Bacterium-Dependent Induction of Cytokines in Mononuclear Cells and Their Pathologic Consequences In Vivo

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Viridans streptococci are a heterogeneous group of gram-positive bacteria that are normal inhabitants of the mouth. These organisms are thought to contribute significantly to the etiology of infective endocarditis, although recently they have been implicated in serious infections in other settings. Another group of oral bacteria, gram-negative anaerobes, is associated with chronic dental infections, such as periodontal diseases or endodontic lesion formation. We evaluated the ability of the oral pathogens Streptococcus mutans and Porphyromonas endodontalis to induce a pathogenic response in vivo, with the goal of quantifying the inflammatory response in soft tissue by measuring leukocyte recruitment and hard tissues by measuring osteoclastogenesis. S. mutans induced a strong inflammatory response and was a potent inducer of osteoclast formation, while P. endodontalis was not. To further study the mechanisms by which P. endodontalis and S. mutans elicit significantly different levels of inflammatory responses in vivo, we tested the capacity of each to induce production of cytokines by mononuclear cells in vitro. S. mutans stimulated high levels of interleukin-12 (IL-12), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α), all of which are associated with inflammation, enhanced monocyte function, and generation of a Th1 response. In contrast, P. endodontalis stimulated production of IL-10 but not of TNF-α, IL-12, or IFN-γ. These results demonstrate that oral pathogens differ dramatically in their abilities to induce inflammatory and immunoregulatory cytokines. Moreover, there is a high degree of correlation between the cytokine profile induced by these bacteria in vitro and their pathogenic capacity in vivo.

Several different bacteria have been associated with oral infections (1, 27, 34). However, the mechanisms by which specific bacteria cause pathogenic lesions are not completely understood. Bacterial contamination of the dental pulp in decayed teeth inevitably leads to pulpitis and necrosis. The pathogens that cause pulpitis are derived from dental plaque, and are, for the most part, gram-positive bacteria such as viridans streptococci. Oral streptococci are also important agents in infective endocarditis and are suspected to contribute to the etiology in over 50% of cases (16, 31). Approximately 20% of viridans streptococcus endocarditis are caused by Streptococcus mutans (5). In addition, viridans group streptococci have been described as responsible for the alpha-streptococcal shock syndrome in neutropenic patients (19). The mechanisms by which viridans streptococci cause bacteremia associated with severe clinical manifestations have not been elucidated. In contrast to oral streptococci, which appear to cause acute or subacute infections, gram-negative anaerobes are primarily associated with chronic oral infections such as periodontal disease or endodontic lesions. For example, Porphyromonas endodontalis has been found in half of chronic endodontic lesions and is almost exclusively found in infections of endodontic origin, suggesting a specific association of P. endodontalis and the dental pulp (4, 32).

The innate immune response associated with infections is linked to the adaptive immune response by cytokine production. For example, bacteria can initiate cell-mediated immunity by stimulating macrophages to produce interleukin-12 (IL-12), which can then promote gamma interferon (IFN-γ) production by natural killer cells. IFN-γ-activated macrophages then secrete more IL-12 as a result of positive feedback, leading to cell-mediated Th1 responses. IFN-γ also activates macrophages to produce tumor necrosis factor alpha (TNF-α), which in turn maintains the activated state of the macrophage (16). TNF-α is a potent proinflammatory cytokine that can stimulate chemokine production by endothelial cells and fibroblasts in vitro (25). In addition to its protective role in the host defense against infectious agents, TNF-α contributes to bone resorptive activity and has been implicated in pathologic bone resorption (11, 21, 28). An effective host defense against bacterial invasion is characterized by the vigorous recruitment and activation of inflammatory cells, which are modulated by the coordinated expression of both pro- and anti-inflammatory cytokines. IL-10, which is produced by monocytes and Th2 lymphocyte subsets, inhibits IFN-γ synthesis by Th1 cells and inhibits the production of proinflammatory cytokines such as IL-12 and TNF-α. The inhibiting effects of IL-10 on cytokine production correlate with their antiinflammatory effects in vivo (9). Therefore, IL-10, IFN-γ, IL-12, and TNF-α have important and cross-regulatory roles in infection (29).

In data presented here, we examined the capacities of S. mutans and P. endodontalis to stimulate leukocyte recruitment and osteoclastogenesis in vivo and cytokine production by mononuclear cells in vitro. This was accomplished in an animal model that has recently been shown to be an effective in describing the host response to oral pathogens (35). The results demonstrate that S. mutans stimulates considerable inflammatory cell recruitment and osteoclast formation in vivo and is a strong inducer of proinflammatory cytokine induction in vitro whereas P. endodontalis is not. These results should provide insight into the inflammatory response of S. mutans and its capacity to stimulate the host in oral infections and in bacterial endocarditis.
Materials and Methods

Bacteria. *P. endodontalis* ATCC 35406 (generously provided by Ann Tanner [Forsyth Dental Center, Boston, Mass.]) was cultured under strict anaerobic conditions (85% N2, 10% H2, and 5% CO2) on Trypticase soy-brain-heart infusion-yeast extract-blood agar containing hemin and vitamin K3 (Northwest Laboratory, Waterville, Maine). *S. mutans* LMT7 and OMZ175, grown in Todd-Hewitt broth (Becton Dickinson, Cockeysville, Md.), were generously donated by F. Oppenheim (Boston University Goldman School of Dental Medicine, Boston, Mass.). The bacteria, used at early- to mid-log-phase growth, were heat killed by boiling for 10 min and then washed three times with phosphate-buffered saline (PBS) as described previously (7). The amount of each bacterium was quantitated by colony formation on bacterial plates.

Mononuclear cell stimulation. Human peripheral blood was obtained from healthy volunteers by venipuncture followed by the addition of heparin (100 U per 50 ml of blood). Peripheral blood mononuclear cells (PBMC) were isolated from the leukocyte-richuffy coat following centrifugation over a gradient of Ficoll-Hypaque. Autologous serum was obtained by centrifugation of nonheparinized venous blood. PBMC (> 106 per well) were incubated in polypropylene cell culture plates containing the indicated stimulus in a final volume of 250 μl of RPMI tissue culture medium supplemented with 5% autologous serum. All solutions and reagents had nondetectable endotoxin levels (<30 pg/ml) as determined by the Limulus amebocyte lysate assay (Sigma, St. Louis, Mo.). After 24, 48, 72, or 96 h of stimulation, supernatants were harvested for evaluation of induced cytokine levels by enzyme-linked immunosorbent assay (ELISA).

ELISAs for cytokines. Antibody pairs for human IL-12 p70 (R&D Systems, Minneapolis, Minn.), IL-10, TNF-α, and IL-10 (PharMingen, San Diego, Calif.) were used in the sandwich ELISA. These assays were sensitive to 10 pg/ml of cytokine per ml. Other ELISA reagents were purchased from Kiekegaard & Perry Laboratories (Gaithersburg, Md.). ELISAs were performed as we described previously (6). Briefly, 96-well plates were incubated with capture monoclonal antibody at 4°C overnight. Nonspecific bind sites were blocked by 1% bovine serum albumin for 1 h at room temperature. After being rinsed with PBS-Tween 20 buffer, the samples were added and incubated at room temperature for 1 h. Biotinylated detecting antibody to the cytokine was added to the samples and incubated for 1 h after the plate was washed with PBS-Tween buffer. Horseradish peroxidase-streapavidin and color metric system (tetramethylbenzidine) were used as described by the manufacturer (KPL, Kiekegaard & Perry Laboratories). The plates were read at 450 nm within 30 min with an ELISA plate reader (Molecular Devices).

Bacteria inoculation and immunohistology studies. The host response to bacteria was investigated in both soft and hard tissue, using the calvarial model that was recently shown to be effective in characterizing the response to oral pathogens (35). CD-1 outbred mice, 12 to 14 weeks of age, were obtained from Charles River Laboratories (Boston, Mass.). Mice were inoculated with 106 live *P. endodontalis* or *S. mutans* in 50 μl of PBS. Bacteria were applied by supraperiosteal injection in the mid-calvarial region after complete removal of hair with a microshaver (Conair). Vehicle (PBS) alone was injected as a negative control. Mice were sacrificed 2, 5, and 10 days after injection of bacteria. The calvarial samples with attached scalp were harvested, fixed in 4% paraformaldehyde in 24 h at 4°C, and decalcified in EDTA-glycerin-PBS (pH 7.0). After decalcification, sections were immersed in 30% sucrose-PBS overnight at 4°C, snap frozen, and cut into 5-μm sections with a cryostat according to published procedures (12). The tissue sections were stained with hematoxylin and eosin to count inflammatory cells or tartrate-resistant acid phosphatase (TRAP) to aid in identifying osteoclasts.

Statistical analysis. Statistical analysis was performed with one-way analysis of variance with Duncan's post hoc test, to determine the significance of cytokine production between control and bacteria-stimulated samples. Significance was determined at *P* < 0.05.

Results

The response to *P. endodontalis* and *S. mutans* in vivo. To determine whether different oral bacteria stimulate disparate host responses, *P. endodontalis* and *S. mutans* were injected into the mouse calvaria (scalp), adjacent to the periosteum. The number of inflammatory cells recruited and number of osteoclasts formed were quantified. The results demonstrate that *S. mutans* elicits a pronounced inflammatory infiltrate in mouse calvarial soft tissue, while *P. endodontalis* elicits a relatively mild inflammatory cell infiltrate (Fig. 1). While both *S. mutans* and *P. endodontalis* stimulated a peak of inflammatory cell recruitment at 2 days, *S. mutans* produced a much more sustained inflammatory infiltrate. At 5 and 10 days, *S. mutans* enhanced inflammatory cell recruitment 30- and 20-fold, respectively, while *P. endodontalis* stimulated increases of only 5- and 3-fold, respectively, compared to vehicle alone.

To assess the impact of *S. mutans* or *P. endodontalis* infection on hard tissue, we measured osteoclastogenesis and the percent bone surface covered by osteoclasts. *S. mutans* induced formation of a large number of osteoclasts, which were identified as large multinucleated TRAP-positive cells in lining the bone surface. *S. mutans* stimulated a sevenfold-greater increase in both the number of osteoclasts and the percent bone surface covered by osteoclasts compared to *P. endodontalis* at the 5-day time point (Fig. 2 and 3). Thus, the magnitude of the induced osteoclastogenesis agrees well with the results obtained by measuring the recruitment of inflammatory cells, indicating that *S. mutans* stimulates a more vigorous host response than *P. endodontalis*. With the negative control (vehicle alone), there was virtually no stimulation of osteoclast formation.

In the experiments described above, we used heat-killed bacteria to simplify interpretation of the experimental results since the confounding influence of bacterial survival in vivo is eliminated. Since it is possible that heat inactivation of the bacteria may have altered their true capacity to induce a host response, we repeated the experiments with live bacteria in vivo. Similar results were obtained, with *S. mutans* inducing a greater degree of inflammatory response (tissue necrosis and...
inflammatory cell infiltration) and osteoclast activity (data not shown). Thus, the host response in vivo induced by inoculation with either heat-killed or live S. mutans is dramatically more pronounced than that stimulated by P. endodontalis. Other investigators have found that injection of either heat-killed or live oral bacteria in this model produces similar inflammatory responses (35).

**Bacterium-stimulated cytokine production.** To explore the mechanisms to account for differences in the inflammatory response and osteoclast activation induced by S. mutans or P. endodontalis infection, we examined the capacities of these bacteria to stimulate production of a proinflammatory cytokine profile by mononuclear cells in vitro. Since IL-12 is produced by bacterium-stimulated macrophages and bridges the innate immune response and cell-mediated immune response, we examined IL-12 production stimulated by these two bacteria. The results demonstrate that S. mutans significantly increased IL-12 production compared to unstimulated controls at ratios of 1, 10, and 100 bacteria per PBMC. In contrast, P. endodontalis stimulated only a minimal increase in IL-12 production compared to unstimulated controls. Since IFN-γ is a powerful activator for macrophages and IL-12 increases inflammatory responses by enhancing the production of IFN-γ (8, 17), we examined bacterium-stimulated IFN-γ production. Figure 5 shows that S. mutans stimulated high levels of IFN-γ by PBMC. S. mutans stimulated IFN-γ production approximately 22-fold over unstimulated controls at a ratio of 10 bacteria per PBMC, while P. endodontalis induced none.

TNF-α is an important proinflammatory cytokine. It can promote inflammatory cell infiltration leading to a vigorous host response and can directly stimulate osteoclast formation and activity (24). Therefore, we examined TNF-α induction by putative endodontic pathogens and found that S. mutans stimulated high levels of TNF-α production by PBMC whereas P. endodontalis was a relatively weak inducer (Fig. 6). IL-10 inhibits IL-12, IFN-γ, and TNF-α production and is considered an antiinflammatory cytokine. In contrast to the above findings, P. endodontalis stimulated high levels of IL-10 production (2,600 pg/ml) whereas S. mutans stimulated low levels (178 pg/ml) (Fig. 7). Compared to S. mutans, P. endodontalis stimulated 15-fold more IL-10 production.

To further investigate the capacity of each of the two bac...
teria to stimulate production of an inflammatory cytokine profile, time-dependent cytokine induction was assessed over periods of 1, 4, and 7 days. This would take into account potential cell-to-cell interactions that occur from different leukocyte subsets found in mononuclear cell populations. Figures 8 to 11 demonstrate that the patterns of cytokine induction by both bacteria are consistent with time. *S. mutans* stimulated high levels of IL-12 (Fig. 8) and IFN-γ (Fig. 9) at all time points, while *P. endodontalis* did not. The levels of TNF-α production decreased with time for both bacteria but kept the same order of potency. Thus, *S. mutans* was a more efficacious stimulator than *P. endodontalis* (Fig. 10). For IL-10 induction, the order was reversed, as *P. endodontalis* stimulated high levels and *S. mutans* stimulated low levels of IL-10 (Fig. 11). These findings are not restricted to the strain of *P. endodontalis* used here (OMZ175), since similar results were obtained with another strain, LM7 (data not shown).

**DISCUSSION**

We demonstrate here that two putative oral pathogens are considerably different in their capacities to stimulate human PBMC to produce inflammatory and immunoregulatory cytokines. Interestingly, *S. mutans*, which stimulates high levels of Th1 cytokines such as IL-12 and IFN-γ, also stimulates high levels of the proinflammatory cytokine TNF-α. In contrast, *P. endodontalis*, which induces little or none of the above cytokines, appears to be potent in inducing IL-10, an anti-inflammatory cytokine. It is possible that IL-10 induced in response to *P. endodontalis* inhibits expression of other cytokines by the mononuclear cells. Regardless of the mechanism, it is clear that the inherent properties of an invading bacterium influence the cytokine profile that is ultimately produced.

The proinflammatory cytokine profile that was induced in human PBMC corresponded well with both quantitative and qualitative measures of the inflammatory response observed in vivo in mice. That human and murine leukocytes would behave similarly is not surprising given the large body of evidence indicating similarities in various immune responses (15, 23). When injected in vivo, *S. mutans* induced formation of a dense inflammatory cell infiltrate and bone resorptive activity compared to *P. endodontalis*. This finding is consistent with the production of TNF-α and IFN-γ stimulated by *S. mutans* and the relative lack of their expression induced by *P. endodontalis*.

*S. mutans* gained notoriety in the 1960s as a cariogenic pathogen; its virulence factors related to caries formation have...
been extensively studied (2). Bacteriemia of *S. mutans* are capable of causing significant morbidity and mortality through bacterial endocarditis or other complications such as respiratory distress syndrome (14, 22, 30). The mechanisms by which *S. mutans* produce an inflammatory response and causes tissue damage have not been elucidated. Our results demonstrated that *S. mutans* is able to elicit cell-mediated Th1 responses by stimulating expression of IL-12, IFN-γ, and TNF-α in mononuclear cells. We postulate that the exuberant production of these cytokines stimulated by *S. mutans* may contribute to pathological process of the infection since Th1 cytokines can exacerbate inflammatory pathology by enhancing chemokine expression and recruitment of inflammatory cells (3, 10, 18, 20, 26).

In contrast to *S. mutans*, *P. endodontalis* (which is associated with chronic infections of the dental pulp) has been found to be of low virulence in experimental monoinfections but seems to play an essential role in anaerobic mixed infection (33). The reasons for this interesting phenomenon have not been explained. In the present study, we demonstrate that *P. endodontalis* induces production of high levels of IL-10 but low levels of IL-12, IFN-γ, and TNF-α. Consistent with this cytokine profile, in vivo, *P. endodontalis* elicits mild inflammatory infiltration and bone resorption activity. It is possible that the cytokine profile induced by *P. endodontalis* (i.e., IL-10) contributes to its low virulence in monoinfections. However, in mixed infections, the “stealth” property of this microorganism may help itself and other bacteria to invade the host without causing strong host responses, so that the pathogens can reside in infected sites for long periods and grow to large numbers. The stealth properties of *P. endodontalis* are consistent with finding that a bacterium of the same genus, *Porphyromonas gingivalis*, has similar properties. *P. gingivalis* is poorly recognized by cells of the innate host defense system (13), and over 1,000 times more bacteria are required to stimulate high levels of chemokine production compared to *S. mutans* (6). Thus, both *P. endodontalis* and *P. gingivalis* seem to possess stealth properties, which may provide a selective advantage to the bacteria by enabling them to avoid host defense mechanisms. It is possible that this represents an important aspect of their pathologic potential.

From our results, we conclude that specific bacteria can induce the synthesis of a profile that is consistent with up-regulation of the host response or one that is not. Thus, the type of host response generated is considerably different for specific bacteria and is reflected by the cytokine profile induced in vitro. This conclusion is supported by results demonstrating that the nature of the cytokine profile induced in vitro is consistent with the capacity of the bacteria to produce an inflammatory infiltrate and stimulate pathologic osteoclast activity in vivo.

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**REFERENCES**