Coinvasion of Dentinal Tubules by Porphyromonas gingivalis and Streptococcus gordonii Depends upon Binding Specificity of Streptococcal Antigen I/II Adhesin

ROBERT M. LOVE,1 MALCOLM D. McMILLAN,1 YOONSUK PARK,2 AND HOWARD F. JENKINSON1*

School of Dentistry, University of Otago, Dunedin, New Zealand; Department of Oral Biology, University of Washington, Seattle, Washington; and Department of Oral and Dental Science, University of Bristol Dental School, Bristol, United Kingdom

Received 24 August 1999/Returned for modification 3 November 1999/Accepted 23 November 1999

Cell wall-anchored polypeptides of the antigen I/II family are produced by many species of oral streptococci. These proteins mediate adhesion of streptococci to salivary glycoproteins and to other oral microorganisms and promote binding of cells to collagen type I and invasion of dentinal tubules. Since infections of the root canal system have a mixed anaerobic bacterial etiology, we investigated the hypothesis that coadhesion of anaerobic bacteria with streptococci may facilitate invasive endodontic disease. Porphyromonas gingivalis ATCC 33277 cells were able to invade dentinal tubules when cocultured with Streptococcus gordonii DL1 (Challis) but not when cocultured with Streptococcus mutans NG8. An isogenic noninvasive mutant of S. gordonii, with production of SspA and SspB (antigen I/II family) polypeptides abrogated, was deficient in binding to collagen and had a 40% reduced ability to support adhesion of P. gingivalis. Heterologous expression of the S. mutans SpaP (antigen I/II) protein in this mutant restored collagen binding and tube invasion but not adhesion to P. gingivalis or the ability to promote P. gingivalis coinvasion of dentin. An isogenic afimbrial mutant of P. gingivalis had 50% reduced binding to S. gordonii cells but was unaffected in the ability to coinvasive dentinal tubules with S. gordonii wild-type cells. Expression of the S. gordonii SspA or SspB polypeptide on the surface of Lactococcus lactis cells endowed these bacteria with the abilities to bind P. gingivalis, penetrate dentinal tubules, and promote P. gingivalis coinvasion of dentin. The results demonstrate that collagen-binding and P. gingivalis-binding properties of antigen I/II polypeptides are discrete functions. Specificity of antigen I/II polypeptide recognition accounts for the ability of P. gingivalis to coinvasive dentinal tubules with S. gordonii but not with S. mutans. This provides evidence that the specificity of interbacterial coadhesion may influence directly the etiology of pulpal and periapical diseases.

Infections of the tooth pulp (pulpitis) can develop in several ways: through oral bacterial penetration of dentinal tubules opened by caries, restorative procedures, dental trauma, or tooth wear; from direct bacterial contamination of an exposed pulp subsequent to caries or trauma; or by infection of lateral canals from a deep gingival pocket. Studies of the dynamics of root canal infections show that facultative anaerobes (mainly streptococci) are usually the dominant species in the early stages of infection (13). After longer periods of infection (for example, 6 months or so), facultatively anaerobic bacteria become progressively outnumbered by obligate anaerobes (12, 13). These are the predominant organisms in chronic infections and include species of Fusobacterium, Peptostreptococcus, Prevotella, and Porphyromonas (28, 41, 42). Attempts have been made to correlate the presence of certain bacterial species with clinical symptoms. Although direct relationships are hard to prove, species of Porphyromonas (especially Porphyromonas gingivalis) and Prevotella have been associated with acute symptoms of infection (16, 44).

Studies investigating the bacterial flora of carious coronal dentin and infected root canal dentin consistently show that gram-positive bacteria are predominant (3, 11, 38). In vitro studies have demonstrated that oral streptococci penetrate dentinal tubules over several days and remain viable for prolonged periods (30). The invasion of human dentinal tubules by cells of Streptococcus gordonii and Streptococcus mutans depends upon the production by these bacteria of cell wall-anchored polypeptides of the antigen I/II family. These polypeptides contain approximately 1,500 amino acid (aa) residues and bind salivary components, collagen, and other oral microorganisms (20). Isogenic mutants of S. gordonii that are deficient in production of the antigen I/II polypeptides SspA and SspB are unable to invade dentinal tubules. Likewise, an isogenic mutant of S. mutans deficient in SpaP (antigen I/II) expression is also unable to invade dentin (30). Evidence suggests that the recognition, by antigen I/II family polypeptides, of collagen type I present within the tubules (9) is essential for bacterial invasion and for intratubular growth of streptococci (30).

By contrast to results obtained with gram-positive bacteria, monocultures of gram-negative anaerobic bacteria do not invade dentinal tubules (1, 37). This has led to the notion that dentinal tubules may in some way be selective for streptococci and other facultatively anaerobic bacteria. However, gram-negative bacteria must be able to gain entrance into the root canal system from the oral cavity via the dentinal tubules. This is because these organisms can be found in the root canal systems of teeth that are clinically sound but nonvital owing to trauma (28, 43). Since coadhesion of oral bacteria is recognized as an important mechanism in the development of the dental plaque communities (46), it seemed likely that bacterial cell-cell interactions might play a role also in invasion of dentinal tubules. Accordingly, we hypothesized that a noninvasive
organism, such as *P. gingivalis*, might be able to penetrate tubules in association with a coadhering invasive partner organism, such as *S. gordoni*.

Coadhesion of *P. gingivalis* and *S. gordoni* is reasonably well characterized at the molecular level and involves cell surface proteins on both partners (22, 24). These molecules include the 44-kDa fimbriilin subunit (FimA) and a 35-kDa outer membrane protein of *P. gingivalis* (22, 25) and the streptococcal antigen I/II polypeptides SspA (1,528 aa residues) and SspB (1,448 aa residues) (5, 20, 24). Expression of SspB on the surface of *Enterococcus faecalis* cells confers on these cells the ability to bind *P. gingivalis* (24). Purified recombinant SspA or SspB polypeptide of *S. gordoni* binds FimA, whereas recombinant PAC (antigen I/II) from *S. mutans* does not (5). The *P. gingivalis* binding domain of SspB is defined by amino acid residues 1167 to 1250 (5) and is identical in SspA (10). By contrast, this region within the *S. mutans* antigen I/II polypeptide differs in primary sequence and in predicted secondary structure (5).

In this study we have investigated the role of streptococcal antigen I/II family polypeptides in promoting invasion of dental tubules by an otherwise nonpenetrating organism. The results demonstrate that adhesion of *P. gingivalis* to *S. gordoni* confers on the porphyromonads the ability to coinvade dental tubules. Conversely, invasion of dentin by *P. gingivalis* does not occur in the presence of *S. mutans* cells or in the presence of *S. gordoni* sspA sspB cells expressing heterologous SpaP from *S. mutans*. Thus, specificity of coadhesion may determine the ability of oral bacteria to colonize new environments and influence both the etiology and outcome of oral diseases.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture media.** *S. gordoni* DL1 (Challis) (35), *S. mutans* NG9 (kindly provided by A. S. Blewies, University of Florida, Gainesville), and isogenic derivatives (27, 30) were cultivated at 37°C in brain heart infusion medium (Difco Laboratories, Detroit, Mich.) containing 0.5% (wt/vol) yeast extract (BHY medium). Kanamycin (250 μg/ml) was included when appropriate to ensure maintenance of replicating plasmid pSMII/3 (18) in *S. gordoni*. Lactococcus lactis MG1363 and derivatives carrying plasmid pTREX1-sspA or pTREX1-sspB were cultivated at 30°C in M17 medium (Difco) containing 0.5% (wt/vol) glucose. *P. gingivalis* ATCC 33277 or YPF1 cells were cultivated in anaerobic BHY medium containing (5 μg/ml) and menadione (1 μg/ml). All cultures were inoculated from stock cell suspensions stored at −80°C in BHY medium containing 15% (vol/vol) glycerol and were grown in closed tubes or bottles without shaking. Bacterial cells were radioactively labeled by growth in BHY medium containing [methyl-3H]thymidine (8 μCi/ml [85 Ci/μmol]; Amersham Corp., Arlington Heights, Ill.) (21) to a specific radioactivity of between 1 × 103 and 3 × 103 cpm per cell.

**Construction of *P. gingivalis* albimutante.** *P. gingivalis* YPF1 fimA was generated by homologous recombination between *P. gingivalis* ATCC 33277 chromosomal DNA and a suicide plasmid carrying an internal fragment of the fimA gene as follows. The 0.65-kb PstI-HincII internal fragment of the fimA gene (14) was removed and subcloned into pBluescript SK− (19) to produce pBluFIM. The XbaI-SphI fragment of this plasmid containing the internal fimA fragment was subcloned into the suicide vector pJR/D/E (47). The resultant plasmid, pJR/D/E/FIM, was then introduced by conjugal transfer into *P. gingivalis* ATCC 33277 (36). The ensuing gene disruption generated a FimA-deficient mutant (YPF1) as confirmed by Western immunoblot analysis of cell extract proteins with anti-FimA antibodies and by reverse transcription-PCR analysis (data not shown).

**Adhesion assays.** Bacterial adhesion to acid-soluble collagen type I immobilized onto the wells of Maxi-Sorb microtiter plates (A/S Nunc, Kamstrup, Denmark) was measured as described previously (30). Binding of radiolabeled 105 cells [3H]thymidine (800 × g for 5 min] onto gelatin-coated slides, and purified for counting (21). Fewer than 1% of input *P. gingivalis* cells bound to blocked (control) wells. All data were analyzed by using Student’s t test.

### RESULTS

#### Role of antigen I/II family polypeptides in adhesion of *P. gingivalis* to oral streptococci.

*S. gordoni* strains DL1 and M5 express two antigen I/II family polypeptides designated SspA and SspB. These are the products of tandemly arranged chromosomal genes that are independently transcribed (10, 18, 21). In contrast, *P. gingivalis* cells were not able to bind to wild-type streptococci (Table 1). We have not yet determined the ability of oral bacteria to colonize new environments and influence both the etiology and outcome of oral diseases.

#### Invasion of dental tubules.

Noncarious, unrestored human teeth with single root canals were used for invasion experiments. Two root specimens were prepared from each of >100 teeth collected from numerous subjects, with the specimens being resin-coded so that for any one experiment complimentary specimens were avoided. Roots were prepared as previously described (29, 30). For each invasion experiment, six roots were selected at random and incubated at 37°C fully submerged with their pulpal surfaces uppermost in BHY medium containing bacterial cells for 14 days (29). At intervals of 3 days, the culture medium was removed short just exposing the roots and replaced with fresh medium. The viability of cells and purity of cultures were continually monitored (29). Coinvasion experiments were initiated with cultures containing equal numbers (2 × 108 cells/ml) of each bacterial species. BHY medium containing hemin and menadione (see above) was used for cocultivating *P. gingivalis* and streptococci, while M17 medium containing glucose, hemin, and menadione was employed for coculturing *P. gingivalis* and *L. lactis* at 30°C. Antibiotics were not included in these media. Analysis of bacterial samples, taken during and at the end of experiments, for plasmid-encoded antibiotic resistances (17, 30) and for antigen I/II polypeptide expression on Western immunoblots (30) confirmed that the plasmids were maintained in the absence of antibiotic selection.

**Light microscopy and assessment of invasion.** Infected roots were fixed, dehydrated in 10% (wt/vol) formic acid containing 2% (wt/vol) formalin, neutralized, washed, dehydrated, and blocked in wax (29). Consecutive transverse sections (6 μm) were cut from the coronal root dentin cervically and stained (5), and viewed at a standard magnification (×100). The extent of invasion was expressed as the tubeule invasion index (TI), which was calculated as described previously (30). On a linear scale, a TI of <0.2 was classified as nil invasion, while a TI of >2.0 was classified as heavy invasion. Data were analyzed by using Student’s t test. Sections that exhibited external root resorption were disregarded, and further sections were cut until the defect was removed. For the immunohistochemical detection of bacterial antigens, sections of wax-blocked teeth were dewaxed, and paraffin sections were cut, picked up onto gelatin-coated slides, and stained (5), and viewed at a standard magnification (×100). The extent of invasion was expressed as the tubeule invasion index (TI), which was calculated as described previously (30). *S. gordoni* cells were detected with rabbit polyclonal antibodies to a cell surface protein extract of *S. gordoni* cells (19), and antigen I/II polypeptides were detected with rabbit polyclonal antibodies to SpaP (29) (from N. A. Jacques, Institute of Dental Research, University of Sydney, Sydney, Australia). *P. gingivalis* cells were detected with rabbit antibodies raised to whole cells of *P. gingivalis* (from R. J. Lamont, University of Washington, Seattle). Primary antibodies were diluted in the normal rabbit serum (1:40 to 1:500) and binding was determined by incubation with primary antibodies as previously detailed (30). *S. gordoni* cells were detected with rabbit antibodies raised to whole cells of *P. gingivalis* (from R. J. Lamont, University of Washington, Seattle). Primary antibodies were diluted in the normal rabbit serum (1:40 to 1:500) and binding was determined by incubation of specimens with peroxidase-conjugated antirabbit antibodies and development with 3,3′-diaminobenzidine (30). Negative controls (no primary antibodies) were included in every experiment.

#### Infection of dental tubules.

Noncarious, unrestored human teeth with single root canals were used for invasion experiments. Two root specimens were prepared from each of >100 teeth collected from numerous subjects, with the specimens being resin-coded so that for any one experiment complimentary specimens were avoided. Roots were prepared as previously described (29, 30). For each invasion experiment, six roots were selected at random and incubated at 37°C fully submerged with their pulpal surfaces uppermost in BHY medium containing bacterial cells for 14 days (29). At intervals of 3 days, the culture medium was removed just short of exposing the roots and replaced with fresh medium. The viability of cells and purity of cultures were continually monitored (29). Coinvasion experiments were initiated with cultures containing equal numbers (2 × 108 cells/ml) of each bacterial species. BHY medium containing hemin and menadione (see above) was used for cocultivating *P. gingivalis* and streptococci, while M17 medium containing glucose, hemin, and menadione was employed for coculturing *P. gingivalis* and *L. lactis* at 30°C. Antibiotics were not included in these media. Analysis of bacterial samples, taken during and at the end of experiments, for plasmid-encoded antibiotic resistances (17, 30) and for antigen I/II polypeptide expression on Western immunoblots (30) confirmed that the plasmids were maintained in the absence of antibiotic selection.
plasmid pSMII-3 (18) in strain OB220 enhanced the ability of OB220 spaP mutant cells to bind collagen type I (Table 1) to above wild-type binding levels and restored significantly the ability of strain OB219 cells to bind collagen (Table 1) (30). However, expression of SpaP (P1) protein on the surface of these S. gordoni mutants (OB573 and OB576) did not enhance significantly (P > 0.05) their abilities to support adhesion of P. gingivalis ATCC 33277 cells (Table 1). This confirms previous results suggesting that S. mutans Pac protein, which is 99% identical in amino acid sequence to SpaP (P1), is poorly recognized by P. gingivalis cells (5). Levels of binding of P. gingivalis to S. mutans NG8 and 834 spaP were <40% of those to S. gordoni DL1 (Table 1).

P. gingivalis fimbriae mediate coadhesion of cells with S. gordoni. An afimbriate isogenic mutant of P. gingivalis ATCC 33277 was generated as described in Materials and Methods. Loss of the major surface fimbriae in strain YPF1 resulted in approximately a 50% reduction in numbers of porphyromonad cells bound to S. gordoni DL1 (Table 1). Binding of P. gingivalis YPF1 fimA cells to S. gordoni DL1 spaP was equivalent to only 32% of the P. gingivalis wild-type numbers bound to S. gordoni DL1. As before, levels of adhesion of P. gingivalis to S. gordoni strains expressing heterologous SpaP (P1) protein were not increased significantly (Table 1). Abrogation of FimA expression also resulted in approximately 50% reduced numbers of P. gingivalis cells bound to S. mutans NG8 cells (Table 1). The slight reduction in numbers of YPF1 cells bound to strain 834 cells, compared with NG8 (Table 1), was not statistically significant (P > 0.05). These data demonstrate that P. gingivalis fimbriae are involved in the binding of porphyromonad cells to S. gordoni and to S. mutans. They also suggest that binding of fimbriae to streptococci occurs independently of interaction of P. gingivalis cells with the SpaA and SspB polypeptides.

Invasion of dentinal tubules by P. gingivalis. Previous studies have established experimental conditions under which cells of oral streptococci reproducibly invade dentinal tubules (29). Randomly selected human tooth roots were incubated with bacterial cultures at 37°C for 14 days and then removed, washed, fixed, sectioned, and visualized by light microscopy. More than 50% of root tubules were invaded by S. gordoni

<table>
<thead>
<tr>
<th>Bacterial strain and antigen III polypeptide phenotype</th>
<th>Mean no. of cells ± SD (10⁶) adhered to collagen (n = 4)</th>
<th>Mean no. of cells ± SD (10⁶) bound (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. gordoni DL1 (wild type) (SspA⁺) SspB⁻</td>
<td>4.68 ± 0.57</td>
<td>2.38 ± 0.21</td>
</tr>
<tr>
<td>OB220 spaP spaB⁻</td>
<td>2.73 ± 0.21</td>
<td>1.76 ± 0.19</td>
</tr>
<tr>
<td>OB219 spaP spaB⁻</td>
<td>1.59 ± 0.17</td>
<td>1.43 ± 0.15</td>
</tr>
<tr>
<td>OB573 spaP (SpaP⁺) OB576 spaP (SpaP⁺)</td>
<td>5.46 ± 0.58</td>
<td>1.83 ± 0.20</td>
</tr>
<tr>
<td>S. mutans NG8 (wild type) (SpaP⁺) 834 spaP</td>
<td>3.72 ± 0.38</td>
<td>1.55 ± 0.17</td>
</tr>
<tr>
<td>spaP</td>
<td>0.39 ± 0.02</td>
<td>0.69 ± 0.05</td>
</tr>
</tbody>
</table>

*Five micrograms of collagen type I digest was immobilized on microtiter plate wells, and the numbers of radioactively labeled streptococcal cells adhered (input, 3 × 10⁶ cells) were measured as described in Materials and Methods. 5 × 10⁶ cells of each streptococcal strain were immobilized onto microtiter plate wells, and the numbers of radioactively labeled P. gingivalis cells adhered (input, 5 × 10⁶ cells) were measured as described in Materials and Methods.

**TABLE 2. Coinvasion of dentinal tubules by P. gingivalis and streptococci**

<table>
<thead>
<tr>
<th>Bacterial strain(s)</th>
<th>Mean TI ± SD (n = 6)</th>
<th>Immunohistochemical detection* with antibodies to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. gingivalis ATCC 33277</td>
<td>S. gordoni DL1</td>
<td>S. gingivalis extract or SpaP⁺</td>
</tr>
<tr>
<td>OB576</td>
<td>2.73 ± 0.21</td>
<td>1.69 ± 0.13</td>
</tr>
<tr>
<td>OB219</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>OB573</td>
<td>2.21 ± 0.34</td>
<td>1.56 ± 0.45</td>
</tr>
<tr>
<td>OB576</td>
<td>2.07 ± 0.26</td>
<td>1.06 ± 0.32</td>
</tr>
<tr>
<td>OB219</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
</tr>
<tr>
<td>OB573</td>
<td>2.24 ± 0.19</td>
<td>0.67 ± 0.18⁴</td>
</tr>
<tr>
<td>OB576</td>
<td>2.26 ± 0.28</td>
<td>0.59 ± 0.09⁴</td>
</tr>
<tr>
<td>S. mutans NG8</td>
<td>&lt;0.10</td>
<td>ND⁵</td>
</tr>
<tr>
<td>S. mutans 834</td>
<td>1.67 ± 0.19</td>
<td>0.51 ± 0.09⁴</td>
</tr>
<tr>
<td>S. mutans OB219</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
</tr>
</tbody>
</table>

*Calculated as described in Materials and Methods. TI of <0.2, nil invasion. ⁴ Transverse sections of infected coronal root dentin were stained for bacteria and viewed under ×100 magnification. ⁵ Demineralized specimens were fixed, blocked in wax, sectioned, and reacted with primary antiserum, and antibody bound to cells was detected as described in Materials and Methods. ⁶ S. gordoni cells expressing SpaP and S. mutans cells were detected with SpaP (P1) antibodies.

DLI or S. mutans NG8 cells under these conditions, with penetration of bacterial cells to 200 μm or more (30). The degree of invasion was quantified by assessing microscopically the numbers of tubules infected per unit area and was expressed as the TI (see Materials and Methods). Streptococcal cells were detected either by staining (6) or by incubating sections with antibodies reacting with the S. gordoni cell surface, in which case bound antibodies were detected immunohistochemically (30). The TI values obtained for wild-type S. gordoni cells by the two methods differed somewhat, with the antibody detection method being less sensitive and thus giving lower TI values (Table 2).

To determine the ability of P. gingivalis cells to invade dentinal tubules, human tooth roots were incubated with P. gingivalis cultures in BHY medium supplemented with hemin (5 μg/ml) and menadione (1 μg/ml). Although this medium supported vigorous growth of P. gingivalis, no penetration of dentinal tubules by P. gingivalis cells was observed following staining of sections (Table 2). We then investigated the ability of P. gingivalis and S. gordoni growing in coculture to penetrate dentinal tubules. After 14 days of incubation, heavy invasion of dentinal tubules by bacteria was evident (TI = 2.21 ± 0.34) as detected by direct staining of microbial cells within sections (Table 2; Fig. 1A). To differentiate clearly between the bacterial types invading the tubules, serial sections were incubated either with S. gordoni cell surface antibodies or with antibodies raised to whole cells of P. gingivalis. Subsequent immunohistochemical detection of bound antibodies demonstrated that infected tubules contained both S. gordoni (Fig. 1B) and P. gingivalis (Fig. 1C) cells. Antibodies to P. gingivalis did not bind to streptococci (Table 2). The TI value for the mixed infection as determined by direct staining was not significantly different from that for S. gordoni alone (Table 2). This indicated that the numbers of tubules invaded by bacteria in coculture were similar to those for S. gordoni in monoculture, implying that P.
*P. gingivalis* and *S. gordonii* occupied the same tubules. These experiments did not allow accurate estimations of the relative proportions of the two bacterial cell types present within infected dentinal tubules.

**Antigen I/II binding specificity determines *P. gingivalis* invasive ability.** When *P. gingivalis* was cocultured with *S. gordonii* OB220 sspA, invasion levels of both streptococcal and porphyromonad cells were reduced (Table 2). When *P. gingivalis* cells were cocultured with strain OB219 cells, which were deficient in production of both the SspA and SspB proteins, no invasion of dentinal tubules by either organism was observed (Table 2; Fig. 1D, E, and F). These observations appear to rule out the possibility that streptococcal cell growth simply modifies the environment in some way so as to allow *P. gingivalis* to penetrate dentin. To determine if *P. gingivalis* invasion depended upon specificity of antigen I/II binding, cells were cocultured with *S. gordonii* OB576 sspA sspB expressing SpaP from *S. mutans* cocultured with *P. gingivalis*. *P. gingivalis* cells are detected in panel C only, even though tubule invasion by streptococci is restored in panel G. Bar, 50 μm.

**FIG. 1.** Transverse sections of human roots showing the invasion of dentinal tubules from the pulpal side by *S. gordonii* in coculture with *P. gingivalis*. Bacterial cells were stained (6) (A, D, and G) or detected immunohistochemically with antibodies to *S. gordonii* surface proteins (B, E, and H) or with antibodies to *P. gingivalis* cells (C, F, and I). (A, B, and C) Wild-type *S. gordonii* DL1 cocultured with *P. gingivalis* ATCC 33277; (D, E, and F) *S. gordonii* OB219 sspA sspB cocultured with *P. gingivalis*; (G, H, and I) *S. gordonii* OB576 sspA sspB expressing SpaP from *S. mutans* cocultured with *P. gingivalis*. *P. gingivalis* cells are detected in panel C only, even though tubule invasion by streptococci is restored in panel G. Bar, 50 μm.

1362 LOVE ET AL. INFECT. IMMUN.

Heterologously expressed *S. gordonii* antigen I/II polypeptides promote coadhesion and coinvasion. We have previously demonstrated that heterologous expression of *S. gordonii* SspA or SspB polypeptide on the surface of the food-grade bacterium *L. lactis* MG1363 confers on these cells the ability to bind salivary glycoproteins and collagen type I (17). Lactococci expressing SspA or SspB were also found to bind twofold-higher numbers of *P. gingivalis* cells than did *L. lactis* MG1363. In
binding assays, $2.55 \times 10^6 \pm 0.21 \times 10^6$ P. gingivalis cells bound to $1 \times 10^7$ immobilized cells of L. lactis expressing SpS protein, and $2.40 \times 10^6 \pm 0.20 \times 10^6$ P. gingivalis cells bound to L. lactis cells expressing SpB protein. We then determined if expression of antigen I/II polypeptide in L. lactis might also confer on these cells the ability to invade dentinal tubules. Wild-type lactococci did not invade dentinal tubules, although some cells appeared to attach to the dentin surface (Fig. 2A). Conversely, cells of L. lactis expressing SpS (or SpB) were able to invade dentinal tubules, with a TI of $1.14 \pm 0.29$ calculated from stained sections (results not shown). Co-cultivation of P. gingivalis with L. lactis(pTREX1-sspA) cells expressing SpS polypeptide demonstrated bacterial invasion of tubules to $100 \, \mu m$ or more (Fig. 2B). Reaction of root sections with antigen I/II polypeptide antibodies demonstrated that lactococcal cells were present within dentinal tubules and that SpS polypeptide was expressed in situ (Fig. 2C). Incubation of root sections with P. gingivalis-specific antibodies showed clearly that P. gingivalis cells co-invaded with lactococci expressing SpS (Fig. 2D). L. lactis(pTREX1-sspB) cells expressing SpB polypeptide also promoted P. gingivalis invasion of dentin (results not shown).

**DISCUSSION**

Interbacterial binding is considered to be a significant factor in the development of dental plaque, promoting both selective and temporal acquisition of microorganisms to form complex communities (46). While a considerable amount of information on bacterial cell-cell interactions has been obtained from in vitro studies, it has been difficult to demonstrate the ecological significance of interbacterial adhesion reactions in vivo. Model systems have been developed to approach such considerations, and one of these recently has been used to investigate the effects of oxygen (aerobiosis) on the growth in planktonic or biofilm phase of a defined consortium of oral microorganisms (4). In a two-stage continuous culture, survival of anaerobic bacteria in the second (aerobic) vessel depended upon the ability of Fusobacterium nucleatum to simultaneously coaggregate with both anaerobic (P. gingivalis and Prevotella) and facultatively anaerobic bacteria. In the absence of F. nucleatum, numbers of anaerobic bacteria were significantly reduced, indicating that coaggregation facilitates survival of anaerobes in aerated environments (4). We have now extended the information on the ecological significance of coaggregation (cohesion) by showing that successful invasion of dentinal tubules by the obligate anaerobe P. gingivalis depends upon adhesion to an invasive partner organism. This process, designated co-invasion, establishes coadhesion as a potential determinant of endodontic disease etiology.

Previous light microscopy studies have demonstrated that several gram-negative anaerobic bacterial species, such as Prevotella melanogena (1) and Prevotella intermedia (37), were unable to invade dentin. On the other hand, it has been reported that Porphyromonas endodontalis BN11a-f and P. gingivalis ATCC 33277 in monoculture were able to invade bovine dentinal tubules, although very few tubules were infected (40). While the number, distribution, and size of bovine dentinal tubules are similar to those in human dentin, Siqueira et al. (40) removed the cementum from the dentin blocks in their experiments, and this artificially enhances bacterial penetration. Thus, the reported penetration of very few tubules may have occurred by nonspecific incorporation and not through cell division and growth along the tubule length. We have never observed invasion of human dentinal tubules by P. gingivalis cells in monoculture, even over a wide range of different culture conditions of medium, redox potential, and temperature. We have previously provided evidence that the ability of S. gordonii or S. mutans cells to invade dentinal tubules depends upon collagen recognition by antigen I/II polypeptides (30). Although P. gingivalis cells also bind to collagen type I deposited onto hydroxylapatite surfaces (32, 33), it would appear that the ability to bind type I collagen, present within dentinal tubules (9), is not sufficient to promote invasion of tubules by these bacteria in monoculture.

The antigen I/II polypeptides produced by oral streptococci have been shown to have a range of adhesive functions. Sequences within the N-terminal alanine-rich repeat blocks, and sequences C-terminal to the proline-rich repeats in the central region of the polypeptide, bind salivary glycoproteins (20). Sequences within the N-terminal region are also believed to be involved, at least in part, in binding to collagen (39), while an 84-aa sequence downstream of the proline-rich repeats, and present in SspA and SspB proteins from S. gordonii, is implicated in binding to P. gingivalis cells (5, 20). The results in this paper provide further evidence that the regions within the antigen I/II polypeptides that mediate adhesion to collagen or to P. gingivalis are distinct. Binding to collagen appears to be a general property of antigen I/II family polypeptides produced by S. gordonii and S. mutans, while binding to P. gingivalis is a specific property of the SspA and SspB polypeptides (5). There is no evidence to suggest that the SspA and SspB polypeptides differ in their relative affinities of binding to P. gingivalis cells (5). This was confirmed in that similar numbers of P. gingivalis cells bound to lactococcal cells expressing SspA or SspB proteins. On the other hand, the SspA and SspB polypeptides appear to have different binding affinities for salivary agglutinin glycoprotein and for collagen (17).

Previously we have shown that S. gordonii SspA and SspB polypeptides confer on L. lactis cells the ability to bind collagen type I (17). Remarkably, surface expression of these proteins in L. lactis also conferred on lactococci the abilities to penetrate dentinal tubules and to support P. gingivalis co-invasion. These observations support the conclusion that it is the specificity of...
interaction of *P. gingivalis* cells with antigen I/II polypeptide that allows coaggregation of dentin by this organism. Bacteria such as *S. mutans* may colonize tooth surfaces and invade dentinal tubules in an antigen I/II (P1)-dependent mechanism (8), but they do not appear to promote coaggregation. The abilities of other streptococcal species, for example *Streptococcus oralis*, to enhance *P. gingivalis* invasion of dentin, might therefore depend upon their antigen I/II polypeptide binding specificities. *P. gingivalis* coaggregates with *S. oralis* (2, 26), but the role of antigen I/II polypeptide SoaA (20) in this reaction and whether SoaA binds *P. gingivalis* cells are not known. We are currently investigating the abilities of different streptococcal species to promote coaggregation of dentinal tubules by *P. gingivalis* and by periodontal pathogens associated with root canal infections. This will provide information on the extent to which the specificities of other interbacterial adhesion reactions might determine the etiologies of endodontic infections.

Interbacterial coaggregation or coadhesion between *P. gingivalis* and *S. gordonii* has been shown recently to enhance colonization of both *P. gingivalis* and streptococci in a biofilm model of plaque formation (7). *S. gordonii* outcompetes *P. gingivalis* for attachment sites in salivary pellicle, and substantial mixed bacterial biofilms develop on saliva-coated glass slides only when deposited *S. gordonii* cells provide an attachment substrate for *P. gingivalis* (7). The results in the present paper extend these observations and suggest that specificity of coadhesion may initiate invasive infections of endodontic and periodontal tissues by these organisms. The occurrence of coadhesive interactions in vivo might explain why typically a limited number of species are found in an infected root canal.

The presence of gram-negative bacteria in infected dentin (3, 11, 15, 34) and within root canals with intact dentin walls (28, 40, 41, 42, 43) suggests that these bacteria can penetrate dentin in vivo. Associations between gram-negative bacteria and other bacteria that are capable of dentinal tubule invasion may thus allow invasion by the gram-negative organisms.

In summary, invasion of dentinal tubules by streptococci depends upon multiple recognition functions of antigen I/II proteins. When *P. gingivalis* cells are present in association with streptococci, the ability of *P. gingivalis* cells to coaggregate depends upon the species specificity of antigen I/II recognition, with SspA or SspB proteins on *S. gordonii* (or on heterologous lactococci) providing attachment sites for *P. gingivalis*. Binding of *P. gingivalis* cells to intratabular collagen may assist the invasion process. The production of fimbriae by *P. gingivalis*, while contributing to coaggregation with *S. gordonii* in vitro, is not necessary for coaggregation of dentinal tubules with *S. gordonii*. This is in direct contrast to the requirement for fimbriae in *P. gingivalis* invasion of gingival epithelial cells (45). Coadhesions of *P. gingivalis* and *S. gordonii* therefore functions to extend the invasive potential of *P. gingivalis*. This property of coaggregation of dentin by oral bacteria has not previously been investigated. Some other gram-negative periodontopathogenic bacteria, such as *Actinobacillus actinomycetemcomitans*, have a high capacity to invade and replicate within epithelial cells (31), as does *P. gingivalis* (23, 26). In light of the results presented here, it is possible that coaggregation may also promote coaggregation of epithelial cells, thus enhancing the pathogenic potential of otherwise noninvasive organisms.

ACKNOWLEDGMENTS

We are most grateful to A. S. Bleiweis, N. A. Jacques, R. J. Lamont, and S. F. Lee for providing bacterial strains, plasmids, or antibodies. We thank R. A. Baker and A. Samways for excellent technical assistance and A. R. Holmes and R. J. Lamont for helpful advice and discussions.

The support of the New Zealand Health Research Council, The Wellcome Trust (London, United Kingdom), and NIDCR (grant no. DE12505) is gratefully acknowledged.

REFERENCES

25. Lamont, R. J., G. W. Hsiao, and S. Gil. 1994. Identification of a molecule of

26. Lamont, R. J., and H. F. Jenkinson. 1998. Life below the gum line: patho


Editor: J. D. Clements