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Immunization with *Porphyromonas (Bacteroides) gingivalis* Fimbriae Protects against Periodontal Destruction

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Adhesive fimbriae from *Porphyromonas gingivalis* are cell surface structures which may be important in the virulence of this oral pathogen and thus may serve as a critical or target antigen. Immunization with highly purified 43-kDa fimbrial protein protected against periodontal tissue destruction when tested in the *P. gingivalis*-infected gnotobiotic rat model. A similarly highly purified 75-kDa cell surface component did not provide protection. Heat-killed whole-cell and sonicated cell surface extracts which contain the 43-kDa protein as well as the 75-kDa component were protective also. This study indicates that the fimbrial protein may serve as a model for the development of effective vaccines against periodontitis, a major human oral disease.

Anaerobic black-pigmented bacteroides including *Porphyromonas (Bacteroides) gingivalis* have been associated with periodontal destruction in humans (39, 40, 44), monkeys (14, 20, 42), dogs (28, 48), and monoinfected rats (17). Patients with destructive periodontitis often harbor *P. gingivalis*, while those with little or no disease harbor few or none of these microorganisms (3, 9, 38, 39, 47, 57). Patients with destructive periodontitis also have a markedly elevated serum and gingival fluid antibody response to *P. gingivalis* (7, 8, 31, 33, 51). *P. gingivalis* fimbria-specific antibody-secreting cells have been demonstrated in gingival tissues (35), and serum antibodies against the 75-kDa outer membrane protein and 43-kDa fimbria have been demonstrated in serum of patients with periodontitis (6, 53, 55).

It may be important to investigate the possibilities for immunological prophylaxis of periodontal disease. However, selection of antigens for a periodontitis vaccine is difficult, since several oral bacteria may be associated with the disease (29, 41), as well as *P. gingivalis* (9, 29, 44). Recently, periodontal destruction induced in rats by monoinfection with *P. gingivalis* was shown to be reduced by immunization with heat-killed intact *P. gingivalis* cells (17). Since the many antigenic determinants present on whole-cell preparations increase the risks for adverse side effects, more homogeneous and defined antigen preparations are desirable for vaccination. Fimbriae from *P. gingivalis* are well characterized (23, 54), highly immunogenic (17), and important for the adhesion of *P. gingivalis* to oral tissues (24, 36). The purpose of the present study is to investigate the effect of immunization with various fimbrial and cell surface antigen preparations on *P. gingivalis*-associated periodontal disease in gnotobiotic rats.

MATERIALS AND METHODS

Microbiology. *P. gingivalis* 381, originally obtained from S. Socransky at the Forsyth Dental Center, and strain 2561 (ATCC 33277) were used in these studies. *P. gingivalis* 381, used for infection and whole-cell antigen preparation, was cultured on modified 5% sheep blood agar (Trypticase soy agar base supplemented with 0.5% yeast extract, 5 μg of hemin per ml, and 5 μg of menadione per ml) (56) grown at 37°C in an anaerobic chamber (Forma Scientific, Marietta, Ohio) for 48 h. Estimates of cell numbers were made by measuring turbidity with a spectrophotometer at 480 nm and comparing with known standards obtained by counting *P. gingivalis* cell dilutions in a Petroff-Hauser chamber. *P. gingivalis* 2561, used for purified antigen preparation, was grown according to procedures previously described (45, 46).

Animals. Male germfree Sprague-Dawley rats (Taconic Farms, Germantown, N.Y.) were kept under gnotobiotic conditions in plastic cages with stainless steel tops in positive-pressure plastic inflatable film isolators (Standard Safety Equipment Co., Palatine, Ill.). Each group of eight animals was kept in a separate isolator. Food pellets (diet L-485; Teklad, Madison, Wis.) and water were available ad libitum. Cage bedding consisted of 1/4-in. (1 in. = 2.54 cm) granules (Bed o' Cobs; Anderson, Inc., Maumee, Ohio) and was changed twice a week to minimize trauma to periodontal tissues from impaction of hair and bedding. All equipment, food, bedding, and water were sterilized by autoclaving. Materials entering the isolator were sprayed with 2% peracetic acid. Fecal and surface swab samples from the isolators were cultured once a week by using both aerobic and anaerobic incubation conditions.

Experimental design. Six groups of eight rats each were used. Group A was sham-immunized, uninfected animals used as negative controls, and group B was *P. gingivalis* sham-immunized, infected animals used as a positive control for infection. The other four groups (C to F) were immunized with selected *P. gingivalis* antigens and subsequently monoinfected with *P. gingivalis* 381. Group C was immunized with heat-killed whole cells; group D was immunized with highly purified 43-kDa fimbrial protein from *P. gingivalis*; group E was immunized with highly purified 75-kDa cell surface component from *P. gingivalis*; and group F was immunized with a cell surface protein fraction which included the 43-kDa protein, the 75-kDa protein, and other proteins extracted during the purification process.

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Antigen purification. Cell surface components used as antigens were sheared from *P. gingivalis* 2561, a *P. gingivalis* strain similar to strain 381 (26), by mild sonication and then precipitated by ammonium sulfate at a 40% concentration. Both of the components were further purified by differential precipitation. The 43-kDa major structural subunit of *P. gingivalis* fimbriae was purified by precipitation in the presence of 1% sodium dodecyl sulfate (SDS) and 0.2 M $MgCl_2$ at pH 6.5, and highly pure, homogeneous preparations of the component were obtained by repetitive precipitation steps (45). Purification of the 75-kDa outer membrane component was achieved by differential precipitation of the 75-kDa protein at pH 5.0 and subsequently resolubilized by adjusting the pH to 7.4. By repeated cycles of isoelectric precipitation at pH 5.0, a homogeneous pure 75-kDa component was prepared (46).

Immunization. Four-week-old germfree rats were immunized by subcutaneous injection with 10^{10} heat-killed whole cells of *P. gingivalis* or 20 μg of soluble cell surface protein antigens in Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, Mo.) per rat. Each rat received 0.4 ml in the scruff of the neck and 0.1 ml in the vicinity of the submaxillary salivary glands. The latter route was used to increase the specific salivary antibody response (43, 49).

Infection. Bacteria used for infection were suspended in 5% carboxymethyl cellulose (low viscosity; Sigma), and each rat received 0.5 ml (1.5×10^{12} cells per ml by gavage three times) at 48-h intervals. Forty-two days after the last infection, the rats were sacrificed and exsanguinated under anesthesia. Before sacrifice, subgingival plaque samples were obtained from all animals by gently inserting a sterile paper point (2720XX Fine; Johnson Dental Products Co., East Windsor, N.J.) into the subgingival area mesial to the upper second molars. The paper points were streaked across the media surface and then laid on the blood agar plates and incubated anaerobically as described above.

Antibody assays. Immediately prior to sacrifice, blood and saliva samples were obtained for antibody measurements. Approximately 0.5 ml of whole saliva was obtained from each rat after subcutaneous injection of 1% pilocarpine nitrate (0.1 ml/100 g of body weight). Blood was obtained by cardiac puncture, and the serum was separated by centrifugation and stored at −20°C.

Antibody levels in rat sera and saliva were measured by using particle concentration fluorescence-linked immunoabsorbent assay as developed by Jolley et al. (16). Sera were diluted 1:200 and saliva was diluted 1:10 in phosphate-buffered saline (PBS; pH 7.0) prior to analysis. Antigens used were purified 43-kDa fimbria and 75-kDa cell surface component covalently linked to Fluoricon carboxyl-polystyrene assay particles (IDEXX Corp., Portland, Maine) by using EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; Sigma). The covalent coupling procedure consisted of reacting 1 mg of protein antigen per ml with 0.5 mg of EDC per ml in 3 ml of 0.1 M phosphate buffer (pH 5.5) containing carboxyl-polystyrene beads for 1 h at room temperature. The beads were washed two times in PBS (pH 7.0) and resuspended to a concentration of 0.25% (wt/vol) in PBS containing 10% bovine serum albumin (BSA) to block unreacted sites.

Antigen-coated beads were incubated (15 min) with the rat serum (1:200 dilution) or saliva (1:10 dilution). During the second step the antibody-antigen-complexed beads were incubated for 15 min with a 1:35 dilution of goat anti-rat immunoglobulin G (IgG; Kirkegaard-Perry Laboratories, Inc., Gaithersburg, Md.). All sera and saliva dilutions were made in PBS (pH 7.0). The beads were separated, washed, and read at 485/535 in the IDEXX Screen Machine. All results were expressed as relative fluorescence units, on a linear scale ranging from 0 to 99,999.

In order to further characterize the antibody response to antigens, sera were subjected to immunoblot analysis (Western immunoblot). A partially purified fimbrial preparation rich in 43- and 75-kDa components was used as the antigen source and was separated by SDS–10% polyacrylamide gel electrophoresis (PAGE). The protein antigens were then transferred electrophoretically to nitrocellulose paper (Bio-Rad Laboratories, Richmond, Calif.). The paper was blocked by incubation for 30 min with 1% PBS–Tween 20 containing 1% BSA and then incubated with a 1:50 dilution of rat serum or 1:5 dilution of rat saliva in PBS-Tween-BSA for 1.5 h. The nitrocellulose paper was then washed with PBS-Tween 20 buffer (30 min, three times) and incubated for 1.5 h with a 1:500 dilution of horseradish peroxidase-conjugated goat anti-rat IgG (Southern Biotechnology Associates, Inc., Birmingham, Ala.). Internal molecular weight standards (Pierce, Rockford, Ill.) were run simultaneously on each gel. Localized antigens were detected by the addition of 4-chloryl-1-naphthol substrate solution (Bio-Rad).

Periodontal bone level. The periodontal bone level was assessed on defleshed rat jaws by morphometric and radiographic methods, as described recently (18, 19). Briefly, the morphometric procedure is as follows (19). The distance from the cementoenamel junction to the alveolar bone crest was measured under a dissecting microscope (30× magnification) by means of a scale in the eyepiece calibrated in fractions of a millimeter. The smallest discernible distance measured was 0.037 mm. Each jaw was oriented so that buccal and lingual cusps were superimposed prior to measurement. Measurements were made on the buccal root surfaces of the maxillary molar teeth. In all, 14 measurements were made on three separate occasions for each rat (one recording per root), with the jaws coded. The average bone loss per site was calculated and expressed in millimeters as the mean distance per site from the cementoenamel junction to the alveolar bony crest.

A second estimate of destructive periodontitis was made by using radiographic measurements. This technique was previously shown to be correlated with alveolar bone loss measured morphometrically (18). The radiographic method estimates interproximal bone loss. On magnified radiographs, the distances from the apex to the deepest bony defect (*AB*) and from the apex to cusp tip (*AC*) were measured distal to the first mandibular molars, and the result was calculated as $AB/AC \times 100$. Results are expressed as percentages. The means of the right and left ratios for each rat were used as a measure for periodontal bone support. These recordings were made in a blinded fashion on coded jaws and performed in duplicate on separate days.

Gingival enzyme activity. Collagenase activity in rat gingival tissue was measured by using procedures described previously (11, 12, 17, 37, 59). In brief, the buccal gingiva were dissected from the upper and lower jaws (about 20 mg [wet weight] per rat) and pooled by group, since the amount of gingival tissue from each rat is not sufficient to carry out the extraction and subsequent collagenase assay. The tissues were minced and extracted, and the extracts were precipitated and concentrated by ammonium sulfate (11, 12, 17, 37). Aliquots of gingival extracts were measured for protein by using a dye-binding protein assay (1) and were also measured for collagenase (37), gelatinase, and cystine proteinase activities (17).

Collagenase assay. The degradation of collagen was measured by using salt-soluble, lathyritic rat skin type 1 collagen (labelled in vitro with [^{3}H]formaldehyde) as a substrate. Seventy microliters of the gingival extract was incubated with 10 μl of [*methyl*-^{3}H]collagen (10 μg) at 27°C for 18 h. Aminophenylmercuric acetate was added to the reaction mixture at a final concentration of 1.4 mM to activate latent collagenase. The reaction was stopped with 10 μl of 1,10 phenanthroline, and the undigested collagen was precipitated by the addition of 1,4-dioxane (50% final concentration) and 10 μl of methylated nonradioactive carrier collagen (20 μg). After centrifugation, 50 μl of the supernatant was counted in a liquid scintillation spectrophotometer. Spontaneous breakdown of the tritiated methyl collagen after 18 h of incubation was less than 1.0%. Trypsin (final concentration, 5 μg/ml) degraded less than 5% of the collagen substrate. The collagenase activity is expressed as micrograms of collagen degraded per hour per milligram of protein. In addition, aliquots of the gingival extract were incubated with the [*methyl*-^{3}H]collagen at 22°C for 24 h, and the collagen degradation products were characterized by a combination of SDS-PAGE and fluorography as described previously (10, 17).

Gelatinase and cathepsin B and L assays. The details of the gelatinase activity (with thermally denatured type I collagen as the gelatin substrate) and the cystine proteinase (cathepsin B and L) activity (with the synthetic substrate Phe-Arg-*N*-methyl coumarin as the substrate) were also described in detail by us previously (17).

Statistical analysis. Differences among experimental groups were analyzed by one-way analysis of variance with multiple comparisons made by using Tukey's HSD test. For rejection of hypotheses, the 5% level of significance was chosen (58).

RESULTS

Microbiologic findings. *P. gingivalis* was recovered from infected rats at the initiation and during the experiment for an average of 3 to 4 weeks following the initial infection. No bacteria could be cultivated from noninfected controls. No contaminating microorganisms were found during culture of the isolators at any time throughout the study.

Measurement of periodontal destruction. From Fig. 1 it can be seen that the uninfected animals (i.e., the germfree sham-immunized animals) had a mean bone distance from the cementoenamel junction of 0.29 ± 0.01 mm, whereas the animals that were infected with *P. gingivalis* and sham immunized had a mean bone level of 0.39 ± 0.02 mm, indicating significant alveolar bone loss ($P < 0.05$). Animals immunized with whole cells of *P. gingivalis* showed alveolar bone levels comparable to those of the uninfected controls and comparable to those we have reported previously (17). Whole-cell-immunized animals, therefore, can be considered a positive control for vaccination. Animals immunized with the 43-kDa purified fimbriae and subsequently infected with *P. gingivalis* were protected against periodontal bone loss, since they had levels of 0.26 ± 0.01 mm, which are comparable to those of the uninfected sham-immunized group. In contrast, animals immunized with another surface antigen of *P. gingivalis*, the 75-kDa outer membrane component, had bone levels of 0.35 ± 0.02 mm, comparable to levels found in the sham-immunized infected animals. This result indicates that the 75-kDa antigen did not induce protective immunity. The final group was infected and immunized with a crude fimbrial preparation which contained the 43- and 75-kDa antigens and other components. It can be seen that the bone level (0.28 ± 0.02 mm) in this group was comparable to that of the noninfected control, indicating that a protective immune response was also induced in these animals.

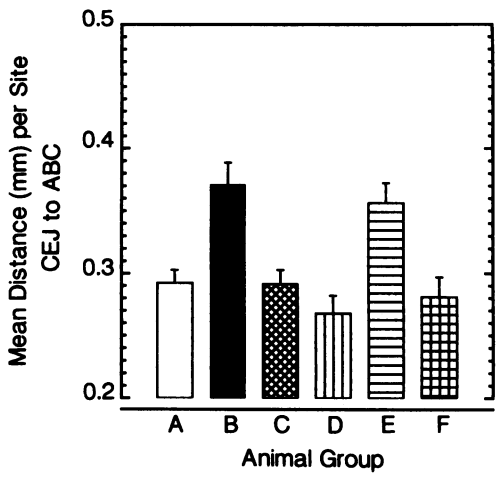

FIG. 1. Morphometric bone levels. Distance from cementoenamel junction (CEJ) to the alveolar bone crest (ABC) of maxillary molars is given as the mean difference between the CEJ and the ABC in millimeters per site for each group ± standard error of the mean. Group A, germfree, sham immunized; group B, infected with *P. gingivalis*, sham immunized; group C, infected, immunized with whole cells of *P. gingivalis*; group D, infected, immunized with 43-kDa fimbriae; group E, infected, immunized with the 75-kDa component; group F, infected, immunized with a crude fimbrial extract. The level for group B was significantly greater than that for group A ($P < 0.005$). The levels for groups C, D, and F were significantly lower than that for group B, with $P < 0.005$, $P < 0.001$, and $P < 0.01$, respectively. The level for group E was not significantly different from that for group B ($P > 0.50$).

From the radiographic measurements shown in Fig. 2, it can be seen that the group of animals which is germfree and sham injected had bone support of 55.2% ± 0.88%. Infected, sham-immunized animals had significantly less bone support (51.8% ± 0.84%) than germfree, sham-immunized rats. Animals immunized with whole cells (55.4% ± 0.62%) or 43-kDa fimbrial antigen (54.9% ± 0.43%) had bone support similar to those of germfree, sham-immunized rats, indicating that immunization with these preparations was protective. In contrast, groups immunized with the 75-kDa antigen (53.7% ± 0.99%) and crude fimbriae (54.0% ± 0.57%) were not significantly different from infected, sham-immunized rats.

Gingival enzyme measurements. An additional estimate of destructive periodontal disease in the rat model is obtained by measuring tissue enzymatic activities, i.e., collagenase, cathepsin B and L activity (cystine proteinase), and gelatinase activity. The gingival collagenase and cathepsin B and L have been previously reported to reflect the experimental periodontal disease seen in these animals (17). From Fig. 3A (and Fig. 4 [see below]), it can be seen that minimal collagenase activity was evident in the pooled gingival tissue extracts from the sham-immunized, noninfected animals. However, in the infected animals, the collagenase activity was increased about 10-fold (compared with that in the noninfected controls). The collagenase activity was reduced by about 72, 68, and 64% in the animals immunized with whole cells, the 43-kDa fimbrial antigen, and crude fimbriae, respectively, and reduced by 39% in animals immunized

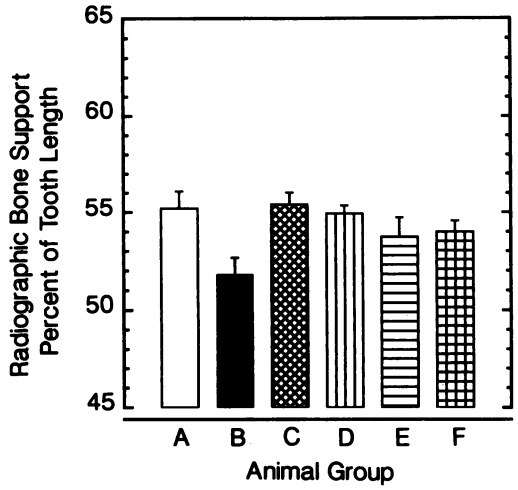

FIG. 2. Radiographic bone support. Radiographic measurement of interproximal bone level between mandibular 1st and 2nd molars is given as a mean percentage of tooth height for each group ± standard error of the mean. Group A, germfree, sham immunized; group B, infected with *P. gingivalis*, sham immunized; group C, infected, immunized with whole cells of *P. gingivalis*; group D, infected, immunized with 43-kDa fimbriae; group E, infected, immunized with the 75-kDa component; group F, infected, immunized with a crude fimbrial extract. Group B showed less interproximal bone support than group A ($P < 0.025$). Groups C and D showed significantly greater bone support than group B with $P < 0.025$ and $P < 0.01$, respectively. Groups E and F were not significantly different than group B ($P > 0.20$).

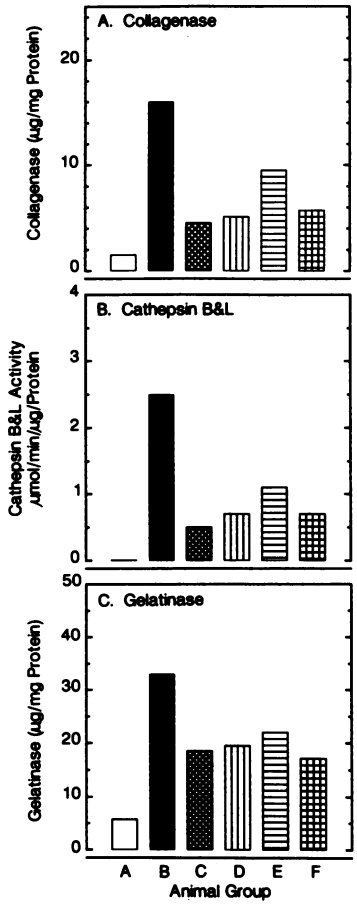

FIG. 3. Gingival tissue enzyme levels. Group A, germfree, sham immunized; group B, infected with *P. gingivalis*, sham immunized; group C, infected, immunized with whole cells of *P. gingivalis*; group D, infected, immunized with 43-kDa fimbriae; group E, infected, immunized with the 75-kDa component; group F, infected, immunized with a crude fimbrial extract.

with the 75-kDa protein. A similar pattern of change was seen in the fluorogram (Fig. 4), which also demonstrated that the collagen breakdown products (α_1^a and α_2^a) were generated by a mammalian collagenase. By using SDS-PAGE and fluorography, it can be seen that there is virtually no collagen destruction in the sham-immunized, noninfected animals, whereas in sham-immunized, infected animals, the production of α_1^a and α_2^a collagen cleavage fragments becomes quite marked. In the lanes showing tissue collagenase activity from whole-cell- and 43-kDa-antigen-immunized animals, there is a reduced production of these specific collagen breakdown fragments. Tissue from 75-kDa-antigen-immunized animals showed similar results. However, animals immunized with crude fimbriae demonstrated marked collagenase activity.

Similar results can be seen in Fig. 3B, in which gingival cathepsin B and L activity is assessed. Six germfree sham-immunized animals showed no detectable gingival cathepsin B and L activity, whereas sham-immunized animals infected with *P. gingivalis* showed levels in the range of 2.5 µmol of cathepsin B and L substrate degraded per min per µg of protein. The activities were low in animals immunized with whole cells (0.5 µmol/min/mg), 43-kDa protein (0.7 µg/min/mg), and crude fimbrial antigen (0.7 µg/min/mg). Animals immunized with the 75-kDa component showed intermediate levels of gingival cathepsin B and L activity (1.1 µmol/min/µg), suggesting some level of protection, but not the low levels seen in fully protected animals immunized with whole cells or the 43-kDa fimbriae.

Figure 3C shows the gingival gelatinase activity. Low levels of gingival gelatinase are seen in animals that were germfree and sham immunized (5.7 µg degraded per mg of protein), whereas the animals that were sham immunized and infected with *P. gingivalis* showed levels at 33 µg/mg of protein. A marked reduction in gingival gelatinase was seen upon immunization with whole cells of *P. gingivalis* and with crude fimbriae (18.5 and 17.1 µg/mg, respectively), whereas intermediate levels of reduction were seen with animals immunized with the 75-kDa component (22 µg/mg). The results of these measures of gingival enzymes generally paralleled the direct measurements of alveolar bone loss made morphometrically and radiographically. Moreover, the enzyme inhibition profiles of *P. gingivalis* gelatinase and collagenase were different from these tissue-extracted enzyme activities, indicating that their origin was mammalian and not bacterial (data not shown).

Antibody measurements. In the untreated, germfree rats' serum antibody levels against the 43-kDa fimbrial component from *P. gingivalis* were at low or undetectable levels (Fig. 5A). In contrast, animals infected with *P. gingivalis* showed detectable but low levels of antibody to fimbriae. Animals immunized with whole cells or with 43-kDa antigen showed markedly elevated responses to 43-kDa antigen, as expected, as did the animals immunized with crude fimbriae. Animals immunized with the 75-kDa component showed what appears as a modest but statistically significant ($P <$

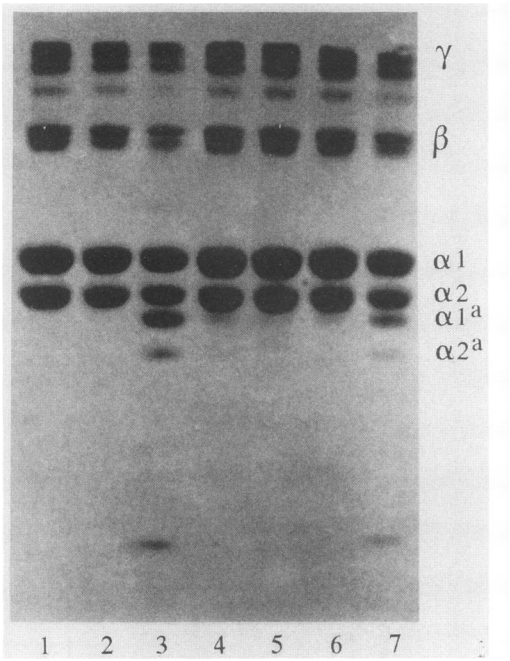

FIG. 4. Collagen gel. Lane 1, collagen blank; lane 2, gingival tissue from sham-infected animals; lane 3, tissue from *P. gingivalis* 381-infected-only animals; lanes 4, 5, 6, and 7, tissue from whole-cell-, 43- and 75-kDa-antigen-, and crude fimbrial extract-immunized animals, respectively.

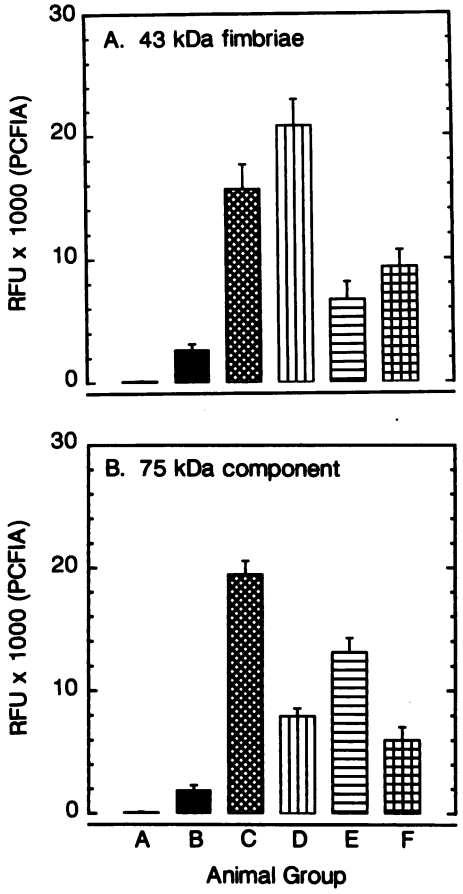

FIG. 5. Serum antibody levels to the 43-kDa fimbriae (A) and the 75-kDa outer membrane component (B) of *P. gingivalis*. All sera were tested at a 1:200 dilution by the particle concentration fluorescence immunoassay (PCFIA), with antigens covalently bound to the solid phase. Group A, germfree, sham immunized; group B, infected with *P. gingivalis*, sham immunized; group C, infected, immunized with whole cells of *P. gingivalis*; group D, infected, immunized with 43-kDa fimbriae; group E, infected, immunized with the 75-kDa component; group F, infected, immunized with a crude fimbrial extract. RFU, relative fluorescence unit.

0.05) increase in antibodies to the 43-kDa fimbriae above that antibody induced by infection only.

The uninfected, germfree animals had little or no detectable serum antibody, whereas *P. gingivalis* infection alone induced a detectable but low immune response (Fig. 5B). Animals immunized with whole cells, with the 75-kDa antigen, or with the crude fimbrial preparation have an antibody response to the 75-kDa antigen at levels higher than those of the infected animals. The highest serum antibody responses to the 75-kDa antigen were seen in whole-cell- and 75-kDa-antigen-immunized rats.

Similar results (data not shown) were seen when the sera were tested against crude extracts of the bacteria containing a mixture of 43-kDa, 75-kDa, and other cell surface antigens. In these studies, little or no antibody response was seen in the animals that were sham immunized and uninfected, and significantly higher levels of antibodies were seen in all other groups including the infected-only group. The highest levels were seen in the groups that were immunized with whole cells or 43-kDa, 75-kDa, or crude fimbriae, confirming (as expected) that immunized animals had a higher antibody response than the sham-immunized, uninfected animals.

The antibody responses to the fimbriae and the 75-kDa antigens were further evaluated by immunoblot analysis to confirm the specificity for antigen, and typical results are shown in Fig. 6. Uninfected animals showed no detectable antibody response to any of the antigens in crude fimbrial extracts, as can be seen in patterns for group A. However, infection alone induced antibodies reactive with the 75-kDa antigen and weakly reactive with the 43-kDa antigen. When animals were immunized with whole cells (Fig. 6, group C), they reacted very strongly with the 75- and 43-kDa antigens as well as other antigens present in the extract. In animals immunized with purified 43-kDa antigen there is a major antibody response seen to the 43-kDa antigen and antibodies are also seen to the 75-kDa antigen at a level comparable to those seen in the *P. gingivalis*-infected sham-immunized animals. On the other hand, animals immunized with the 75-kDa antigen gave a very strong reaction to the 75-kDa antigen and a very weak reaction in the 43-kDa region, comparable to that seen in the infected sham-immunized animals. Animals immunized with the crude fimbrial preparation gave reactions to both antigens that are comparable to those of the whole-cell-immunized rats (group F).

No detectable salivary antibodies to 43-kDa antigen were seen in the germfree sham-immunized animals (Fig. 7A). However, animals that were infected showed detectable but low levels of antibody to *P. gingivalis* in the saliva. Animals that were immunized with whole cells showed higher levels, but the highest levels of antibody to 43-kDa component were found in the animals immunized with 43-kDa component. These levels were three to four times greater than those seen even with whole-cell immunization or in animals immunized with 75-kDa or crude fimbrial antigen preparations, suggesting that 43-kDa antigen induces a salivary antibody re-

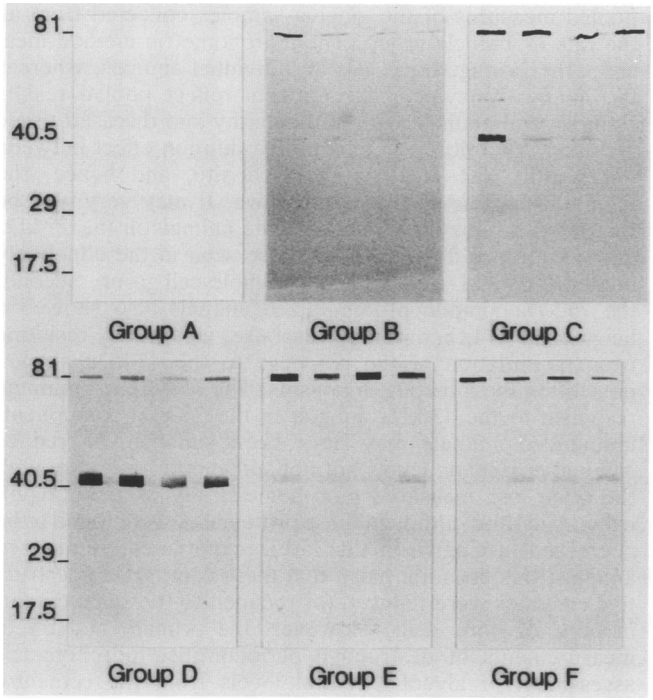

FIG. 6. Immunoblot analysis of serum antibody responses of rats to antigens in an extract of cells of *P. gingivalis*. Rat sera were tested at a 1:50 dilution; and anti-rat IgG-horseradish peroxidase conjugate was used at a 1:35 dilution. Group A, germfree, sham immunized; group B, infected with *P. gingivalis*, sham immunized; group C, infected, immunized with whole cells of *P. gingivalis*; group D, infected, immunized with 43-kDa fimbriae; group E, infected, immunized with the 75-kDa component; group F, infected, immunized with a crude fimbrial extract.

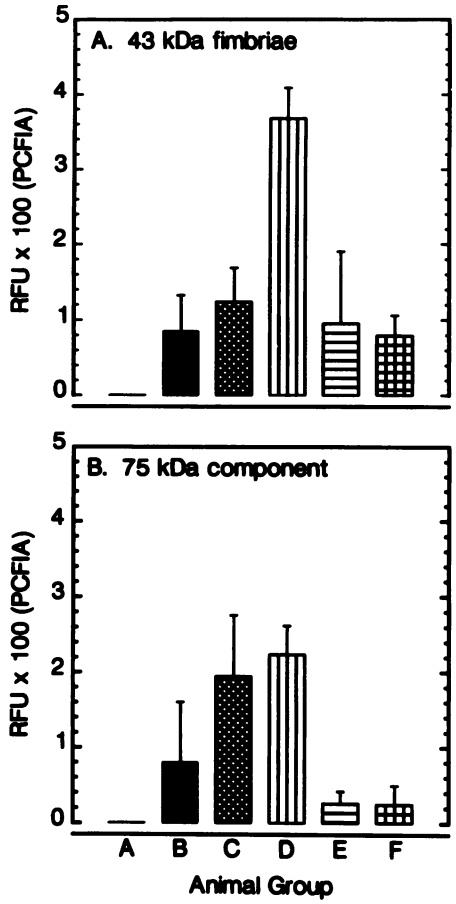

FIG. 7. Salivary antibody levels to the 43-kDa fimbriae (A) and 75-kDa outer membrane components (B) of *P. gingivalis*. All rat saliva was diluted to 1:5 prior to testing by PCFIA. Group A, germfree, sham immunized; group B, infected with *P. gingivalis*, sham immunized; group C, infected, immunized with whole cells of *P. gingivalis*; group D, infected, immunized with 43-kDa fimbriae; group E, infected, immunized with the 75-kDa component; group F, infected, immunized with a crude fimbrial extract. RFU, relative fluorescence unit.

sponse. In Fig. 7B the salivary antibody response to the 75-kDa component is depicted. The animals that were germfree or uninfected showed little or no detectable response; animals that were infected showed detectable responses, but the responses to the 75-kDa antigen were two to two and a half times higher in animals immunized with either whole cells or 43-kDa components. The animals immunized with 75-kDa or crude fimbrial component showed lower but detectable levels of antibody in saliva.

The salivary response to *P. gingivalis* antigens was also evaluated by immunoblot (Fig. 8). As can be seen from lanes 1, 2, and 3, the animals immunized with whole cells had a significant response to the 43- and 75-kDa components. Animals immunized with 43-kDa antigen, representatives of which are shown in lanes 4, 5, and 6, also showed a significant antibody response to the 43-kDa component. Animals immunized with the 75-kDa component (results from three of them are shown in lanes 7, 8, and 9) showed detectable levels of salivary antibodies to the 75-kDa component as well as to the 43-kDa component. Thus, both the levels and the specificity of salivary antibodies reflect the induction of an immune response by infection or deliberate immunization.

DISCUSSION

In the present study we report that immunization of monoinfected rats with whole cells and soluble antigens of *P. gingivalis* protects against periodontal destruction induced by *P. gingivalis*. Furthermore, we found that immunization with highly purified *P. gingivalis* fimbrial preparations, which retain nativelike structure, was protective against periodontal bone destruction. These findings suggest that fimbriae are protective or critical antigens, an immune response to which moderates or prevents *P. gingivalis*-induced destructive periodontal disease. This conclusion is supported by the finding of high levels of antibodies to the 43-kDa fimbrial preparations in protected rats immunized with whole cells, purified 43-kDa fimbriae, or soluble antigens of *P. gingivalis* containing the 43-kDa fimbrial component. In contrast, immunization with a 75-kDa outer membrane component was not protective or was only marginally protective, although it induced a vigorous antibody response. These results suggest that a specific immune response to *P. gingivalis* fimbriae protects against *P. gingivalis*-associated periodontal destruction.

The gnotobiotic rat model is a relatively simple in vivo model in which a single periodontal organism infects the rat, leading to destructive periodontitis. Interference with this process can be studied in the absence of confounding factors

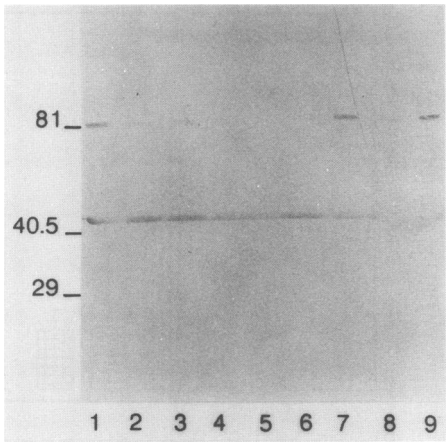

FIG. 8. Immunoblot analysis of salivary antibody responses of representative rats to antigens of an extract of whole cells of *P. gingivalis*. Rat saliva was tested at a 1:5 dilution, and anti-rat IgG-horseradish peroxidase conjugate was used at a 1:500 dilution. Lanes 1 to 3, whole cell immunized and infected with *P. gingivalis*; lanes 4 to 6, 43-kDa-antigen immunized and infected; lanes 7 to 9, 75-kDa-component immunized and infected with *P. gingivalis*.

produced by other members of the flora which would be present in the conventional or specific pathogen-free rat. It is likely that the protective immune response seen here is directed to early events, possibly oral colonization by *P. gingivalis*, since fimbriae of bacteria are often critically involved in colonization of mucosal surfaces (24, 36). This interpretation is supported by the finding of high levels of salivary antibodies to the 43-kDa fimbria in protected rats. It has been shown that antibodies directed to fimbriae of *P. gingivalis* inhibit adherence of this organism to epithelial cells in vitro (15). It is possible, however, that the protection afforded by immunization with the *P. gingivalis* fimbriae may be mediated by antibodies to fimbriae which block other fimbrial functions. It is also possible that induction of T-cell immunity to fimbriae may offer protection against periodontal destruction caused by *P. gingivalis* in this model.

In the gnotobiotic rat model used here, periodontal destruction is directly measured by two independent but correlated methods (18). The first is morphometric (4, 18) and measures horizontal bone loss on the buccal surfaces of the molars. The second is radiographic and estimates vertical bony changes occurring between the molars. We also assessed periodontal destruction indirectly by measuring changes in gingival enzymes which are induced by *P. gingivalis* infection. All of these measurements show essentially the same result, i.e., that destruction of the periodontium induced by *P. gingivalis* in monoinfected rats was inhibited by immunization with whole cells and by highly purified fimbriae from *P. gingivalis*.

It is of considerable interest that immunization with the 75-kDa outer membrane component found on the surface of *P. gingivalis* did not protect against periodontal bone destruction as measured by the morphometric technique. In contrast, when the indirect measures of periodontal disease, i.e., the gingival enzymes, were analyzed, there appeared to be a reduction of enzyme levels when the 75-kDa component, as well as whole cells or purified fimbriae, was used as the immunogen. There are several possible explanations for the differences in findings. First, the gingival enzyme measurements were not statistically analyzed, since they were pooled measures of the gingiva samples collected from all the rats in a single group. The morphometric method measures the average bone loss in individual animals, whereas the gingival enzyme measurements reflect pooled results from several animals with both healthy and diseased tissue included. Therefore, there can be a dilution effect between the healthy site and the diseased site, and hence, the variability in this system is unknown. It may very well be that levels of enzymes seen in some animals in the 75-kDa group were not different from those seen in the effectively immunized 43-kDa-component or whole-cell group. Second, the 75-kDa-component-immunized animals also showed a heightened 43-kDa antibody response, most likely resulting from potentiation of the response to the 43-kDa antigen present on the infecting organisms. The heightened immune response to the 43-kDa antigen in the 75-kDa-component-immunized animals may have been sufficient to reduce gingival enzymatic levels but not sufficient to interfere with the bone loss measured morphometrically or radiographically. And third, although these proteinases were found to be decreased in gingiva in the 75-kDa-component-immunized animals, this does not mean that these same tissue-destructive enzymes were reduced (or reduced to the same extent) in bone or bone cells. However, the primary and direct measurements of destructive periodontitis, morphometric assessment of alveolar crestal levels from the cemento-enamel junction, and radiographic interproximal bony measurements clearly show that immunization with purified 43-kDa fimbriae from *P. gingivalis* is protective, whereas immunization with the purified 75-kDa outer membrane component is not protective.

Although indicative of infection and immunization, the role of serum antibodies in modulating periodontal infection seen in this model, although likely important, is not clear. It is reasonable to expect that salivary antibodies play a role in modulating *P. gingivalis* infection in the rat, possibly by acting locally to inhibit colonization, a phenomenon that has been seen in rats immunized and infected with *Streptococcus mutans* (27, 30, 49). Salivary antibodies to *P. gingivalis* found by both the solid-phase assay and immunoblot assay in general reflected the immunization regimes. No detectable antibody was seen in the saliva of animals who were sham immunized but not infected with *P. gingivalis*; however, animals infected with *P. gingivalis* had low but detectable levels of antibodies to antigens of *P. gingivalis*. The salivary immune response to the 43-kDa antigen in the 43-kDa antigen-immunized animals was approximately three to four times greater than that seen in any other group of animals, indicating that a marked salivary antibody response was induced by active immunization. When salivary antibodies in animals immunized with 75-kDa antigen were tested, these animals gave a low but detectable response. The failure of animals immunized with the 75-kDa antigen to make salivary antibodies was unexpected, especially in light of the fact that these same animals had a vigorous serum antibody response to the 75-kDa antigen. No detectable antibody to the 75-kDa antigen was seen in the sham-immunized but noninfected animals, whereas infection alone did induce a substantial antibody response to the 75-kDa antigen. Immunoblots also confirmed levels of antibody in the saliva to the 43-kDa antigen in the whole-cell- and 43-kDa-antigen-immunized animals and levels of antibody to the 75-kDa antigen in animals immunized with preparations containing that antigen.

From the study of serum and salivary antibodies it then appears that there is a good correlation between protection

and levels of antibodies to the 43-kDa antigen in the serum and whole saliva. That is, animals with high levels of antibodies to whole cells and the 43-kDa antigen were protected. On the other hand, animals with high levels of antibody to 75-kDa antigen, such as seen in the 75-kDa-antigen-immunized groups were not protected, possibly because adequate levels of antibody were not achieved in their saliva to the 75-kDa component of *P. gingivalis*.

The correlation between high levels of antibody to the 43-kDa fimbriae and protection against *P. gingivalis*-induced periodontal destruction provides strong evidence that the fimbriae or fimbria-associated antigens are protective or critical antigens in *P. gingivalis* infection. The mechanism by which this immunity provides protection is not known. Possible protective mechanisms operative in this model include inhibition of colonization by secretory antibodies, opsonization of *P. gingivalis*, inhibition of bacterial toxins, or bacterium-induced triggering of host destructive mechanisms. Further experiments will be necessary to unravel the mechanism(s) responsible for protection in this model. Once known, this mechanism(s) may provide leads to investigate protective immunity to *P. gingivalis*-associated periodontal disease in man.

It should be noted that the fimbrial preparations used here contained only the monomer of *P. gingivalis* fimbriae at detectable levels; however, the participation of minor fimbrial adhesins at levels below detection by biochemical methods, but still able to induce an immune protection, cannot be ruled out at this time. The fimbriae were purified by a procedure which resulted in retention of at least partial native fimbrial qualities (45). Fimbriae or fimbria-associated components are then protective antigens against *P. gingivalis*-induced periodontal disease in the rat. It is likely, however, that the 43-kDa monomeric unit itself, which appears to be the basic structural monomeric unit of the fimbriae, may mediate *P. gingivalis* adhesion to oral surfaces. We have recently described the ability of the 43-kDa adhesin to inhibit binding of *P. gingivalis* to saliva-coated hydroxyapatite (24). The binding of *P. gingivalis* to hydroxyapatite is likely promoted by salivary proline-rich proteins (2). Such a reaction, i.e., binding of *P. gingivalis* via the fimbriae to the pellicle-covered tooth, may very well be operative in the gnotobiotic rat model. In this case, the 43-kDa antigen would be expected to be protective and, therefore, should contain protective epitopes. Investigation of the immune responses to synthetic peptides of the 43-kDa fimbrial monomeric unit are needed to determine if there are indeed protective epitopes.

Fimbriae of *P. gingivalis* may play a role in other reactions of importance in periodontal disease. For example, they have been shown to stimulate the release of interleukin-1 (IL-1) from mouse monocytes (13). IL-1 has many biologic activities, including stimulation of osteoclastic bone resorption, which may play a role in periodontal disease. It is possible that immunization with fimbriae may induce a protective immune response which would interfere with such mechanisms. For example, antibodies directed to fimbriae may inhibit their ability to stimulate IL-1 production by monocytes. Other potential virulence properties of fimbriae include mediation of adherence to tissue components such as collagen (32) and fibrinogen (21, 22).

These studies point to an important role for the fimbriae in the virulence of *P. gingivalis* and point to experiments directed at systematic evaluation of the mechanisms by which fimbriae act as virulence factors. Further, since the fimbriae induce a strong immune response in humans with periodontitis who are infected with *P. gingivalis* (6, 35, 55), evaluation of the functional role of the antibodies in the serum and saliva of these patients may be helpful for better understanding of the role of the immune response to fimbriae in modulation of periodontal disease.

Rat saliva contains both IgG and secretory IgA (50), and high levels of either could conceivably affect the colonization of *P. gingivalis* by binding to fimbriae and blocking fimbria-mediated adherence. Definitive proof awaits more information about the actual function of the salivary antibodies, whether they are directed to the adherence epitopes of the fimbriae or adhesins associated with the fimbriae, and direct measurement of the effect of these antibodies on *P. gingivalis* colonization.

Other immune reactions that may explain the protective effects include T-cell-mediated immunity, immediate hypersensitivity, mucosal immunity, or combinations of these immune responses. Further studies are necessary to distinguish these potential explanations for the protection observed.

Variations in the gnotobiotic rat model of periodontal destruction will be needed to fully assess the role of other protective antigens and to evaluate the effects of specific immunization when a more complex flora exists. Furthermore, understanding the effect of immune responses on late events in periodontal destruction where *P. gingivalis* colonizes the deep pockets and the bacteria or bacterial products invade the gingiva may be assisted by use of other models including the mouse (34, 52), dog (5, 25), or primates (14).

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REFERENCES

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
2. **Brissette, R., R. J. Gibbons, D. I. Hay, and S. Schluckebier.** 1987. Attachment of *Bacteroides gingivalis* 381 to salivary proline-rich proteins on hydroxyapatite surfaces, abstr. 1433. J. Dent. Res. **66**:286.
3. **Christersson, L. A., J. J. Zambon, R. G. Dunford, S. G. Grossi, and R. J. Genco.** 1989. Specific subgingival bacteria and diagnosis of gingivitis and periodontitis. J. Dent. Res. **68**:1633–1639.
4. **Crawford, J. M., M. A. Taubman, and D. J. Smith.** 1978. The natural history of periodontal bone loss in germ-free and gnotobiotic rats infected with periodontopathic microorganisms. J. Periodontal Res. **13**:316–325.
5. **Dahlen, G., L. Heijl, J. Lindhe, and A. Moller.** 1982. Development of plaque and gingivitis following antibiotic therapy in dogs. J. Clin. Periodontol. **9**:223–228.
6. **De Nardin, A. M., H. T. Sojar, S. G. Grossi, L. A. Christersson, and R. J. Genco.** 1991. Humoral immunity of older adults with periodontal disease to *Porphyromonas gingivalis*. Infect. Immun. **59**:4363–4370.
7. **Ebersole, J. L., M. A. Taubman, D. J. Smith, and D. E. Frey.** 1986. Human immune response to oral microorganisms: patterns of systematic antibody levels to *Bacteroides* species. Infect. Immun. **51**:507–513.
8. **Genco, R. J., J. Slots, C. Mouton, and P. Murray.** 1980. Systemic immune response to oral anaerobic organisms, p. 277–293. *In* D. W. Lambe, Jr., R. J. Genco, and K. J. Mayberry-Carson (ed.), Anaerobic bacteria: selected topics. Plenum Publishing Corp., New York.
9. **Genco, R. J., J. J. Zambon, and L. A. Christersson.** 1988. The

origin of periodontal infections. Adv. Dent. Res. **2:**245–259.
10. **Golub, L. M., S. Ciancio, N. S. Ramamurthy, M. Leung, and T. F. McNamara.** 1990. Low-dose doxycycline therapy: effect on gingival and crevicular fluid collagenase activity in humans. J. Periodontal Res. **25:**321–330.
11. **Golub, L. M., H. M. Lee, G. Lehrer, A. Nemiroff, T. F. McNamara, R. Kaplan, and N. S. Ramamurthy.** 1983. Minocycline reduces gingival collagenolytic activity during diabetes. Preliminary observations and a proposed new mechanism of action. J. Periodontal Res. **18:**516–526.
12. **Golub, L. M., M. Wolff, H. M. Lee, T. F. McNamara, N. S. Ramamurthy, J. Zambon, and S. Ciancio.** 1985. Further evidence that tetracyclines inhibit collagenase activity in human crevicular fluid and from other mammalian sources. J. Periodontal Res. **20:**12–23.
13. **Hanazawa, S., Y. Murakami, K. Hirose, S. Amano, Y. Ohmori, H. Higuchi, and S. Kitano.** 1991. *Bacteroides* (*Porphyromonas*) *gingivalis* fimbriae activate mouse peritoneal macrophages and induce gene expression and production of interleukin-1. Infect. Immun. **59:**1972–1977.
14. **Holt, S. C., J. Ebersole, J. Felton, M. Brunsvold, and K. S. Kornman.** 1988. Implantation of *Bacteroides gingivalis* in nonhuman primates initiates progression of periodontitis. Science **239:**55–57.
15. **Isogai, H., E. Isogai, F. Yoshimura, T. Suzuki, W. Kagota, and K. Takano.** 1988. Specific inhibition of adherence of an oral strain of *Bacteroides gingivalis* 381 to epithelial cells by monoclonal antibodies against the bacterial fimbriae. Arch. Oral Biol. **33:**479–485.
16. **Jolley, M. T., C.-H. J. Wang, S. J. Ekenberg, M. S. Zuelke, and D. M. Kelso.** 1984. Particle concentration fluorescence immunoassay (PCFIA): a new rapid immunoassay technique with high sensitivity. J. Immunol. Methods **67:**21–35.
17. **Klausen, B., R. T. Evans, N. S. Ramamurthy, L. M. Golub, C. Sfintescu, C., J.-Y. Lee, G. S. Bedi, J. J. Zambon, and R. J. Genco.** 1991. Periodontal bone level and gingival proteinase activity in gnotobiotic rats immunized with *Bacteroides gingivalis*. Oral Microbiol. Immunol. **6:**193–201.
18. **Klausen, B., R. T. Evans, and C. Sfintescu.** 1989. Two complementary methods of assessing periodontal bone level in rats. Scand. J. Dent. Res. **97:**494–499.
19. **Klausen, B., C. Sfintescu, and R. T. Evans.** 1991. Asymmetry in periodontal bone loss of gnotobiotic Sprague-Dawley rats. Arch. Oral Biol. **36:**685–687.
20. **Kornman, K. S., S. C. Holt, and P. B. Robertson.** 1981. The microbiology of ligature-induced periodontitis in the cynomologus monkey. J. Periodontal Res. **16:**363–371.
21. **Lantz, M. S., R. D. Allen, L. W. Duck, J. L. Blume, L. M. Switalski, and M. Hook.** 1991. Identification of *Prophyromonas gingivalis* components that mediate its interaction with fibronectin. J. Bacteriol. **173:**4263–4270.
22. **Lantz, M. S., R. D. Allen, T. A. Vail, L. M. Switalski, and M. Hook.** 1991. Specific cell components of *Bacteroides gingivalis* mediate binding and degradation of human fibrinogen. J. Bacteriol. **173:**495–504.
23. **Lee, J.-Y., H. T. Sojar, G. S. Bedi, and R. J. Genco.** 1991. *Porphyromonas* (*Bacteroides*) *gingivalis* fimbrilin: size, amino-terminal sequence, and antigenic heterogeneity. Infect. Immun. **59:**383–389.
24. **Lee, J.-Y., H. T. Sojar, G. S. Bedi, and R. J. Genco.** 1992. Synthetic peptides analogous to the fimbrillin sequence inhibit adherence of *Porphyromonas gingivalis*. Infect. Immun. **60:** 1662–1670.
25. **Lindhe, J., S.-E. Hamp, H. Loe, and C. Rindom-Schiott.** 1970. Influence of topical application of chlorhexidine on chronic gingivitis and gingival wound healing in the dog. Scand. J. Dent. Res. **78:**471–478.
26. **Loos, B. G., D. Mayrand, R. J. Genco, and D. P. Dickinson.** 1990. Genetic heterogeneity of *Porphyromonas* (*Bacteroides*) *gingivalis* by genomic DNA fingerprinting. J. Dent. Res. **69:** 1488–1493.
27. **Ma, J. K.-C., R. Smith, and T. Lehner.** 1987. Use of monoclonal antibodies in local passive immunization to prevent colonization of human teeth by *Streptococcus mutans*. Infect. Immun. **55:**1274–1278.
28. **Mikx, F. H. M., D. N. B. Ngassapa, F. M. J. Reijntjens, and J. C. Maltha.** 1984. Effect of splint placement on black-pigmented *Bacteroides* and spirochetes in the dental plaque of beagle dogs. J. Dent. Res. **63:**1284–1288.
29. **Moore, W. E. C.** 1987. Microbiology of periodontal disease. J. Periodontal Res. **22:**335–341.
30. **Morisaki, I., S. M. Michalek, C. C. Harmon, M. Torii, S. Hamada, and J. R. McGhee.** 1983. Effective immunity to dental caries: enhancement of salivary anti-*Streptococcus mutans* antibody responses with oral adjuvants. Infect. Immun. **40:**577–591.
31. **Mouton, C., P. G. Hammond, J. Slots, and R. J. Genco.** 1981. Serum antibodies to *Bacteroides asaccharolyticus* (*Bacteroides gingivalis*): relationship to age and periodontal disease. Infect. Immun. **31:**182–192.
32. **Naito, Y., and R. J. Gibbons.** 1988. Attachment of *Bacteroides gingivalis* to collagenous substrata. J. Dent. Res. **67:**1075–1080.
33. **Naito, Y., K. Okuda, and I. Takazoe.** 1987. Detection of specific antibody in adult human periodontitis sera to surface antigens of *Bacteroides gingivalis*. Infect. Immun. **55:**832–834.
34. **Neiders, M. E., P. B. Chen, H. Suido, H. S. Reymonds, J. J. Zambon, M. Shlossman, and R. J. Genco.** 1989. Heterogeneity of virulence among strains of *Bacteroides gingivalis*. J. Periodontal Res. **24:**192–198.
35. **Ogawa, T., M. L. McGhee, Z. Moldoveanu, S. Hamada, J. Mestecky, J. R. McGhee, and H. Kiyono.** 1989. *Bacteroides*-specific IgG and IgA subclass antibody-secreting cells isolated from chronically inflamed gingival tissues. Clin. Exp. Immunol. **76:**103–110.
36. **Okuda, K., J. Slots, and R. J. Genco.** 1981. *Bacteroides gingivalis*, *Bacteroides asaccharolyticus*, and *Bacteroides melaninogenicus* subspecies: cell surface morphology and adherence to erythrocytes and human buccal epithelial cells. Curr. Microbiol. **6:**7–12.
37. **Ramamurthy, N. S., and L. M. Golub.** 1983. Diabetes increases collagenase activity in extracts of rat gingiva and skin. J. Periodontal Res. **18:**23–30.
38. **Savitt, E. D., and S. S. Socransky.** 1984. Distribution of certain subgingival species in selected periodontal conditions. J. Periodontal Res. **19:**111–123.
39. **Slots, J.** 1977. Microflora in the healthy gingival sulcus in man. Scand. J. Dent. Res. **85:**247–254.
40. **Slots, J.** 1986. Bacterial specificity in adult periodontitis. A summary of recent work. J. Clin. Periodontol. **13:**912–917.
41. **Slots, J., and R. J. Genco.** 1984. Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in colonization, survival, and tissue destruction. J. Dent. Res. **63:**412–421.
42. **Slots, J., and E. Hausmann.** 1979. Longitudinal study of experimentally induced periodontal disease in *Macaca arctoides*: relationship between microflora and alveolar bone loss. Infect. Immun. **23:**260–269.
43. **Smith, D. J., M. A. Taubman, and J. L. Ebersole.** 1980. Local and systemic antibody response to oral administration of glucosyltransferase antigen complex. Infect. Immun. **28:**441–450.
44. **Socransky, S. S., and A. D. Haffajee.** 1990. Microbiological risk factors for destructive periodontal diseases, p. 79–90. *In* J. D. Bader (ed.), Risk assessment in dentistry. University of North Carolina Dental Ecology, Chapel Hill.
45. **Sojar, H. T., J.-Y. Lee, G. S. Bedi, M.-I. Cho, and R. J. Genco.** 1991. Purification, characterization and immunolocalization of fimbrial protein from *Prophyromonas* (*Bacteroides*) *gingivalis*. Biochem. Biophys. Res. Commun. **175:**713–719.
46. **Sojar, H. T., J.-Y. Lee, G. S. Bedi, M.-I. Cho, and R. J. Genco.** 1991. Purification, characterization and localization of a major envelope protein antigen from *Porphyromonas* (*Bacteroides*) *gingivalis*. Biochem. Int. **25:**437–446.
47. **Spiegel, C. A., S. E. Hayduk, G. E. Minah, and G. N. Krywolop.** 1979. Black pigmented bacteroides from clinically characterized periodontal sites. J. Periodontal Res. **14:**376–382.

48. **Svanberg, G. K., S. A. Syed, and B. W. Scott, Jr.** 1982. Differences between gingivitis and periodontitis associated microbial flora in the beagle dog. Relationship of plaque parameters to histological parameters of periodontal disease. J. Periodontal Res. **17**:1–11.
49. **Taubman, M. A., and D. J. Smith.** 1974. Effects of local immunization with *Streptococcus mutans* on induction of salivary immunoglobulin A antibody and experimental dental caries in rats. Infect. Immun. **9**:1079–1091.
50. **Taubman, M. A., D. J. Smith, and J. L. Ebersole.** 1980. Conventional and specialized rodent models for studies of immune mechanisms and dental caries, p. 439–450. *In* J. M. Tanzer (ed.), Animal models in cariology. Information Retrieval, Inc., Washington, D.C.
51. **Tew, J. G., D. R. Marshall, J. A. Burmeister, and R. R. Ranney.** 1985. Relationship between gingival crevicular fluid and serum antibody titers in young adults with generalized and localized periodontitis. Infect. Immun. **49**:487–493.
52. **van Steenbergen, T. J. M., P. Kastelein, J. J. A. Touw, and J. de Graaff.** 1982. Virulence of black-pigmented *Bacteroides* strains from periodontal pockets and other sites in experimentally induced skin lesions in mice. J. Periodontal Res. **13**:236–244.
53. **Yoshimura, F., T. Sugano, M. Kawanami, H. Kato, and T. Suzuki.** 1987. Detection of specific antibodies against fimbriae and membrane proteins from the oral anaerobe *Bacteroides gingivalis* in patients with periodontal diseases. Microbiol. Immunol. **31**:935–941.
54. **Yoshimura, F., T. Takasawa, M. Yoneyama, T. Yamaguchi, H. Shiokawa, and T. Suzuki.** 1985. Fimbriae from the oral anaerobe *Bacteroides gingivalis*: physical, chemical, and immunological properties. J. Bacteriol. **163**:730–734.
55. **Yoshimura, F., K.-I. Watanabe, T. Takasawa, M. Kawanami, and H. Kato.** 1989. Purification and properties of a 75-kilodalton major protein, an immunodominant surface antigen, from the oral anaerobe *Bacteroides gingivalis*. Infect. Immun. **57**:3646–3652.
56. **Zambon, J. J., H. S. Reynolds, P. Chen, and R. J. Genco.** 1985. Rapid identification of periodontal pathogens in subgingival dental plaque. Comparison of indirect immunofluorescence microscopy with bacterial culture for detection of *Bacteroides gingivalis*. J. Periodontol. **56**(Suppl.):32–40.
57. **Zambon, J. J., H. S. Reynolds, and J. Slots.** 1981. Black-pigmented *Bacteroides* spp. in the human oral cavity. Infect. Immun. **32**:198–203.
58. **Zar, J. H.** 1984. Biostatistical analysis, 2nd ed., p. 186–190. Prentice-Hall, Inc., Englewood Cliffs, N.J.
59. **Zucker, S., R. M. Lysik, N. S. Ramamurthy, L. M. Golub, J. M. Wieman, and D. P. Wilke.** 1985. Diversity of melanoma plasma membrane proteinases: inhibition of collagenolytic and cytolytic activity by minocycline. JNCI **75**:517–525.