivo mouse muscle model and selection of oligonucleotide probes and examination of their ability to detect inflammatory mediators in the mouse model. Mouse muscle was used as a model of acute inflammation since it is an organ in which whole bacteria or purified LPS may become entrapped and induce a host response. The gastrocnemius muscle was chosen since it is easily isolated for injection and later for excision. Three amounts of LPS, 0.002, 0.02, and 0.2 mg, were tested in this model. A total of 0.2 mg of LPS was used per injection, since this amount of *E. coli* LPS induced a strong inflammatory cell infiltrate. Four separate experiments were performed with 0.2 mg in a total of 21 *E. coli* LPS-injected mice and 13 *P. gingivalis* LPS-injected mice. Mice were sacrificed at either 4 or 24 h. The 4-h time point is reported here, since cellular infiltration, indicating cell activation, was observed at this time after injection of *E. coli* LPS. Furthermore, expression of selectins and MCP-1 and FIC appeared to be similar at the 4- and 24-h time points.

To detect inflammatory mediators in this model, we used specific oligonucleotide oligonucleotides. Oligonucleotides were selected to be compatible for in situ hybridization. Since a cocktail of three probes was used, the oligonucleotides were designed to be nonoverlapping and identical in length and G+C content (Table 1). RT-PCR was used to test the specificity of our oligonucleotides used for in situ hybridization and to compare the relative amounts of selectin and chemokine expression in *E. coli* LPS-treated and control muscles. For these studies, the 3’ oligonucleotide probes used for in situ hybridization were paired with 5’ oligonucleotide primers to generate PCR products of less than 1 kb. Figure 1 shows DNA fragments of the predicted size from all primer pairs except E-selectin 2, for which no band was observed. The relative amount of each band increased in cDNA from *E. coli* LPS-injected muscle when compared with PBS-injected muscle. In the case of P-selectin, an additional band approximately 400 bp smaller than the expected size was detected. This additional band may represent DNA from an alternatively spliced mRNA transcript or the result of one of the primers binding to a second site. β2-Microglobulin, used as a control for mRNA and cDNA synthesis, yielded bands of equal intensity for both *E. coli* LPS- and PBS-injected muscle samples.

Induction of inflammation by *E. coli* but not *P. gingivalis*. Figure 2 shows the histopathological features of muscles 4 h after the injection of PBS or 0.2 mg of LPS. Multiple sections...
of each muscle sample were examined. Sections taken from PBS-injected muscle (Fig. 2A) or *P. gingivalis* LPS-injected muscle (Fig. 2C) displayed normal histology. This included the absence of infiltrating leukocytes in the vasculature. In contrast, a section of *E. coli* LPS-injected muscle showed massive infiltration of leukocytes in the epimysium and perimysium (Fig. 2B). Large numbers of leukocytes were observed in venules as well. High magnification identified these cells as mononuclear cells/macrophages and polymorphonuclear leukocytes (Fig. 2D).

**Localization of selectin mRNAs.** In situ hybridization demonstrated the absence of hybridization for P-selectin in control sections of muscles 4 h after the injection of PBS (Fig. 3A and B) or 0.2 mg of *P. gingivalis* LPS (Fig. 3C and D). Hybridization
for E-selectin mRNA was also absent in serial sections from these muscle samples. In contrast, expression for P-selectin mRNA was observed in venules from sections of muscle 4 h after the injection of 0.2 mg of *E. coli* LPS (Fig. 3E and F). High magnification confirmed the expression of this adhesion molecule in endothelial cells in postcapillary venules. E-selectin mRNA was also observed in endothelial cells from venules in serial sections (results not shown). As an additional control,
serial sections of muscle given an injection of \textit{E. coli} LPS were probed with an oligonucleotide to nicotinic acetylcholine receptor beta subunit mRNA. These samples were negative, although positive hybridization was observed in cells from a section of neurophophysis, demonstrating the specificity of this probe (data not shown).

Localization of MCP-1 and FIC mRNAs. In addition to \textit{E.-} and P-selectin, we examined the expression of two chemokines in this model. In situ hybridization with an RNA probe revealed relatively few or no MCP-1 mRNA-expressing cells in sections of control muscles 4 h after the injection of PBS (Fig. 4A and B) or 0.2 mg of \textit{P. gingivalis} LPS (Fig. 4C and D). However, abundant expression of MCP-1 mRNA was detected in sections of muscles 4 h after the injection of 0.2 mg of \textit{E. coli} LPS (Fig. 4E and F). High magnification demonstrated that this chemokine mRNA was expressed in mononuclear cells which had infiltrated the connective tissue surrounding the muscle fibers and bundles (Fig. 4G). Interestingly, no hybridization was observed in either fibroblasts or endothelial cells. In these studies, a cocktail of three oligonucleotide probes gave similar results (data not shown).

By using a cocktail of oligonucleotide probes and in situ hybridization, only a few FIC mRNA-expressing cells were observed in sections of control muscles 4 h after the injection of PBS (Fig. 5A and B) or 0.2 mg of \textit{P. gingivalis} LPS (Fig. 5C and D). However, abundant expression of FIC mRNA was detected in sections of muscles 4 h after the injection of 0.2 mg of \textit{E. coli} LPS (Fig. 5E and F). High magnification demonstrated that FIC mRNA was expressed only in neutrophils but not fibroblasts or endothelial cells (Fig. 5G).

Relative expression of selectin and chemokine RNAs. We further evaluated the apparent lack of selectin and chemokine mRNA expression in response to \textit{P. gingivalis} LPS by the sensitive technique of RT-PCR. Using RT-PCR, we were able to detect even minute amounts of mRNAs being transcribed in the entire LPS-injected muscle. We examined muscles from 12 mice that had been given injections of 0.1 mg of \textit{P. gingivalis} and muscles from 6 mice that had been given \textit{E. coli} LPS at 0.1 mg and 6 that had been given \textit{E. coli} LPS at 0.01 mg and the un.injected contralateral muscles from the same \textit{E. coli} LPS-injected mice. In addition, five each of PBS-injected and un.injected muscles were examined. Figure 6 shows a representative gel of two samples per group. In all cases, except for one in which \textit{P. gingivalis} LPS gave a band of equivalent intensity to \textit{E. coli} LPS (not shown in Fig. 6), all samples of \textit{P. gingivalis} LPS produced a relatively reduced signal in response to all mRNAs tested. In addition, an increase in the levels of all RT-PCR products was observed in muscles from mice given 10 and 100 \(\mu\)g of \textit{E. coli} LPS. Interestingly, RT-PCR products were also observed in the uninject ed contralateral muscles from these mice, suggesting a systemic response to the injected LPS. Control cDNA levels from PBS-injected muscles were also low, except for P-selectin, which showed levels above those for \textit{P. gingivalis} LPS but far below those for \textit{E. coli} LPS. Levels of \(\beta_2\)-microglobulin as a control for mRNA and cDNA synthesis were uniform in all tested samples.

DISCUSSION

Recently, our laboratory has shown that \textit{P. gingivalis} was unable to directly stimulate HUVEC to synthesize E-selectin in vitro and was a poor activator of two cytokines which are synthesized by human adherent monocytes and are capable of stimulating human E-selectin (6). We hypothesized that these in vitro data may be indicative of a poor innate host response to this organism, which results in highly localized bacterial colonization of normally sterile tissue. We have developed a mouse model of infection in which we can detect early components of the innate host response to further examine this hypothesis. The mouse muscle provided a local site that could be easily removed and examined. Muscles were examined histologically and by RT-PCR for inflammation 4 h after injection of bacterial LPS. The expression of mouse E- and P-selectins was examined since they have been shown to play a critical role in the early phase of the inflammatory response in mice (15, 18). In addition, the expression of two chemokines, MCP-1 and FIC, was examined to determine if they were involved in the early stages of the innate host response to bacterial LPS in vivo.

In this study, we observed an acute inflammatory response after injection of \textit{E. coli} LPS. Upon histological examination, a strong inflammatory infiltrate consisting of neutrophils and monocytes was observed. In situ hybridization analysis revealed expression of E- and P-selectins which was confined to postcapillary endothelium. Levels of both selectin mRNAs were increased as determined by RT-PCR. Therefore, it appears that selectin mRNA expression correlates with the cellular infiltrate and occurs early in an acute inflammatory reaction. These events are consistent with the well-known ability of \textit{E. coli} LPS to elicit an inflammatory response (for a review, see reference 19) and the role of mouse E- and P-selectins during inflammation (2, 10).

In addition to selectins, an increase in the expression of the chemokine MCP-1 was observed in response to \textit{E. coli} LPS. In our model, MCP-1 mRNA expression was evident in monocytes. This is particularly relevant since reports concerning the ability of LPS to stimulate MCP-1 expression in monocytes in vitro have given conflicting results (3, 5). MCP-1 expression may have occurred either from direct activation of LPS or indirectly by the induction of other inflammatory mediators known to increase expression of this chemokine (5). Our in situ analysis could not distinguish between these possibilities. Regardless of the mechanism, monocyte MCP-1 expression appears to be involved in the early mouse response to bacterial infection. Interestingly, the expression of MCP-1 mRNA was not observed in other cell types normally found in muscle tissues, such as endothelial cells and fibroblasts. In vitro studies have demonstrated that LPS can induce the expression of MCP-1 in human endothelial cells (4) and in human fibroblasts (13). These results may not necessarily correlate with the ability of mouse endothelial cells and fibroblasts localized in skeletal muscle to express MCP-1 in response to \textit{E. coli} LPS. Alternatively, the lack of MCP-1 expression in these cell types may be due to lack of presentation because of efficient removal of the LPS by serum or other cellular components. A novel finding from these studies was that FIC mRNA was
FIG. 5. Localization of FIC mRNA in LPS-injected sections of muscle. LPS- or PBS-injected mouse muscle samples were excised, embedded in paraffin, sectioned, probed by in situ hybridization, and stained with hematoxylin and eosin. (A to F) Muscles were given injections of either PBS (A and B), 0.2 mg of \textit{P. gingivalis} LPS (C and D), or 0.2 mg of \textit{E. coli} LPS (E and F). Magnification, \times150. (G) High-magnification photomicrograph (\times470) showing hybridization of the probe for FIC mRNA in neutrophils. (A, C, E, and G) Bright-field photomicrographs; (B, D, and F) dark-field photomicrographs.
expressed by neutrophils after injection of *E. coli* LPS in the mouse muscle model. To date, only the expression of FIC by NH 3T3 cells has been reported (11). Except for its action on basophils (1) (chemotaxis and histamine release), the function of FIC is unknown. Since monocytes and endothelial cells are reported to have receptors for FIC (11), we can only speculate that in our model neutrophils may be activating these cells as part of the innate host defense against *E. coli* LPS stimulation.

In contrast to the data obtained with *E. coli* LPS, *P. gingivalis* LPS failed to stimulate the production of inflammatory mediators in the mouse model. Histological examination revealed the lack of a cellular inflammatory infiltrate, and in situ hybridization analysis did not detect either *E- or P-selectin* mRNAs in endothelial cells. In addition, in situ hybridization analysis of resident leukocytes did not detect mRNAs for MCP-1 and FIC. This was consistent with the RT-PCR analysis of numerous samples, which failed to amplify these RNAs from excised muscle samples. The inability of *P. gingivalis* LPS to activate inflammatory mediators in the mouse model was not due to a general lack of biological activity of our preparation since it displayed biological activity in human cells. In a previous report, we demonstrated that *P. gingivalis* LPS blocked *E. coli* LPS from stimulating E-selectin expression on HUVEC and, although not nearly as potent as *E. coli* LPS, was able to stimulate tumor necrosis factor alpha and interleukin-1β secretion by human peripheral blood monocytes (6). Our observations in the mouse model described here are also consistent with reports which have shown that *P. gingivalis* has a low biologically reactive LPS in other animal models (22).

One clear advantage of an in vivo evaluation of inflammatory mediator production is that both direct and indirect activation of inflammatory mediators could occur. It is significant, therefore, that within the first 4 h after LPS injection, there was such a significant difference in the host response to these two LPS preparations. The inability of *P. gingivalis* LPS to stimulate inflammatory mediator production in the mouse muscle model is consistent with a role of LPS of low biological reactivity in promoting tissue colonization. It is important to point out, however, that the mouse muscle is sufficiently different from the human periodontium with respect to both normal bacterial exposure and the constitutive expression of innate host defense components (20). Conclusions that can be drawn from these studies with respect to the importance of inflammatory evasion must be limited. Nevertheless, it is apparent that the initial inflammatory host response can be significantly affected by the type of LPS to which the tissue is exposed.

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REFERENCES


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